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MUC18, a marker of tumor progression in human melanoma, shows sequence similarity to the neural cell adhesion molecules of the immunoglobulin superfamily

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ABSTRACT **The MUC18 antigen is an integral membrane glycoprotein of 113 kDa whose expression on primary human melanomas correlates with poor prognosis and the development of metastatic disease. MUC18 is expressed only sporadically in benign melanocytic nevi and thin primary melanomas that have a low probability of metastasizing. However, with increasing tumor thickness, MUC18 expression becomes more frequent and it is found on 80% of advanced primary tumors and metastases. MUC18-encoding cDNA clones were obtained by screening a human melanoma phage λ expression library with monoclonal antibodies produced against the denatured antigen. The deduced sequence of 603 amino acids consists of a signal peptide, five immunoglobulin-like domains, a transmembrane region, and a short cytoplasmic tail. The highest sequence similarity is with a group of nervous system cell adhesion molecules, which includes neural cell adhesion molecule (N-CAM). The close structural relationship with these molecules suggests that MUC18 may also be a developmentally regulated cell adhesion molecule.**

Malignant cells are not inherently metastatic but appear to develop this capacity during tumor progression as they acquire new characteristics through a multistep process of selection from continuously arising variants (1). Analysis of *in situ* phenotypic changes that correlate with the progression of cutaneous malignant melanoma has led to the identification of molecules that may contribute to the metastatic potential of this tumor (2–5). Two of these molecules are HLA-DR and intracellular adhesion molecule 1, which are not found on thin tumors that have a good prognosis but begin to be expressed on primary tumors ≥1 mm thick and appear to be coordinately expressed (6). A third melanoma antigen whose expression has been shown to correlate with tumor progression is the 113-kDa cell surface glycoprotein MUC18 (7). Although the expression of MUC18 is independent of HLA-DR and intracellular adhesion molecule 1, it is also not expressed on benign melanocytic lesions or thin tumors with a good prognosis. Here, we report the cDNA cloning and sequencing of MUC18 from melanoma cells.‡ The derived amino acid sequence indicates that MUC18 is a member of the immunoglobulin superfamily and shows the greatest sequence similarity to a group of neural cell adhesion molecules expressed during organogenesis. On the basis of this sequence similarity, we speculate that MUC18 may also be developmentally regulated and mediate intercellular adhesion. Since the expression of MUC18 in normal adult tissues appears limited to vascular smooth muscle (7), expression of this molecule by melanoma cells may contribute to their interaction with elements of the vascular system, an essential step in the dissemination of tumor cells.

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MATERIALS AND METHODS

Antibodies and Cells. Monoclonal antibody (mAb) MUC18 (IgG2a) has been described (7) and mAb 96.5 (anti-melanotransferrin, ref. 8) was kindly provided by I. Hellström (Oncogen, Seattle), and the myeloma protein UPC10 (IgG2a) was purchased from Sigma. Cell lines were obtained from the American Type Culture Collection, through exchange, or were established in our laboratory.

Immunohistochemistry and Flow Cytometry. Indirect immunoperoxidase staining on frozen tissues was performed as described (7). Tissues were regarded as positive when ≥5% of the tumor cells were stained. The following numbers of primary melanomas were examined: 17 at ≤0.75 mm and 15 at 0.76–1.5 mm, 15 at 1.51–3.0 mm, and 15 at ≥3.0 mm. For flow cytometry, cells were incubated with mAb followed by fluorescein isothiocyanate-conjugated second antibody and analyzed using a FACScan (Becton Dickinson).

Selection of the Antibodies for Western Blot Analysis. mAbs MUCBA18.1–18.5 (all IgG1) were produced from a (C57BL/6 × BALB/c)F_1 mouse injected i.p. with 100 mg of lipid A (9) followed 14 days later by heat-denatured immunoprecipitated MUC18 in 0.1% SDS. Three injections of immunoprecipitates from 3×10^9 Mel-JuSo cells with Freund's complete adjuvant, Freund's incomplete adjuvant, and *Bordetella pertussis* adjuvant, respectively, were spaced over 6 weeks. The spleen cells were fused with P3×63Ag8.63 myeloma cells and culture supernatants were screened on MUC18 glycoprotein-loaded nitrocellulose strips. Immunoprecipitates from Mel-JuSo cells (5×10^8 cells per cm of nitrocellulose) were separated by SDS/PAGE under reducing conditions and electrophoretically transferred to nitrocellulose membranes (BA85; Schleicher & Schüll). The nitrocellulose strips were incubated with the mAb followed by peroxidase-conjugated rabbit antiserum to mouse immunoglobulins (Dakopatts, Copenhagen) as described (4).

