The Role of Alternative Pre-mRNA Splicing in Regulating the Structure and Function of Skeletal Protein 4.1 (44347)

JOHN CONBOY[1]

Life Sciences Division, Lawrence Berkeley National Laboratory, University of California, Berkeley, California 94720

Abstract. **Alternative pre-mRNA splicing plays a major role in regulating cell type–specific expression of the protein 4.1 family of skeletal proteins. The biological importance of alternative splicing as a mechanism for 4.1 gene regulation is underscored by studies of the prototypical 4.1R gene in erythroid cells: activation of exon 16 inclusion in mRNA at the erythroblast stage greatly enhances the ability of newly synthesized 4.1R protein to bind spectrin and actin, and thus assemble into a stable membrane skeleton. This gain-of-function has profound effects on the biophysical properties of deformability and membrane strength that are critical to red cell survival in the circulation. Another example of developmentally regulated splicing occurs in differentiating mammary epithelial cells in culture, where cell morphogenesis is accompanied by a splicing switch that reversibly activates inclusion of alternative exon 17B. Tissue-specific splices in the 4.1R gene have also been described in brain and muscle. Few other genes are known to be so richly endowed with regulated switches in pre-mRNA splicing, making the 4.1R gene an interesting paradigm for the role of alternative splicing as a mediator of cell function. Recent evidence that other members of the 4.1 gene family are also regulated by alternative splicing suggests, moreover, that this phenomenon is of general importance in regulating the structure of this class of skeletal proteins.** [P.S.E.B.M. 1999, Vol 220]

Protein 4.1R is a multifunctional structural protein best known for its role in stabilizing the red blood cell membrane skeleton. Through its lateral interactions with spectrin and actin in the skeletal framework and vertical interactions with the cytoplasmic domains of integral proteins, such as glycophorin C and band 3, 4.1R contributes to assembly of a properly functioning red cell membrane skeleton. Biophysical properties such as deformability (important for transit through the smallest capillaries) and mechanical strength (to withstand shear forces in the larger vessels) are dependent on inclusion of 4.1R into this spectrin-based skeletal network. Genetic studies have shown that mutation or deficiency of protein 4.1R leads to production of red cells with morphological abnormalities and mechanically weakened, fragmentable membranes. Molecular cloning studies of reticulocyte mRNAs have elucidated the primary structure of the major 80-kDa isoform of 4.1R (1) and suggested the existence of structural heterogeneity of the protein even in red cells, arising from alternative pre-mRNA splicing (2). Thus, the structure and function of the major red cell form of the protein is relatively well understood.

In nucleated cells of erythroid and nonerythroid origin, the expression of 4.1 proteins is considerably more complex. 4.1-Immunoreactive proteins ranging in size from 30 kDa–210 kDa have been reported (3), and immunofluorescence experiments have suggested that 4.1 proteins may function in diverse intracellular sites including the nucleus (4–6) and centrosomes (7). Molecular cloning of 4.1R cDNA from a T lymphocyte cell line (8) provided early evidence for tissue-specific differences in 4.1R splicing patterns. This review will summarize what is currently known about the diversity in protein 4.1 structure and localization that can be attributed directly to alternative pre-mRNA splicing. Several specific examples of regulated splicing events will be cited, and where possible, the biological significance of the alternative protein products will be noted.

The structure of the complex transcription unit that constitutes the protein 4.1R gene is shown in Figure 1A. In

[1] To whom requests for reprints should be addressed at Life Sciences Division, MS 74-174, Lawrence Berkeley National Laboratory, Berkeley, CA 94720.

