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Two forms of 1B236/myelin-associated glycoprotein, a cell adhesion molecule for postnatal neural development, are produced by alternative splicing

(immunoglobulin super family/myelinogenesis/platelet-derived growth factor/P_0 protein)

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ABSTRACT **The structures of two rat brain-specific 1B236 mRNAs, alternative splice products from a single gene regulated differently during postnatal brain development, were deduced from full-length cDNA clones. The 626- and 582-amino acid-long encoded proteins are indistinguishable from two forms of myelin-associated glycoprotein, a cell adhesion molecule involved in axonal–glial and glial–glial interactions in postnatal brain development, particularly in myelination. The two proteins share a single membrane-spanning domain and a glycosylated N terminus but differ in the structures of their C termini. The N terminus consists of five domains related in sequence to each other and to immunoglobulin-like molecules, especially the neural cell adhesion molecule N-CAM, suggesting a common structure for cell adhesion molecules.**

Rat brain protein 1B236 was originally defined by nucleotide sequence analysis of randomly selected cDNA clones of mRNAs expressed in adult rat brain but not detectable in liver or kidney (1, 2). Antisera to synthetic peptides corresponding to nonoverlapping regions of the partial 1B236 sequence detected a postnatally expressed 100-kDa rat brain protein containing ≈30% N-linked carbohydrate and proteolytic fragments derived from its C-terminal regions (2–5). During early postnatal development, 1B236 is expressed predominantly by oligodendrocytes in myelinating fiber tracts. Subsequently, there is gradual elaboration of the adult pattern in which 1B236 mRNA is detected predominantly in subsets of neurons in grey matter regions and the 1B236 protein is restricted to specific neuronal cell bodies and fibers (refs. 2, 4, and 6; G. A. Higgins, H. Schmale, F.E.B., M. C. Wilson, R.J.M., unpublished data).

We have now analyzed 1B236 expression more completely and report here the structures of two differently regulated, alternatively spliced 1B236 mRNAs that encode proteins with alternative C-terminal tails. The shared N-terminal region consists of five domains that are related in sequence to each other and to proteins of the immunoglobulin super family (8), especially the neural cell adhesion molecule N-CAM (9). We show that the 1B236 protein is indistinguishable from myelin-associated glycoprotein (MAG), a nervous system-specific glycoprotein of 100 kDa that contains ≈30% carbohydrate (10). MAG has been implicated in the interactions between myelinating cells and axons (10) and the formation and maintenance of the periaxonal space (11). In the peripheral nervous system, MAG appears to take over Schwann cell–axon and Schwann cell–Schwann cell interactions initiated by N-CAM and L1/neuron–glia cell adhesion molecule (12). Thus two cell adhesion molecules that act at successive stages of neural development also share significant structural properties.

EXPERIMENTS

Primary Structure of the 1B236 mRNA: Alternative Splicing Produces Two Forms. We described a 1500-nucleotide (nt) partial cDNA clone of the 2500-nt 1B236 mRNA (2). Two additional clones (p1B236-18 and p1B236-20) with apparently full-length inserts were isolated from a rat brain cDNA library and their nucleotide sequences were determined. The sequence of p1B236-18 (nt 1–2389) was identical where it overlapped (nt 1085–2389) with the published partial sequence (2), except for a minor difference (Fig. 1). Primer extension analyses show that clone p1B236-18 is a full-length clone (data not shown). The single open reading frame begins at nt 163, extends to the termination triplet TGA at nt 2043, and can be translated into a 626-residue protein (Fig. 1). The sequence of most of the single rat 1B236 gene has been determined and, when aligned with the p1B236-18 cDNA sequence, reveals the positions of 11 introns (Fig. 1).