Isoelectric Focusing and Peptide Mapping. Cells were labeled using lactoperoxidase–glucose oxidase-catalyzed surface iodination (10) and immunoprecipitates were prepared as described (7). Isoelectric focusing was performed essentially as described by Neefjes *et al.* (11). Peptide mapping by partial proteolysis in gel slices (12) was performed using 100 ng of *Staphylococcus aureus* V8 protease (Sigma).

cDNA Libraries and DNA Sequencing. cDNA expression libraries were constructed in the λgt11 and λZAP vectors (Strategene). cDNA was prepared from Mel-JuSo cells (cDNA synthesis system; BRL) and size-selected [≥1200 base pairs (bp)] by agarose gel electrophoresis. Fusion proteins were induced, blotted on nitrocellulose filters, and

Abbreviation: mAb, monoclonal antibody.

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‡The sequence reported in this paper has been deposited in the GenBank data base (accession no. M28882).

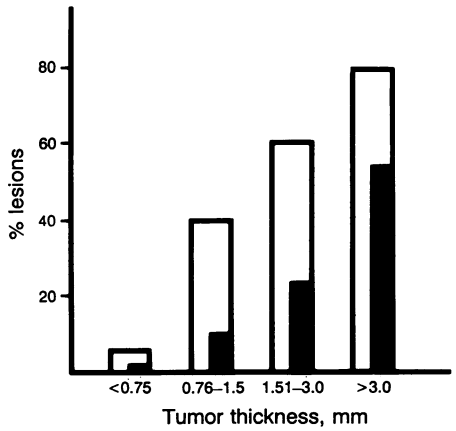


FIG. 1. Reaction pattern of mAb MUC18 with primary melanoma *in situ*. Open bars, percentage of lesions that contained ≥5% stained tumor cells; solid bars, 5-year mortality rate in each group (17).

screened with mAbs MUCBA18.1–18.5 (13). Reactive plaques were detected using peroxidase-coupled second antibody. For sequencing, suitable restriction fragments were subcloned in pUC18 and double-stranded cDNA was sequenced using the dideoxynucleotide chain-termination method (14) with Sequenase (United States Biochemical).

Northern and Southern Blot Analysis. RNA was denatured in formaldehyde, separated on a 1% agarose/formaldehyde gel (15), and transferred to Hybond-N (Amersham). The 1200-bp *Sst* I–*Pst* I cDNA fragment labeled with [α-^{32}P]dCTP (Amersham) by random-priming was used as probe. Membranes were hybridized overnight at 65°C in 6× SSC/5× Denhardt's solution/0.5% SDS/salmon sperm DNA (20 μg/ml). (1× SSC = 0.15 M NaCl/0.015 M sodium citrate, pH 7.0; 1× Denhardt's solution = 0.02% polyvinylpyrrolidone/0.02% Ficoll/0.02% bovine serum albumin.) The filters were washed at 65°C in 3× SSC/0.1% SDS, 1× SSC/0.1% SDS, 0.3× SSC/0.1% SDS, and 0.1× SSC/0.1% SDS. DNA was digested with restriction endonucleases, electrophoresed, transferred to Hybond-N nylon membranes, and hybridized as described above.

