Complex Patterns of Sequence Variation and Multiple 5' and 3' Ends Are Found among Transcripts of the Erythroid Ankyrin Gene*

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The structural protein ankyrin functions in red blood cells to link the spectrin-based membrane skeleton to the plasma membrane. Ankyrin proteins are now known to occur in most cell types, and two distinct ankyrin genes have been identified (erythroid (Ank-1) and brain (Ank-2)). We have characterized transcripts of the mouse erythroid ankyrin gene by cDNA cloning and DNA sequencing. Ank-1 transcripts of 7.5 and 9.0 kilobases are found in erythroid tissues, and a 9.0-kilobase transcript is found in cerebellum. RNA hybridization blot analysis of 13 additional mouse tissues has detected four novel Ank-1 transcripts (5.0, 3.5, 2.0, and 1.6 kilobases in size). Sequencing of Ank-1 cDNA clones isolated from mouse reticulocyte, spleen, and cerebellar libraries has identified (i) multiple 5' ends that indicate possible multiple promoters; (ii) alternative polyadenylation sites that probably account for the 7.5- and 9.0-kilobase size difference; (iii) a variety of small insertions and deletions that could produce transcripts (and ultimately proteins) of nearly identical size, but different functions; and (iv) clones with large deletions of coding sequence that account for the smaller transcripts seen in spleen, skeletal muscle, and heart.

The spectrin-based membrane skeleton forms a supporting protein network underlying the plasma membrane in red blood cells (for review, see Refs. 1 and 2). Erythroid ankyrin functions in the membrane skeleton as a high affinity binding protein linking the β subunit of spectrin to the cytoplasmic domain of band 3, the anion channel (3-6). Once thought to be unique to red blood cells, a spectrin-based membrane skeleton and the linking protein ankyrin are now known to be present in many, if not all, cell types (for review, see Ref. 7). Immunological and biochemical analyses of ankyrin from different cell types have revealed a large family of related proteins. The best characterized members of this family are erythroid and brain ankyrins (ANK-1 and ANK-2, respectively) (8-10). Erythroid and brain ankyrins are coded for by separate genes (11-14), and each gene is known to produce alternatively processed transcripts (15-18). This allows for the production of multiple ankyrin isoforms from a single gene. It is likely that other ankyrin genes exist and will be found as more is learned about the ankyrin proteins in other tissues.

Three laboratories have recently isolated and sequenced overlapping cDNA clones for human and mouse erythrocye ankyrins (15, 16, 18). The three structural domains of ankyrin as defined by chymotrypsin sensitivity (8) have been confirmed by sequence data to include an NH2-terminal 89-kDa domain, a central 62-kDa domain, and a COOH-terminal 55-kDa domain (16). The NH2-terminal domain consists primarily of 22 tandem 33-amino acid repeats and contains band 3 and tubulin binding activity (16). Ankyrin-type repeats have been identified in a variety of other proteins with diverse functions, including cell cycle proteins and transcription factors (for review, see Refs. 20 and 21). The central 62-kDa domain is responsible for β-spectrin and vimentin binding (16). The 89- and 62-kDa domains are relatively well conserved among the erythroid ankyrins sequenced thus far. The COOH-terminal 55-kDa regulatory domain is more variable and appears to function as a modifier of the binding activities of the other two domains (22). Two regions of alternative sequence within the regulatory domain of human erythroid ankyrin are known. The first refers to a 486-base pair (bp)2 segment missing in some clones and accounts for the 2.2 ankyrin isoform found in human red blood cells (16). The second occurs at the COOH terminus where three alternatives have been identified (15, 16). Comparison of mouse Ank-1 sequence (18) to the human sequences shows >95% amino acid identity in the band 3- and spectrin-binding domains. The 55-kDa regulatory domain shows somewhat lower overall homology (79% amino acid identity); however, several segments are 100% conserved, possibly indicating regions essential for ankyrin function in erythroid cells.