The second cDNA (p1B236-20) was identical in sequence with p1B236-18 except that it included an additional 45 nt (Fig. 2*A*) between positions 1878 and 1879 and lacked nt 20–71. The additional 45 nt in clone 20 are flanked in the genomic sequence by introns (Fig. 2*A*). Thus the mRNAs corresponding to clones 18 and 20 differ by the alternate inclusion (Fig. 2*B*) of exons 2 or 12. Exclusion of exon 2 does not affect the coding region since the exon is located in the 5′-noncoding region. However, inclusion of exon 12 introduces a different coding sequence and a termination codon. This produces a 582-residue sequence with the last 9 amino acids distinct from the last 53 residues of the p1B236-18 form.

A cDNA fragment containing exon 12 and flanking regions was subcloned into pT7/T3-19 and used to generate a labeled minus-strand copy (Fig. 3). The inserts from p1B236-18 and p1B236-20 were subcloned into pGEM-4 and used to produce full-length copy RNAs with and without exon 12. When these RNAs were mixed with the labeled probe and the resulting hybrids were treated with RNase A, the clone 20 transcript protected fragments of ≈230 and 120 nt and the clone 18 transcript protected fragments of ≈158 and 147 nt (Fig. 3). No fragments were protected by tRNA. Fragments that comigrated with both the clone 20- and clone 18-protected RNAs were protected by brain RNAs (Fig. 3). Thus mRNAs lacking exon 12 (presumably encoding the 626-residue protein) and mRNAs containing exon 12 (presumably encoding the 582-residue form) are both stable gene products.

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Abbreviations: N-CAM, neural cell adhesion molecule; MAG, myelin-associated glycoprotein; nt, nucleotide(s).