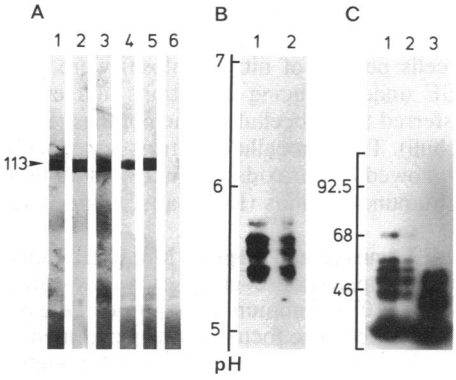
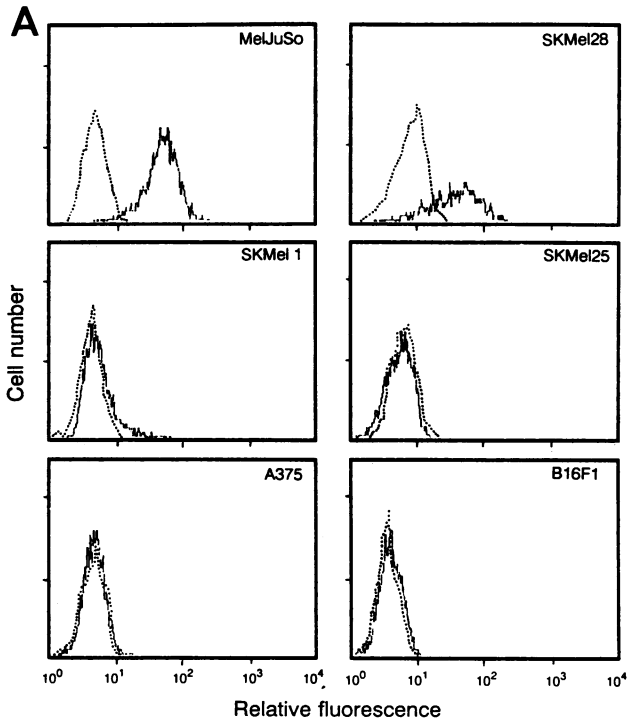


FIG. 2. Biochemical comparison of the molecules recognized by mAbs MUC18 and MUCBA18.1–18.5. (*A*) Immunoblots. The blotted MUC18 immunoprecipitates were probed with mAbs MUCBA18.1 (lane 1), MUCBA18.2 (lane 2), MUCBA18.3 (lane 3), MUCBA18.4 (lane 4), MUCBA18.5 (lane 5), and UPC10 (lane 6). (*B*) Isoelectric focusing banding pattern of the MUC18 immunoprecipitate (lane 1) and the MUCBA18.1 immunoprecipitate (lane 2). Immunoprecipitates were prepared from Mel-JuSo cells labeled by surface iodination. The pH gradient is indicated on the left. (*C*) Peptide mapping. MUC18 immunoprecipitate (lane 1), the MUCBA18.1 immunoprecipitate (lane 2), and, as control, the melanotransferrin immunoprecipitate (p97, lane 3) were partially digested with 100 ng of *Staphylococcus aureus* V8 protease. Immunoprecipitates were prepared from Mel-JuSo cells labeled by surface iodination. The locations of molecular mass standards are indicated on the left in kDa.



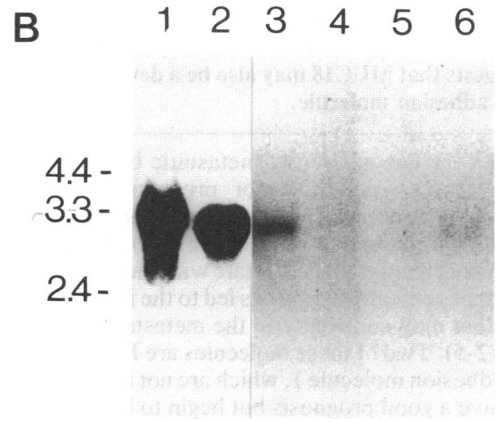


FIG. 3. Flow cytometric analysis of the MUC18 glycoprotein expression and Northern blot analysis. (*A*) Immunofluorescence staining of the melanoma cell lines Mel-JuSo, SK-Mel-28, SK-Mel-1, SK-Mel-25, A375, and B16F1 by mAb MUC18 (dashed lines) and isotype control UPC10 (dotted lines). (*B*) Total RNA (20 μg) from the melanoma cell lines Mel-JuSo (lane 1), SK-Mel-18 (lane 2), SK-Mel-1 (lane 3), SK-Mel-25 (lane 4), A375 (lane 5), and B16F1 (lane 6) was electrophoresed, blotted on nylon membranes, and hybridized with the ^{32}P-labeled MUC18 cDNA probe.