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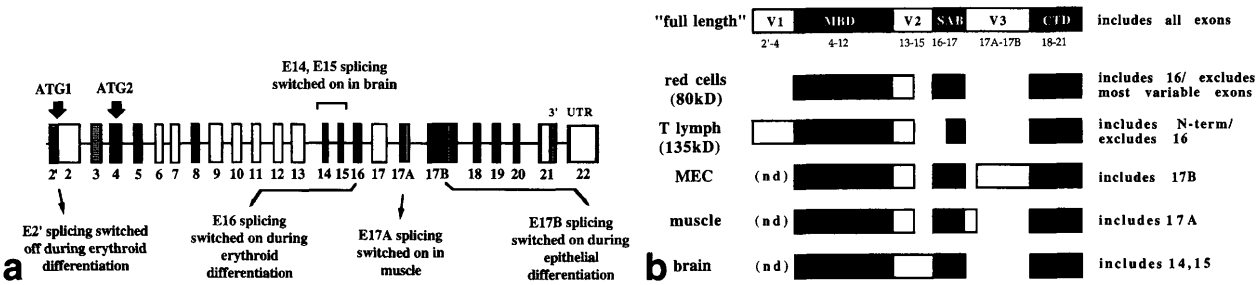


Figure 1. Cell type–specific alternative splicing in the protein 4.1R gene. (A) Exon-intron structure of the gene showing constitutive exons (open boxes) and alternative exons (filled boxes). Cell type–specific splicing events are indicated. (B) Domain structure of predicted protein products, adapted from earlier models (see Ref. 17). The "full length" isoform is a theoretical structure that would result from inclusion of all coding exons in a single transcript. Numbers below the structure indicate exons responsible for encoding each domain. Variable domains (V1–V3) are regions that are poorly conserved among 4.1 genes and/or are excluded from many isoforms by alternative splicing. V1 is the domain previously designated as the N-terminal extension or headpiece of the protein. Conserved domains include the membrane binding domain (MBD), the spectrin-actin binding domain (SAB), and the C-terminal domain (CTD). Below are selected protein isoforms expressed in various cell types, nd, N-terminus not defined in these cells.

mouse and man, the 4.1R gene extends over 200 to 300 kb and contains at least 24 known exons (9–10). Remarkably, about one-half of these are alternatively spliced exons (indicated by filled boxes), and several include internal splice acceptor sites (exon 2) or donor sites (exons 17A and 17B) that further enhance the coding potential of the gene. Combinatorial splicing pathways theoretically could generate an enormous diversity of protein isoforms numbering into the hundreds. More recent studies of the actual complement of 4.1R isoforms expressed in eukaryotic cells have yielded two important conclusions. First, a given cell type expresses at physiologically meaningful levels only a modest subset of the total possible 4.1R isoforms. For example, careful analysis of cloned erythroblast 4.1R mRNAs has shown that seven major isoforms can account for ~87% of the total complement of 4.1R in these cells (11). Second, the complement of 4.1R isoforms being actively expressed can vary widely among different cell types (12–14) due to the existence of cell type–or developmental-specific splicing patterns. These appear most likely to reflect biologically important differences in the function of protein 4.1 isoforms.

Figure 1A is annotated to indicate several major tissue-specific splicing events that are regulated in the 4.1R gene. One of these, involving splice acceptor site utilization in exon 2, contributes directly to the size heterogeneity among 4.1R polypeptides by controlling utilization of alternative translation initiation sites. The remainder of the figure focuses attention on several regulated splicing events involving internal coding exons. These entail inclusion of exon 16 during erythroid differentiation (15, 16), the activation of exon 17B during morphogenesis of mammary epithelial cells (13), the preferential inclusion of exon 17A in muscle (9, 13), and of exons 14 and 15 in brain (10). These splicing switches are described in more detail below.

Figure 1B shows several domain models of the predicted protein products of this complex gene. The first "full length" structure is a theoretical composite protein 4.1R polypeptide derived from an mRNA containing all the known coding exons of the gene. The structural domains depicted in this diagram are adapted from earlier models of the familiar 80-kDa 4.1R protein in red cells (17), updated to incorporate all available information on the coding capacity of the gene. In defining the variable domains, consideration was also given to phylogenetic conservation of orthologous (18) and paralogous 4.1 genes in vertebrate genomes (19, 20).