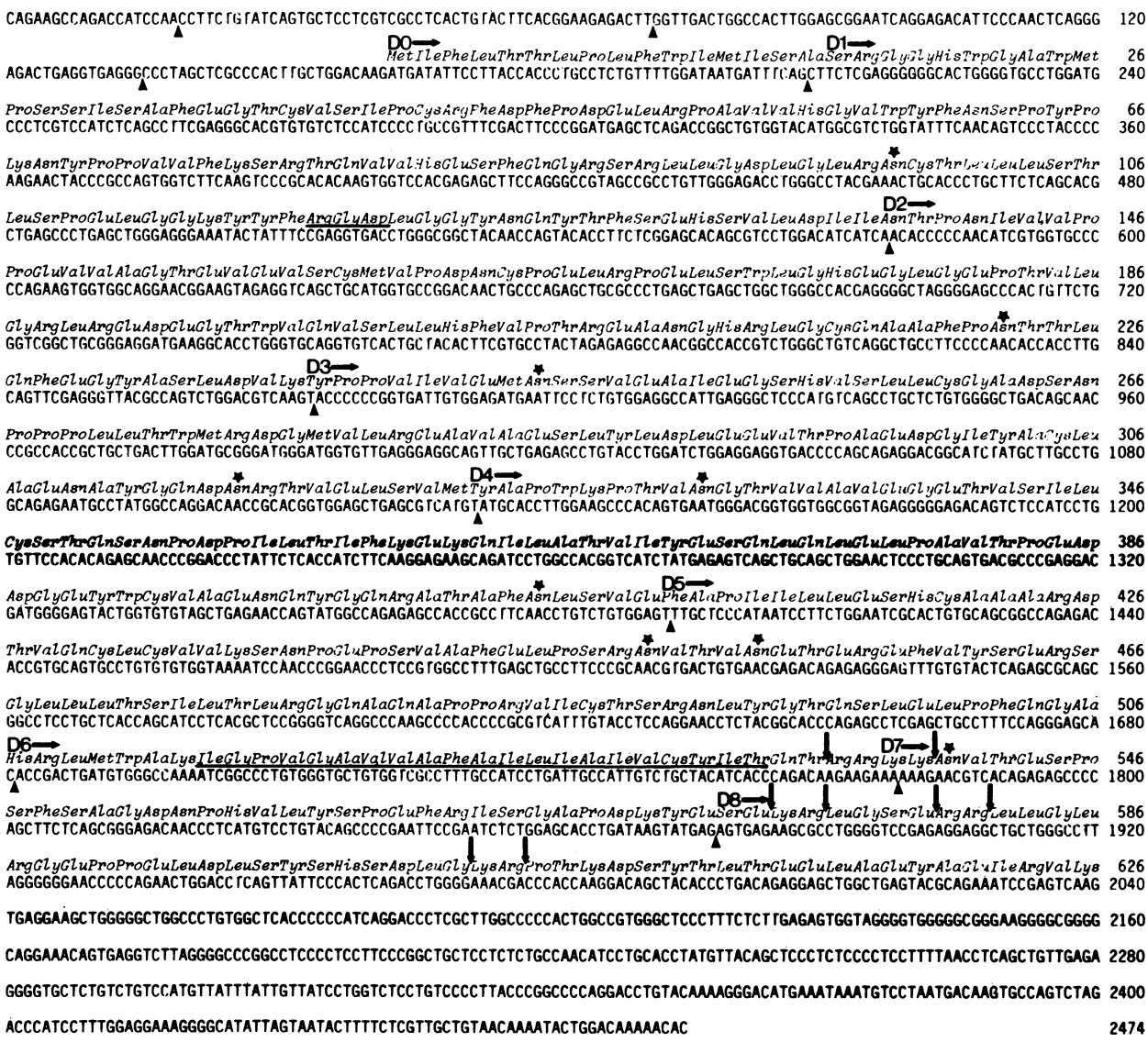

FIG. 1. Nucleotide sequence of cDNA clone p1B236-18 and the translated 1B236 amino acid sequence. Arrowheads under the sequence indicate positions of introns. D0–D8 represent sections of protein defined by intron-exon boundaries. Stars indicate putative sites for N-glycosylation. Underlined residues 118–120 are a possible site for interaction with cell attachment substrates (13). Underlined residues 514–534 represent the putative membrane spanning segment. Vertical arrows near C terminus represent proteolytic cleavage sites (5). In a report (2) nt 1089 and 1090 were clerically transposed, and hence two adjacent amino acids were mistranslated. The poly(A) tail for clone 1B236-18 appears following nt 2389; the poly(A) tail in the original clone appeared after residue 2474.

Fragments that comigrate with those protected by both clone 18 and 20 transcripts are detected with RNA samples from all postnatal ages (Fig. 3). Those corresponding to the clone 18 transcript appear more abundant in the hindbrain at earlier ages and increase to their highest level with RNA from days 17–29 and decrease with day-50 RNA. Fragments comigrating with those protected by the clone 20 transcript are present at low levels in the samples protected by RNA from the younger rats and increase continuously when protected with later hindbrain RNA samples. Thus, the mRNA lacking exon 12 peaks during early postnatal development and decreases, whereas the mRNA containing exon 12, a minor form at early times, becomes the predominant adult species. The two forms do not correlate in a simple way with the oligodendrocyte to neuronal shift in 1B236 expression because antisera to two peptides whose sequence is exclusive to the product of the early mRNA react with neurons in adult rats (6). Similar RNase protection experiments suggest that the inclusion of exon 2 is not coordinately regulated with splicing of exon 12.

1B236 Shares Primary Sequence with MAG. The properties of the 1B236 glycoprotein led us to compare this molecule with MAG, a 100-kDa glycoprotein known to consist of two polypeptides of 67 kDa and 72 kDa (14). Antisera against MAG (15) and 1B236 peptide P5 (2) both detect a 100-kDa band in a purified MAG sample and in brain but not liver or kidney extracts (Fig. 4). Antisera to 1B236 synthetic peptides P6 and P7 (2) gave similar results. With all three anti-peptide sera, reactivity was blocked by the synthetic peptide. Thus molecules defined as MAG contain 1B236 antigenic determinants.