RESULTS AND DISCUSSION

Correlation of MUC18 Expression with the Development of Human Metastatic Melanoma. mAb MUC18 produced against human malignant melanoma was selected because it does not react with benign melanocytic lesions. In contrast 45% of the 62 primary melanomas and 71% of the 31 metastatic melanomas examined expressed the MUC18 antigen (7). The vertical thickness of primary melanomas (the Breslow index) is directly correlated with the probability of metastatic disease (16). Division of the primary melanomas into four evenly

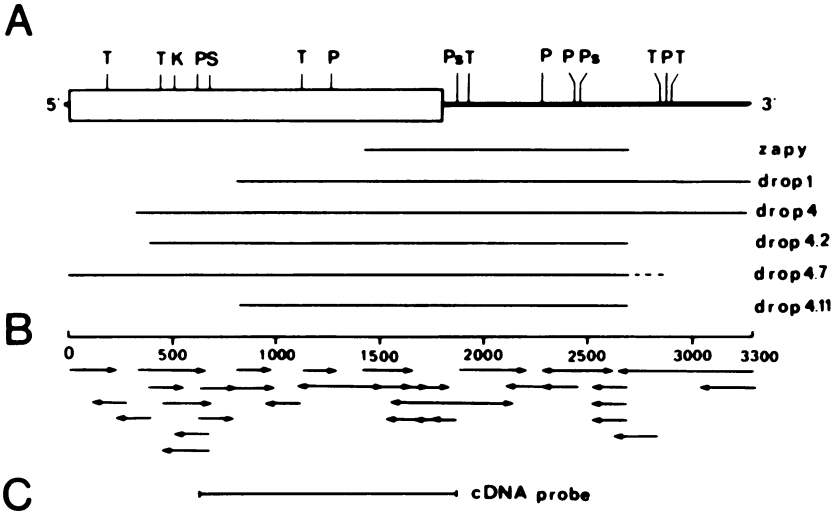


FIG. 4. Structure of the MUC18 glycoprotein mRNA and relationship between the cDNA clones (*A*), sequencing strategy (*B*), and localization of 1300-bp *Sst* I–*Pst* I MUC18 cDNA probe used for Northern and Southern blot analysis (*C*). Three cDNA clones, zapy1, drop1, and drop4, were isolated by using the antibodies used in Western blot analysis as probes and the clones drop4.2, drop4.7, and drop4.11 were isolated by using a ^{32}P-labeled MUC18 cDNA probe derived from the 5′ end of clone drop4. The divergent nucleotide sequence of drop4.7 in the 3′ region is indicated by dashed line. The 1809-bp open reading frame is indicated by the open box. Various restriction sites were used to generate subclones for sequencing. P, *Pvu* II; T, *Taq* I; Ps, *Pst* I; K, *Kpn* I; S, *Sst* I.

matched groups according to their vertical thickness reveals that expression of MUC18 shows a linear correlation with this parameter (Fig. 1). The association of MUC18 expression with tumor thickness suggests that the appearance of this molecule reflects changes in the tumor cells that may contribute to the development of metastatic capacity.

Characterization of the Antibodies for Western Blot Analysis. To isolate MUC18-encoding cDNA clones, five mAbs (MUCBA18.1–18.5) reactive with the denatured MUC18 glycoprotein were produced (Fig. 2*A*). These mAbs precipitated a single protein of 113 kDa from surface-labeled Mel-JuSo cells (data not shown). In addition, MUC18 and MUCBA18.1 immunoprecipitates had identical isoelectric points and the same pattern of microheterogeneity (Fig. 2*B*) as well as identical peptide maps (Fig. 2*C*), indicating that both antibodies recognize the same molecule.