V1

The most N-terminal domain, present only in higher molecular weight isoforms derived from AUG1 in exon 2, has been called the N-terminal extension, or headpiece, of the molecule. Since this domain is not uniformly present among 4.1R protein isoforms in mammalian cells, and its primary sequence is not well conserved in the orthologous frog 4.1R gene or the newly described paralogous 4.1 genes, this region is designated as variable region 1 (V1). V1 is encoded by exon 2 and part of exon 4 (exon 3 is a noncoding exon of uncertain significance that was present in the original erythroid 4.1R cDNA but is rarely expressed).

MBD

Adjacent to this domain is the so-called 30K membrane binding domain (MBD), which contains the sites for interaction with integral membrane proteins such as band 3 and glycophorin C, with p55, and with Ca^{2+}/calmodulin. This is the most conserved domain in the 4.1 superfamily and is found not only in the closely related 4.1 homolog, but also in the more distant ERM (ezrin, radixin, moesin) relatives. This conserved region, which includes part of the previously defined 16K chymotryptic domain, is encoded by exons 4–12. Some variation in MBD structure is possible due to alternative splicing of exon 5 and (very rarely) exon 8.

V2

V2 is defined here as the region consisting of a constitutive but poorly conserved element encoded by exon 13 (the C-terminal portion of the original 16-kDa domain), plus alternative exons 14 and 15.

SABD

The spectrin-actin binding domain (SABD), defined through extensive biochemical study of native and recombinant 4.1R proteins, is highly conserved among 4.1R gene orthologs in avian and amphibian species (18, 19, 22) and in the paralogous 4.1 mammalian genes (19, 20). This domain is encoded by two exons, alternative exon 16 plus constitutive exon 17. As illustrated in Figure 1A, a pre-mRNA splicing switch activates exon 16 inclusion during erythropoiesis.

V3

Variable region 3, encoded by alternative exons 17A and 17B, is expressed only in selected cells where tissue-specific splicing events activate these exons. This region is entirely absent from erythroid 4.1, and its function is unknown. Interestingly, other members of the 4.1R gene family also contain an additional sequence inserted at this position between the SABD and CTD (18), and preliminary results suggest that this region is also alternatively spliced. However, although their relative location within the gene is conserved, their primary sequence is quite divergent.

CTD

The C-terminal domain is highly conserved among 4.1 genes including the Drosophila homolog *coracle*. This region has been reported to bind the nuclear mitotic apparatus protein NuMA (23) and the immunophilin FKBP13 (20), and may thus have multiple functions in eukaryotic cells. The CTD is encoded by exons 18–21 and is subject to extensive alternative splicing, based on the observation that exon 18 or 19 or 20 can be skipped in a small proportion of 4.1R mRNAs. The significance of alternative splicing in this region is unknown.

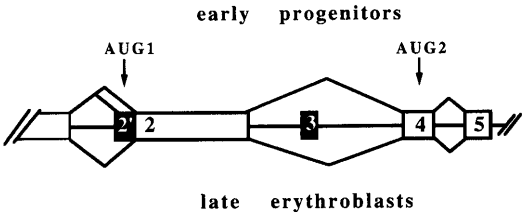
Due to the tissue-specific nature of splicing of several exons, it is likely that no individual protein actually expresses all these domains. Instead, these splicing events create distinct complements of 4.1 mRNAs in different cell types. The remainder of Figure 1B illustrates selected mRNA isoforms that are preferentially expressed in various cell types. It is by no means intended to represent a complete accounting of the isoforms synthesized in these cells. Note that the major red cell isoform represents a truncated form lacking V1 and V3, as well as much of V2. Other cells express specialized forms containing a sequence inserted in one or more of the variable domains.

Alternative Splicing Switches During Erythropoiesis

During mammalian erythropoiesis, early progenitor cells differentiate into erythroblasts and become enucleated as they mature into reticulocytes and ultimately red cells. Many changes in gene expression accompany and promote erythroid morphogenesis. Two major changes that are essential for red cell function include synthesis of globin chains (required for oxygen transport) and assembly of a stable membrane skeleton (vital for acquisition of the membrane deformability and mechanical strength to survive in the circulation). Transcriptional regulation clearly plays a large role in both of these processes, as globin chains are erythroid-specific, and several of the membrane skeletal proteins are encoded by genes with only limited nonerythroid expression.