The insert of p1B236-18, subcloned into pGEM-4, was used to generate an RNA copy of the sense strand. Two truncated 1B236 RNAs lacking different lengths of the C-terminal coding regions were generated by cleaving the template with restriction endonucleases (Fig. 5*A*). The copy RNAs were translated *in vitro*, and the products were immunoprecipitated with polyclonal anti-MAG sera (15), with monoclonal anti-MAG antibodies (17), or with anti-P5 sera directed against the C terminus of 1B236 (2) (Fig. 5*B*). The full-length transcript was translated to give a 70-kDa product that was

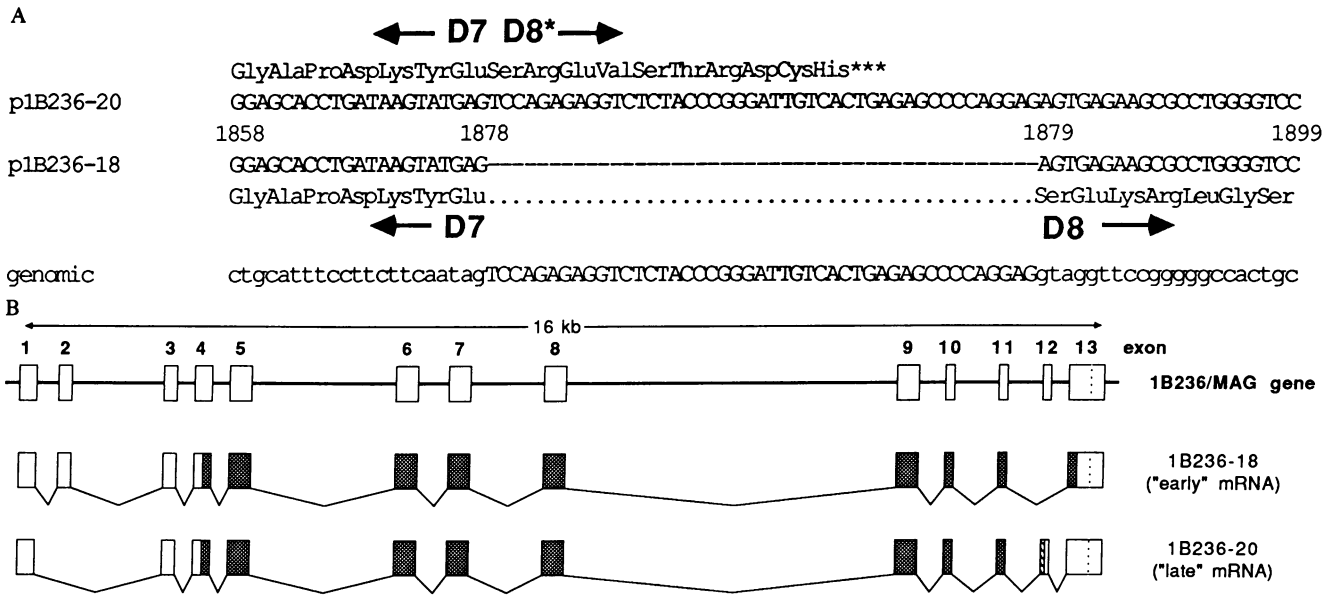

FIG. 2. Structures of alternatively spliced 1B236 mRNAs. (*A*) Comparison of cDNA and encoded amino acid sequences of clones 1B236-20 and 1B236-18, which differ by 45 nt located between positions 1878 and 1879 of the clone 18 sequence (Fig. 1). Sequence of genomic DNA is aligned below, demonstrating that the additional 45 nt (uppercase letters) form an exon flanked by introns (lowercase letters). D7 and D8 refer to the domains defined in Fig. 1; the different portion encoded by 1B236-20 is designated D8*. (*B*) The 1B236/MAG gene contains 13 exons spanning 16 kilobases. Clones 1B236-18 ("early" mRNA) and 1B236-20 ("late" mRNA, see Fig. 3) differ by alternate inclusion of exons 2 and 12. Protein-coding portions of exons are shaded; domains D0–D8 are encoded by exons 4–11 and 13; exon 12 of the "late" mRNA which encodes D8* is shaded differently to indicate the alternative C terminus. Dashed lines in exon 13 indicate that at least two sites are used for polyadenylylation. Sizes of the exons are exaggerated and are not strictly proportional.