The existence of a large family of ankyrin proteins and the observation that more than one type of ankyrin can be expressed in the same cell type suggest that in addition to binding the membrane skeleton to the plasma membrane, ankyrins may have other more specialized functions. For example, some ankyrins have binding affinities for integral membrane proteins other than band 3, notably Na+/K+-ATPase in kidney (23) and the voltage-dependent Na+ channel in brain (24) and the neuromuscular junction (25). Some ankyrins are localized to specialized regions of cells such as Ranvier's nodes in myelinated nerves (26, 27). Studies on mice with a mutation (nb) in the erythroid ankyrin gene have

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† The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EMBL Data Bank with accession number(s) X69063, X69064, and X69065.
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§ The abbreviations used are: bp, base pair(s); kb, kilobase(s); PCR, polymerase chain reaction.

1. Throughout this paper, we have adopted a nomenclature system based upon the conventions used to name mouse ankyrin genes (19). Briefly, the genes are referred to as Ank-1 (erythroid) and Ank-2 (brain). The proteins are ANK-1 and ANK-2. Note that gene names are italicized; protein names are not. A detailed discussion of ankyrin nomenclature is given in Ref. 20.

2. The abbreviations used are: bp, base pair(s); kb, kilobase(s); PCR, polymerase chain reaction.
indicated that the presence of an ANK-1 isoform in Purkinje cells is critical for the survival of these cells in brain (14).

To define the functional significance of the various ankyrin proteins, a clear description of their origin (distinct genes or alternative processing of single gene products), their structure, and their pattern of expression is needed. This study was designed to gain such information about transcripts of the mouse erythroid ankyrin gene Ank-1 on mouse chromosome 8 (11). Two transcripts (9.0 and 7.5 kilobases (kb) in size) are known from erythroid and cerebellar tissues (14). On RNA hybridization blots, we have detected two additional Ank-1-positive tissues (skeletal muscle and heart) and four additional transcripts (5.0, 3.5, 2.0, and 1.6 kb in size). We have prepared mouse reticulocyte and cerebellar cDNA libraries and screened them for erythroid ankyrin. Characterization of the ankyrin clones obtained has produced a full-length coding sequence for a cerebellar transcript and sequence for several partial erythroid and cerebellar transcripts that represent variations from previously described Ank-1 sequence. We have found two distinct NH2 termini and three functional polyadenylation sites and characterized a transcript from spleen that has deleted most of the sequence coding for the spectrin-binding domain. RNA hybridization blot data and domain-specific probes indicate that the small transcripts from skeletal muscle are missing large regions of coding sequence from the band 3- and spectrin-binding domains. Several new inserts and deletions involving small sequence segments are documented. Our data show that the expression of the Ank-1 gene involves complex patterns of sequence variation and suggest the potential for an even greater diversity of ankyrin proteins than previously suspected.

MATERIALS AND METHODS

Animals—All mice used in this study were of the inbred strain C57BL/6J produced in our research colony at the Jackson Laboratory. (The Jackson Laboratory is fully accredited by the American Association of Laboratory Animal Care.)

Hybridization Probes—Mouse erythroid ankyrin clones were detected using the cDNA clone mAnk-1, a 4.6-kb mouse anemic spleen clone isolated and sequenced by White et al. (18). This clone starts within the 15th repeat of the NH2-terminal domain of erythroid ankyrin (bp 1650 of White et al.), continues through the COOH-terminal domain of erythroid ankyrin (bp 1800 of White et al.), continues through the COOH-terminal domain of erythroid ankyrin (bp 1800 of White et al.), and ends with nuclear localization signals for Ank-1 mRNA. Amplification was done by polymerase chain reaction (PCR) techniques (29) using the GeneAmp DNA polymerase (Perkin-Elmer Cetus Instruments). Both probes were 32P-labeled by random oligonucleotide primer extension (30).

Preparation of RNA—When exposed to anemic stress, normal mice respond by production of erythrogenic spleens, bone marrow hyperplasia, and an increase of circulating reticulocytes (31). We exposed mice to anemic stress by injection of the hemolytic agent phenylhydrazine (32). These mice were the source of the reticulocytes used in this study (refer to Fig. 6 for location and sequence of the primers used).