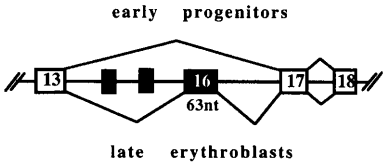
Analysis of protein 4.1 gene expression during erythropoiesis shows that regulated changes in pre-mRNA splicing can also play a critical role in mediating changes in the structure and function of red cell 4.1R. Figure 2 focuses on splice acceptor site utilization in exon 2, which helps control translation initiation site utilization. Two initiation sites are encoded in exon 2 (ATG1) and exon 4 (ATG2); translation from these sites results in synthesis of proteins of ~135 kDa and 80 kDa , respectively. It has long been known that most tissues express a mixture of the two protein 4.1R size classes; in contrast, red cells contain predominantly the 80-kDa forms. This difference is due to a switch in splice acceptor site utilization in exon 2 that occurs during erythropoiesis (15, 24). In most nucleated cells, both acceptor sites at exon 2 are utilized, generating mRNAs that either include AUG1 and synthesize 135-kDa 4.1R, or exclude AUG1 and synthesize 80-kDa 4.1 from AUG2. Maturing erythroid progenitors lose the ability to include AUG1 and therefore express only 80-kDa 4.1R.

Another prime example of this phenomenon is the dramatic change in protein 4.1R function that results from an alternative splicing switch involving exon 16 (Fig. 3). Early erythroid progenitors, like most hematopoietic cells, almost entirely exclude exon 16 from their cytoplasmic 4.1R mRNAs. At the erythroblast stage of differentiation, exon 16 inclusion is activated, resulting in almost complete inclusion of exon 16 in reticulocyte 4.1R mRNA. This phenomenon is observed with cells at different maturational



cell stage	4.1R mRNA structure	4.1R protein synthesized
early progenitors	E2' partially included	-AUG1 -> 135kD -AUG2 -> 80kD
late erythroblasts	E2' skipped	-AUG1 skipped -> no 135kD -AUG2 intact -> 80kD

Figure 2. Switching of translation initiation site usage mediated by alternative splicing. Diagram shows structure of the 5′ exons containing AUG1 and AUG2. Early progenitors utilize both splice acceptor sites at exon 2′/2, leading to synthesis of both 135-kDa and 80-kDa isoforms of the protein. Late erythroblasts splice only to the second acceptor site, excluding AUG1, and leading to synthesis of only 80-kDa isoforms.



cell stage	4.1R mRNA structure	4.1R protein function
early progenitors	E16 skipped	-low affinity binding to spectrin/actin -ineffective in red cell membranes in situ
late erythroblasts	E16 included	-high affinity binding to spectrin/actin -strengthens red cell membrane in situ

Figure 3. A developmental switch in exon 16 splicing alters the structure and function of the SAB domain. The diagram shows the structure of an internal region of the gene around exon 16. Early progenitors splice directly from exon 13 to exon 17, leading to synthesis of 4.1R protein with low affinity for spectrin/actin and with inefficient reconstitution of membrane strength in 4.1R-deficient red cells. Late erythroblasts include exon 16, leading to synthesis of new 4.1R isoforms that bind spectrin/actin with much higher affinity and can effectively strengthen the membrane skeleton. Exons 14 and 15, not labeled, are mostly excluded in erythroid cells.

stages purified from bone marrow (15), and can be reconstituted *in vitro* using mouse erythroleukemia cells induced to differentiate (24). Functional studies with native and recombinant 4.1R proteins have revealed that the inclusion or exclusion of the 21 amino acids encoded by exon 16 dramatically impacts protein 4.1R function. In the absence of this peptide, the SABD binds with low affinity to spectrin and actin (16, 25, 26), and functions poorly in red cell membrane strengthening assays (25, 27). In contrast, inclusion of this critical peptide facilitates high-affinity binding to spectrin and actin, and efficient stabilization of the red cell membrane skeleton when incorporated into 4.1R-deficient membranes that are otherwise quite fragile (25, 27). *In vivo* red cells with deletion or mutation of the SABD are morphologically abnormal and fragment more easily than normal red cells (28).