precipitated by anti-P5 serum and the polyclonal and monoclonal anti-MAG antibodies, but not by control antiserum. The truncated RNAs were translated into products of 53 kDa and 36 kDa: each of these reacted with anti-MAG polyclonal but not with anti-P5 antibodies (Fig. 5*B*). Both anti-MAG monoclonal antibodies reacted with the 53-kDa product but not the 36-kDa product. Hence, the polyclonal anti-MAG antibodies react with antigenic determinants at least in the N-terminal region of the protein encoded by the cDNA clone 1B236-18, while both monoclonal antibodies react with determinants in regions D4 or D5 (defined below). Thus the 1B236 protein contains MAG epitopes not dependent on secondary modifications such as carbohydrate addition. Analogous results were obtained with the p1B236-20 insert subcloned into the pGEM-4 vector: as expected, the translation product was precipitated with anti-MAG and anti-P5 but not anti-P6 or -P7 antibodies.

DISCUSSION

We propose the following structure for the 1B236 proteins (Fig. 6). The predominant early postnatal form of the 1B236 polypeptide is 626 amino acids long; a later developmental product of the same gene has a length of 582 residues. Introns divide the 1B236 protein into several sections, arbitrarily designated as domains D0 to D8 (Fig. 1) and D8* (Fig. 2*A*). The two forms share D0–D7 and differ by either D8 or D8*. Both forms include up to eight N-linked carbohydrate side chains (the site in the putative cytoplasmic tail is probably not used). The N-terminal domain D0 resembles a signal peptide sequence (18); cleavage after alanine-16 would result in polypeptides of 610 and 566 residues with M_rs 67,710 and 62,680. A presumptive internal transmembrane domain (21 uncharged amino acids of D6 underlined in Fig. 1) separates the glycosylated N-terminal region from alternative C-terminal tails of 92 and 48 residues. The C-terminal tails contain sets of tandem basic amino acids that are targets for proteolysis (5). The sequence of D8 suggests that it could possibly be a substrate for other modifications such as phosphorylation.

The N-terminal region is likely to be extracellular. Thus our hypothesis (2) that peptides produced by C-terminal proteolysis of 1B236 might have "neurotransmitter-like" functions seems unlikely, given that the C terminus is probably cytoplasmic. Nevertheless, these peptides might have intracellular signaling functions. Alternatively, modifications of the C terminus by proteolytic cleavage or phosphorylation may modulate binding activity of the N terminus or alter the accessibility of the C terminus to cytoplasmic components. Generation of different C termini by alternative splicing (Fig. 2) emphasizes the potential functional importance of this region of 1B236/MAG.

Arquint *et al.* (19) have isolated a partial cDNA clone encoding a protein that reacts with anti-MAG antibodies and have shown that its sequence overlaps that of 1B236. The identity of the clone with MAG was established by amino acid sequence analysis of fragments from purified MAG. Those data and the results presented here demonstrate that, using the available immunological reagents, 1B236 and MAG are indistinguishable. Thus, 1B236 and MAG are highly related if not identical molecules. There are differences, however, in the known cellular distributions of 1B236 and MAG. In young rats, MAG is predominantly oligodendrocytic (20), and 1B236 protein and mRNA are expressed predominantly in oligodendrocytes (ref. 1 and 4; G. A. Higgins, H. Schmale, F.E.B., M. C. Wilson, and R.J.M., unpublished data). However, in adults, both *in situ* hybridization (G. A. Higgins, H. Schmale, F.E.B., M. C. Wilson, and R.J.M., unpublished data) and immunohistochemistry (6) show that 1B236 is largely, although not exclusively, neuronal, whereas by immunohistochemistry and radioimmunoassay MAG has been found only in oligodendrocytes and myelin sheaths (20–23). One possible explanation for this apparent paradox is as yet unrecognized RNA splicing events that affect the N-terminal region and generate a neuronal molecule lacking the epitopes recognized by some MAG antibodies. A second possibility is differential accessibility in neuronal and glial cells of these N-terminal MAG epitopes in immunohistochemistry and RIA experiments. Thus our work-