In preparation for RNA isolation and characterization of cDNA clones—As a basis
for understanding the clones and sequences reported in this paper, Fig. 2A summarizes known features of erythroid ankyrin. The protein structural domain sizes and primary functions are given above the line, and the locations of the repeat region and known splice regions are indicated below the line. Using the mAnk-1 cDNA clone as a probe to screen the libraries, we isolated 1 clone from the anemic spleen library, 15 clones from the reticulocyte library, and 11 clones from the cerebellar library. All clones were partially sequenced and positioned relative to each other by comparison to the mouse erythroid ankyrin sequence. The amino acid sequences are aligned in Fig. 3. The 3′ splice junction of the 90-bp segment is a 24-bp insert in Cb14/11, coding for 8 amino acids, just 6 residues 5′ to the predicted 89/62-kDa junction (see Cb14 in Fig. 2B and Fig. 3). The amino acid sequence of the insert (TAHISIMG) predicts a neutral peptide with a molecular weight of 829 and a pI of 6.23 and an overall hydrophobic nature.

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A new alternative was found in the 2.2 region of the 55-kDa regulatory domain. Unlike human erythroid ankyrin, no evidence has been found for the occurrence of the 2.2 isoform in mouse. However, we have detected a cDNA clone with a 90-bp deletion within the 2.2 splice region (see Cb14 in Fig. 2B and Fig. 3). The 3′ splice junction of the 90-bp segment.
FIG. 3. Nucleotide and deduced amino acid sequences of Ank-I cerebellar clone Cb14/11. Important features of the nucleotide sequence are in boldface type and underlined. They are the upstream stop codon at bp 319; the start site consensus sequence, including the initiator codon at bp 352; the two stop codons, C1 at bp 5902 and C2 at bp 5955; and the three polyadenylation signals at bp 6910, 7527, and 8040...
Small Transcripts of Spleen and Skeletal Muscle—The anemic spleen clone (Er4) has a completely unique 3' end. This clone begins in the 89-kDa domain at bp 1307 of Er1 (bp 1579 of Cb14/11) and is identical to Er1 until bp 4487 (bp 4783 of Cb14/11), where Er4 substitutes 340 bp of unique sequence. This sequence encodes 38 amino acids and includes 226 bp of 3'-untranslated region, a polyadenylation signal, and a poly(A) tail (Figs. 2B and 6). The predicted peptide has a molecular weight of 4451 and a pI of 6.29 and is very hydrophobic in nature, especially at the COOH terminus. The point of divergence with Er1 coincides with a splice junction identified in the human erythroid ankriyin gene (43). Assuming the same 5' end as that of Er1, the predicted size of the Er4 transcript would be at least 4.5 kb. A search of the GenBank Data Bank did not detect any sequences homologous to the Er4 unique region.

Several experiments were done to verify Er4 as an Ank-1 transcript. A 272-bp PCR fragment was amplified from the Er4 unique region using the oligonucleotide primer pair indicated in Fig. 6. The fragment (Er4/272) was 32P-labeled and used as a probe on DNA and RNA filter blots. Fig. 7A shows a comparison of HindIII and EcoRI restriction fragments detected in mouse genomic DNA by the mAnk-1 and Er4/272 probes. The Er4/272 probe hybridizes to a subset of the fragments detected by mAnk-1, indicating that the Er4 unique sequence is within the Ank-1 gene. On RNA blots of total cellular RNA, Er4/272 detects an ~5.0-kb transcript in anemic spleen, the tissue from which the clone was isolated, but not in the other tissues examined (reticulocytes, cerebellum, and skeletal muscle) (Fig. 7B). Using the same two oligonucleotide primers described above, we were able to reverse-transcribe total cellular RNA and to amplify by PCR (29) the Er4 unique region from anemic spleen RNA, but not from reticulocytes, cerebellum, or skeletal muscle RNA (Fig. 7C). These data strongly support the identity of Er4 as a transcript of the Ank-1 gene.