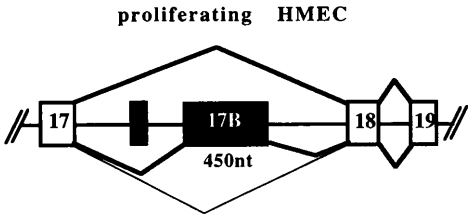
The activation of exon 16 inclusion during erythropoiesis has been shown to occur in both mouse and human systems, and may be inferred to occur (from the observed inclusion in reticulocytes and exclusion in nonerythroid cells) in dogs as well (29). This splicing switch thus appears to be conserved among mammalian species. Interestingly, exon 16 is also an alternative exon, with tissue-specific differences in expression, in the amphibian *X. laevis* (African clawed frog) (18). Based on cDNA cloning and RT/PCR assays, much of the 4.1R mRNA expressed in oocytes fails to include exon 16 (21), whereas the great majority of mRNA in frog red cells includes this exon (18). Such observations suggest that the nuclear splicing machinery responsible for regulation of exon 16 splicing has been evolutionarily conserved.

Although not the focus of this paper, it should also be noted that other alternative splicing events also contribute to proper erythroid differentiation. For example, the form of β-spectrin expressed in erythroid cells differs at the C-terminal end from the major form found in muscle, by virtue of alternative splicing of a distinct set of 3′ exons (30).

An Alternative Splicing Switch in Protein 4.1 RNA During Morphogenesis of Mammary Epithelial Cells

Human mammary epithelial cells (HMEC) growing adherent to plastic culture dishes proliferate in a relatively undifferentiated state, assuming a flattened morphology and failing to express certain differentiated gene products characteristic of mammary epithelial *in vivo*. However, they can be induced to exhibit dramatic changes in morphology and gene expression that mimic certain aspects of differentiation, by culture on a complex extracellular matrix (ECM) substrate, or by culture in suspension (31, 32). The cells cease dividing, assume a more rounded morphology, cluster together, and reorganize their cytoskeletal and nuclear architecture. In addition, the transcription of red cells encoding β-casein and whey acidic protein can be activated under such conditions (33–35). The processes of morphological and functional differentiation are thus intimately related (36, 37).

We have shown that mammary epithelial cell morphogenesis is accompanied by a change in the profile of 4.1R mRNAs expressed in the cells (13). This change, involving expression of a higher molecular weight isoform of the 4.1 protein, is mediated by a differentiation-associated splicing switch involving alternative exon 17B (Fig. 4) (13). RT-PCR analysis of 4.1R mRNAs expressed in HMEC at the two morphogenetically distinct states was used as a simple assay for changes in pre-mRNA splicing. Whereas HMEC proliferating on plastic exhibited 4.1R mRNAs with exon 17 spliced directly to exon 18, their counterparts in more differentiated cells expressed novel transcripts that included exon 17B or (more rarely) exons 17A + 17B. This is an unusually large alternative exon of 450 or 411 nt (depending



cell stage	4.1R mRNA structure	4.1R structure
proliferating	E17B skipped	- 80kD
"differentiated"	E17B partially included	- 80kD + 110kD

Figure 4. A switch in exon 17B splicing associated with morphogenesis of human mammary epithelial cells (HMEC). Cells proliferating on plastic culture dishes in a relatively undifferentiated state skip exon 17B, whereas the more differentiated counterparts cultured in suspension activate exon 17B inclusion and synthesize a higher-molecular-weight isoform of the protein.

on which of two 5′ splice donor sites is utilized) whose expression occurs primarily in epithelial cells. Importantly, this pre-mRNA splicing switch is reversible if the cells are replated on plastic culture dishes to resume proliferation (13). Although the functional significance of this altered expression pattern is unknown at the protein level, the availability of a reversible splicing switch in cultured cells may facilitate analysis of the signals responsible for mediating the switch.