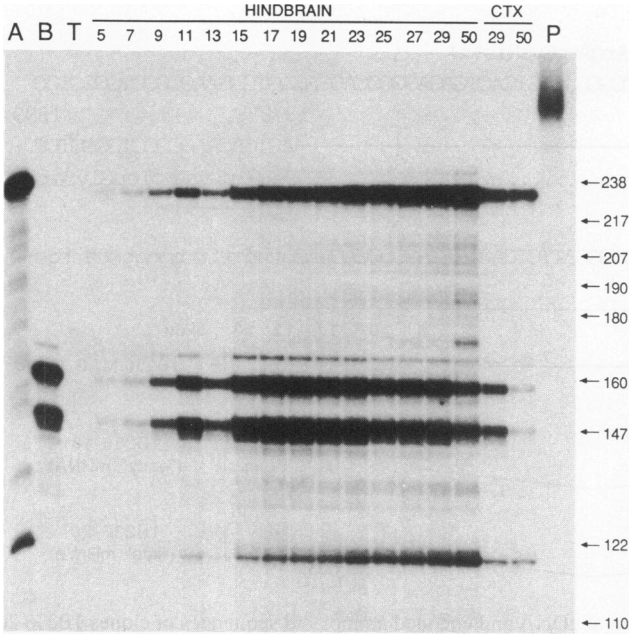

FIG. 3. Developmental expression of alternatively spliced forms of 1B236 mRNAs. Probe (lane P) was made by excising the 231-base-pair *Rsa* I restriction fragment containing exon 12 and flanking regions from p1B236-20 and subcloning it into the *Sma* I site of pT7/T3-19 (Bethesda Research Laboratories). The construct was linearized with *Eco*RI that cuts within the insert, 9 base pairs from its 5′ end. Transcription in the presence of [^{32}P]GTP using T7 polymerase gave a predicted 270-nt species (the insert and 39 nt of vector) that was mixed with RNA samples, digested with RNase A (50 μg/ml, 37°C for 30 min), and loaded onto 8% denaturing acrylamide gel. As controls, complete inserts of p1B236-18 and p1B236-20 were isolated by cleavage with *Bam*HI, subcloned into the *Bam*HI site of pGEM-4 (Promega Biotec, Madison, WI), linearized by digestion with Asp 718, and incubated with T7 RNA polymerase to produce sense-strand transcripts. Lane A shows two RNase A-protected species resulting from hybridization of probe with 1B236-20 RNA (contains exon 12) transcribed *in vitro*. A large band of ≈230 nt represents the fully-protected probe; the small band (120 nt) has not been accounted for. Lane B shows the protected species resulting from hybridization of probe with 1B236-18 RNA (no exon 12) transcribed *in vitro*. Lane T shows protection of probe by 5 μg of tRNA. Remaining lanes each show protection of probe by 1 μg of cytoplasmic poly(A)$^+$ RNA derived from either hindbrain (pons/medulla) postnatal days 5–50 or cortex (CTX) (telencephalon) days 29 and 50. Apparent low day-13 signal is due to less RNA in the hybridization.

ing hypothesis is that 1B236 and MAG are identical or nearly identical products of the single rat 1B236 gene; in mice, this gene has been mapped to within 1 centimorgan of the *Gpi-1* locus on chromosome 7 (C. Blatt, L. Weiner, J.G.S., M. N. Nesbitt, M. I. Simon, unpublished data). Until the exact relationship of 1B236 and MAG is known, we propose the provisional name 1B236/MAG.