Unlike the 5.0-kb transcript, the 3.5-, 2.0-, and 1.6-kb transcripts expressed in skeletal muscle and heart appear to lack large regions of the band 3- and spectrin-binding domains. In experiments using several domain-specific probes against blots of total cellular RNA from skeletal muscle only, the large multidomain probe mAnk-1, a 30-mer oligonucleotide probe from within the COOH-terminal segment (segment B) of Cb14/11 (bp 5815–5844), and a 3'-untranslated region probe from Cb14/11 (bp 6030–6910) detect these transcripts (Fig. 8). A repeat region probe (Cb14/11 bp 819–2488) and a spectrin-binding domain probe (Cb14/11 bp 3164–3932) were negative.

DISCUSSION

Our analysis of transcripts from the mouse Ank-1 gene indicates the potential for a greater complexity of ankriyin protein structure and function than formerly appreciated. Several new features of Ank-1 transcripts are described. Foremost among these is the discovery of multiple NH2 termini and the possibility of gene regulation by multiple promoters. Alternative polyadenylation also occurs, and it is likely that this accounts for the difference in size between the 7.5- and 9.0-kb transcripts. Somewhat surprisingly, the 3'-untranslated regions of mouse and human showed relatively high homology. This could indicate a functional role for some
segments of this region, possibly affecting transcript stability (44) and/or subcellular localization (45).

Our data also define differences among Ank-1 transcripts that involve changes in small segments of coding sequence. We detected, in cerebellar transcripts, a 24-bp insert at the junction of the 89- and 62-kDa domains. This would insert a neutral hydrophobic peptide into a basically charged hydrophilic region and could be functionally significant to the structure of the domain junction. Additional alternatives occur within the regulatory domain. Although no examples of the 2.2 splice are known in mouse, a 90-bp deletion is found within the 2.2 region. In addition to the three described previously, a fourth alternative at the COOH terminus was found. It should be noted that each of these differences would produce transcripts (and proteins) of nearly equal size. The evidence supporting the alternative sequence segments are represented as boxes A–C, as described under “Results.” Open boxes indicate coding regions; hatched boxes indicate 3′-untranslated regions. The lines connecting the boxes show the patterns of sequence segments used. C1 and C2 indicate the alternative stop codons, and the asterisks indicate the stop codon used in each case. B, shown are the amino acid sequences for the two new mouse alternatives described in this report. The segments begin with residue 1813 of Cb14/11. Asterisks indicate the first residue of each segment.

FIG. 5. Graphic representation of known alternative COOH-terminal sequence patterns found among Ank-1 transcripts. A, the alternative sequence segments are represented as boxes A–C, as described under “Results.” Open boxes indicate coding regions; hatched boxes indicate 3′-untranslated regions. The lines connecting the boxes show the patterns of sequence segments used. C1 and C2 indicate the alternative stop codons, and the asterisks indicate the stop codon used in each case. B, shown are the amino acid sequences for the two new mouse alternatives described in this report. The segments begin with residue 1813 of Cb14/11. Asterisks indicate the first residue of each segment.

FIG. 6. Nucleotide and deduced amino acid sequences of clone Er4 unique region. The segment of Er4 shown here starts at bp 4187 of Er1 (bp 4483 of Cb14/11). The asterisk indicates the predicted start of the 55-kDa regulatory domain. The stop codon, the dinucleotide repeat, and the polyadenylation signal of the unique region are underlined. The oligonucleotide primer pair used to amplify by PCR the unique region are overlined. The arrows indicate the direction of primer extension. The unique amino acid sequence appears in boldface type.
whose putative function is to bind P-spectrin to the cytoskeletal transcripts could be responsible for the 43-kDa protein in addition to those described here. It is not clear, for example, whether the bands (−3.5 and 2.0 kb in size) seen in reticulocyte RNA; lanes 2 and 6, 2 µg of anemic spleen RNA; lanes 3 and 7, 2 µg of cerebellar RNA; lanes 4 and 8, 4 µg of skeletal muscle total cellular RNA; lane 9, 4 µg of cerebellar poly(A)⁺ RNA; lane 10, 4 µg of skeletal muscle poly(A)⁺ RNA. Sizes were estimated relative to 28S and 18S RNAs.