Regulation of Exon 16 Splicing

It would be of great interest to understand mechanistically how exon 16 splicing is regulated by the nuclear splicing machinery. Preliminary studies have shown that exon 16 alternative splicing can be reconstituted using a model pre-mRNA derived from minigene constructs containing just three exons: exon 16 itself (flanked by native intron sequences) plus the two nearest constitutive exons, namely exons 13 and 17. Such model pre-mRNAs can be spliced using HeLa nuclear extracts into two RNA products either including or excluding exon 16 (38). Similar results can be obtained by microinjection of the model pre-mRNA into Xenopus oocytes (39), demonstrating that the nuclear machinery that splices endogenous frog 4.1R pre-mRNA can also alternatively splice exogenously introduced mouse 4.1R pre-mRNA.

The ability to reconstitute exon 16 alternative splicing in these systems should facilitate characterization of *cis*-acting RNA regulatory elements that control exon 16 splicing, as well as identification of RNA binding protein/splicing factors that bind to these sites to promote or inhibit recognition and splicing of the exon. Inspection of exon 16 and flanking intron sequences suggest that multiple elements may be involved in the decision to splice exon 16, including a conserved splice acceptor sequence, a purine-rich sequence, and a separate evolutionarily conserved element within the exon (40), and a weak 5′ splice (38, 40). Nothing is known as yet regarding the factors that interact with these sites; however, it is tempting to speculate that one or more members of the SR protein family of splicing factors could interact with the purine rich element and influence exon 16 splicing. Presumably, the concerted action of several factors is required to achieve the developmental regulation of exon 16 splicing that occurs *in vivo*. Much additional work will be required to elucidate this splicing regulatory mechanism.

Future Perspectives

This review has focused on the role of alternative splicing in regulation of 4.1R gene expression. The complex 4.1R gene is shown to utilize an elaborate repertoire of developmentally regulated alternative splicing pathways to express a distinct complement of mRNA isoforms in various cell types. The protein products of these mRNAs exhibit differences in size, protein interactions, and subcellular localization. Although much remains to be learned about the biological significance of the diverse 4.1R protein isoforms, in one case—the activation of exon 16 inclusion during erythropoiesis—the physiological raison d'être is clear: only the 4.1R isoforms synthesized after the splicing switch possess a functional spectrin-actin binding domain, and contribute to the membrane stabilization process that is so critical for red cell survival in the circulation. Alternative splicing thus effects an important change in the skeletal architecture of developing red blood cells.

Several considerations suggest that other eukaryotic cell types may be impacted similarly by changes in expression of 4.1 genes mediated at the level of alternative splicing. This review has already described a pre-mRNA splicing switch associated with morphogenesis of mammary epithelial cells, as well as apparent tissue-specific 4.1R transcripts in brain and muscle. Recent results suggest, moreover, that this phenomenon may have broader significance in regulating expression of other members of the 4.1 superfamily. Three new members of the 4.1 gene family, each with its own unique pattern of tissue-specific expression, have recently been discovered: 4.1G (generally expressed in a wide variety of cells) (19, 20), 41N (neuronal (41)), and 4.1B (brain (42)). These new genes are encoded by distinct genetic loci that map to separate chromosomes but share with 4.1R the propensity to undergo complex alternative splicing. Preliminary results suggest that tissue-specific alternative splicing events exhibit two intriguing features. First, two of these new genes encode an alternative exon whose primary sequence is quite homologous to protein 4.1R exon 16, but whose tissue-specific splicing profile is quite different. This provides an opportunity to examine how a paralogous alternative exon in conserved members of a gene family may have evolved differential splicing controls. Secondly, all three of the new genes appear to express brain-specific splicing events, consistent with earlier reports that neural cells frequently splice pre-mRNAs differently than do other cell types (43, 44).

Much of the work described here was done in close collaboration with my colleagues Joel Chasis and Mohandas Narla, without whose help the studies would not have been possible. I would also like to thank the many members of our laboratory group for their excellent and dedicated contributions to many of the studies discussed here.

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