The shared N-terminal region of 1B236/MAG (Fig. 6) consists of five, roughly equal-sized domains (D1–D5) that are related to each other in sequence and show highly significant alignments with immunoglobulin sequences, with the sequences of other members of the immunoglobulin super family (8) and with three molecules not previously recognized as immunoglobulin super family members: platelet-derived growth factor receptor (25), the product of the oncogene v-*fms* (26), and the peripheral myelin protein P_0 (27). The most striking similarities are found between 1B236/MAG and N-CAM (9). The short Cys-Cys distances of the five 1B236/MAG domains suggest that they fold into the 7-chain, disulfide-bonded, two-layer β-sheet structure characteristic of immunoglobulin constant regions rather than the 9-chain structure of variable regions (8).

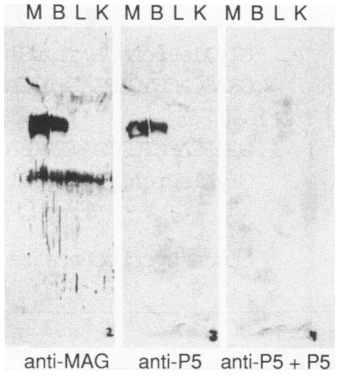

FIG. 4. Immunoblot analysis of 1B236 and MAG. Aliquots of purified MAG (lanes M, 150 ng) made as described (16) and of $NaDodSO_4$ extracts (50 μg) of adult rat brain (lanes B), liver (lanes L), and kidney (lanes K) separated on 7.5% polyacrylamide gels, transferred to nitrocellulose, and assayed with rabbit anti-MAG antiserum (15) (1:500 dilution), anti-P5 serum (2) (1:500 dilution), with or without peptide P5 at 50 μg/ml. Reactive band in all lanes of anti-MAG blot probably reflects detection of a common antigen by other antibodies in the serum and is unrelated to anti-MAG activity.

In particular, 1B236/MAG is highly related in primary sequence to N-CAM, which plays a critical role in neural development (28). N-CAM, L1/neuron–glia cell adhesion mol-

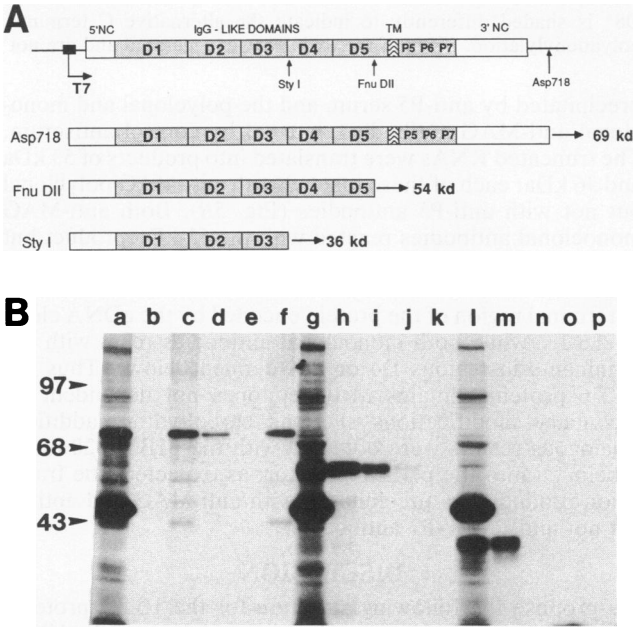

FIG. 5. Immunoprecipitation of *in vitro*-translated 1B236 proteins. (*A*) Schematic drawing illustrating structures of the protein products encoded by the T7 polymerase transcripts from 1B236 transcription templates. Cleavage with Asp 718 generates a transcript encoding the entire open reading frame. Cleavage with *Fnu*DII or *Sty* I generates truncated transcripts encoding proteins of the indicated molecular weight. NC, noncoding; TM, transmembrane; kd, kDa. (*B*) Transcripts were translated *in vitro* using a rabbit reticulocyte lysate system (Promega Biotec) with [^{35}S]methionine. Lanes a, g, and l; products from the full-length transcript, the *Fnu*DII truncate, and the *Sty* I truncate, respectively. Lanes b–f, h–k, m–p; immunoprecipitation of the products shown in lanes a, g, and l, respectively, when incubated with negative control rabbit serum to an unrelated synthetic peptide (lane b), polyclonal anti-MAG(R-31, ref. 15) (lanes c, h, and m), anti-MAG monoclonal B11F7 (17) (lanes d, i, and n), anti-MAG monoclonal D7E10 (17) (lanes e, j, and o), and anti-1B236-P5 (2) (lanes f, k, and p). Samples were fractionated on a 10% $NaDodSO_4$/polyacrylamide gel; positions of molecular size standards in kDa are indicated at left.