FIG. 7. Verification of Er4 as Ank-1 transcript. A, DNA filter blot of 5 µg of HindIII-digested (lanes 1 and 2) and EcoRI-digested (lanes 3 and 4) normal mouse genomic DNA. The blot was probed with the 32P-labeled multidomain probe mAnk-1 (lanes 1 and 3), erased, and replored with the Er4 unique region probe Er4/272 (lanes 2 and 4). B, RNA filter blot of total cellular RNA probed with 32P-labeled mAnk-1 (lanes 1–4) and Er4/272 (lanes 5–8). Lanes 1 and 5, 4 µg of reticulocyte RNA; lanes 2 and 6, 2 µg of anemic spleen RNA; lanes 3 and 7, 2 µg of cerebellar RNA; lanes 4 and 8, 4 µg of skeletal muscle RNA. Band sizes were estimated relative to 28S and 18S RNAs. C, ethidium bromide-stained agarose gel (1.5%) of the products generated by reverse-transcribed PCR amplification (29) from total cellular RNA using the Er4 unique region oligonucleotide primer pair indicated in Fig. 6. Lane 1, 123-bp ladder; lane 2, anemic spleen RNA; lane 3, reticulocyte RNA; lane 4, cerebellar RNA; lane 5, skeletal muscle RNA; lane 6, Er4 DNA control; lane 7, mAnk-1 DNA control. Relevant marker sizes are indicated.

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as a probe (47). It is interesting to speculate that one of these small transcripts could be responsible for the 43-kDa protein whose putative function is to bind β-spectrin to the cytoplasmic domain of the muscle acetylcholine receptor in the neuromuscular junction (25). A clear description of the primary structure of these transcripts should aid our understanding of the structure and function of the spectrin-based membrane skeleton in muscle cells and resolve the question of the gene(s) responsible for their production.

Our results indicate that the Ank-1 gene may produce other transcripts in addition to those described here. It is not clear, for example, whether the bands (~3.5 and 2.0 kb in size) seen in anemic spleen (Fig. 1, lane 1) are the same as those seen in skeletal muscle. Also, there is a small (<1.0-kb) band detected in anemic spleen by the Er4/272 unique region probe (Fig. 7B, lane 6). It is unlikely that all of the Ank-1 transcript alternatives have been discovered. In addition, it is unlikely that all tissues expressing Ank-1 transcripts have been found. The potential for diversity of transcripts from just the Ank-1 gene alone is truly remarkable. This sort of diversity has implications for ankyrin function and lends support to the

FIG. 8. RNA filter blots probed with 32P-labeled region-specific probes. The probes used were as follows: mAnk-1 (multidomain), 89-kDa domain (Chb14/11 bp 819–2488), 62-kDa domain (Chb14/11 bp 3164–3932), 3′-untranslated region (Chb14/11 bp 6030–6910), and Chb14/11 COOH-terminal segment (30-mer oligonucleotide, Chb14/11 bp 5815–5844). Lanes 1, 3, 5, and 7, 1 µg of cerebellar total cellular RNA; lanes 2, 4, 6, and 8, 4 µg of skeletal muscle total cellular RNA; lane 9, 4 µg of cerebellar poly(A)⁺ RNA; lane 10, 4 µg of skeletal muscle poly(A)⁺ RNA. Size was estimated relative to 28S and 18S RNAs.

notion of ankyrins as adaptors that mediate and regulate interactions within integral membrane proteins and other cytoskeletal and cytoplasmic elements (for review, see Refs. 20 and 21). It will be important to determine whether the Ank-1 transcripts we have identified are translated into functional proteins. Our data should aid protein work by alerting investigators to the potential complexity of forms and by defining segments useful for preparing the necessary immunological probes.

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