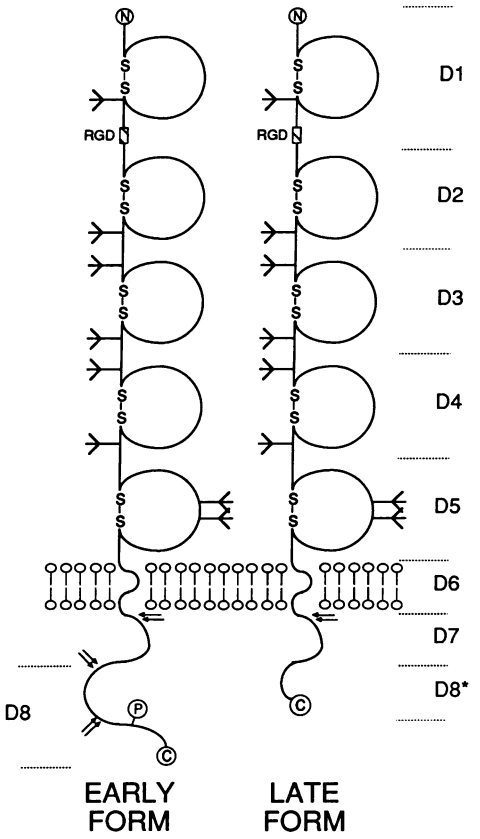

FIG. 6. Models of early and late expressed forms of the 1B236/MAG protein. Five immunoglobulin-like domains are indicated as disulfide-bonded loops and positions of carbohydrate side chains by branched structures. The Arg-Gly-Asp (RGD) sequence may be either in a loop that protrudes from "binding pocket" of domain 1 or in a linker region separating D1 from D2. Transmembrane domain D6 anchors the molecule in the cellular membrane. The two forms differ in the structures of their C termini. The early form contains a potential site (P) for tyrosine kinase phosphorylation within domain D8. Sites for proteolytic cleavage in the C-terminal tails are indicated by arrows.

ecule, and 1B236/MAG share carbohydrate determinants (7, 24, 29) and appear to act sequentially during peripheral myelination as cell adhesion molecules (12). In other neural cell interactions, N-CAM is thought to utilize homotypic reactions in mediating cell adhesion (28). As a cell adhesion molecule, 1B236/MAG may participate in similar homotypic interactions between oligodendrocytes or Schwann cells, particularly between adjacent surfaces of the myelinating cell membranes as these wrap around axons (10, 12). The ability of immunoglobulin domains to interact with each other in a homotypic or heterotypic manner (8) provides a possible structural explanation for the biology of these cell adhesion molecules. P_0 protein is also thought to react homotypically during peripheral myelin compaction (27). The finding that P_0 protein is immunoglobulin-like supports this hypothesis and suggests that a single-domain molecule such as P_0 protein could be similar in structure to the ancestral cell interaction molecule (8). 1B236/MAG may also be involved in heterotypic interactions: other members of the immunoglobulin family—for example, neuronally expressed OX-2, Thy-1, or N-CAM—are potential ligands. The sequence Arg-Gly-Asp, which has been specifically implicated in cell attachment (13), is located in D1 (Fig. 6) and could contribute to such interactions. The finding that 1B236/MAG is expressed in neurons suggests that it may be involved in neuronal as well as glial cell interactions.

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