Isolation of MUC18 cDNA Clones and Identification of MUC18 mRNA. Three immunopositive cross-hybridizing clones were isolated from two human melanoma cDNA λ expression libraries. In addition to reactivity with mAbs

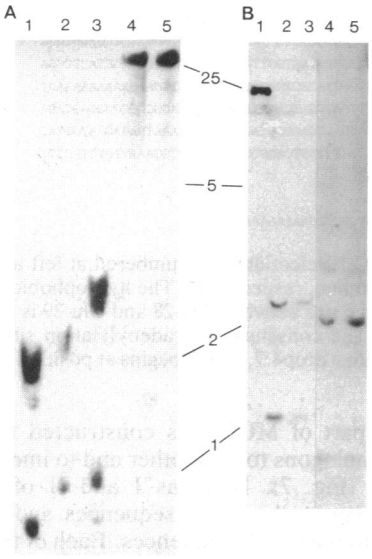


FIG. 5. Southern blot analysis. (*A*) Genomic DNA from Mel-JuSo cells was digested with *Taq* I, *Pvu* II, *Pst* I, *Eco*RI, and *Bgl* II (lanes 1–5, respectively). (*B*) Genomic DNA from peripheral blood lymphocytes of a normal donor was digested with *Eco*RI (lane 1). Genomic DNA from Mel-Ho cells (lanes 2 and 4) and the autologous B-lymphoblastoid cell line (lanes 3 and 5) were digested with *Pvu* II (lanes 2 and 3) or *Taq* I (lanes 4 and 5) and hybridized to the ^{32}P-labeled MUC18 cDNA probe.

MUCBA18.1, -18.2, -18.3, and -18.5, the 3.3-kilobase mRNA detected by these clones demonstrates an expression pattern in human melanoma cell lines that mirrors exactly the binding of the MUC18 mAb (Fig. 3). High levels of MUC18 antigen and the 3.3-kilobase mRNA were seen in Mel-JuSo and Sk-Mel-28 cells, whereas lower levels were seen in Sk-Mel-1 cells and no products were detected in Sk-Mel-25 and A375 cells. The detection of a 3.3-kilobase mRNA in the mouse melanoma cell line B16F1 suggests that the MUC18 gene may be conserved across species, although the particular epitopes defined by the MUC18 antibodies are absent.

Inspection of the cDNA sequence revealed that it was incomplete in the 5′ region. Therefore, the melanoma library was screened with a cDNA probe derived from the 5′ end of clone drop4. Three additional clones were identified and the six overlapping clones correspond to the size of the observed mRNA. The relationship of the various cDNA clones is schematically depicted in Fig. 4*A*.

Analysis of Mel-JuSo DNA digested with various restriction endonucleases showed a pattern consistent with a single-copy gene, as only a single hybridization fragment was obtained (Fig. 5*A*). Comparison of the restriction patterns in melanoma and autologous B-lymphoblastoid cell lines from different patients provides no indication that the gene is rearranged in melanomas or polymorphic in the population (Fig. 5*B*).

Nucleotide and Amino Acid Sequence. The MUC18 cDNA sequence (Fig. 6*A*) reveals a 7-bp 5′ untranslated region, a single open reading frame of 1809 bp, and a 1487-bp 3′ untranslated region, which includes the consensus polyadenylylation signal (AATAAA) at position +3264 followed 15 bases later by a poly(A) tail.

In contrast to this sequence, the cDNA clone drop4.7 contains an unrelated 92-bp 3′ untranslated region that begins at position +2847 (Fig. 6*B*) and lacks the consensus polyadenylylation signal. If not a cloning artifact this cDNA clone may indicate that the expression of the MUC18 molecule is regulated by the production of mRNAs with different 3′ untranslated regions (18).

The translation initiation site was assigned to the first ATG codon of the single open reading frame at position +1, which is surrounded by a nucleotide sequence corresponding to the consensus sequence proposed for putative initiation codons (GGAAGCATGG) (19, 20). Hydrophobicity analysis (21) suggests that this sequence is succeeded by a 28-residue signal peptide sequence, which may be cleaved between alanine-28 and glutamic acid-29 (22). The open reading frame ends with the termination codon TAA at position +1810. Thus, the mature MUC18 molecule appears to consist of 575 amino acid

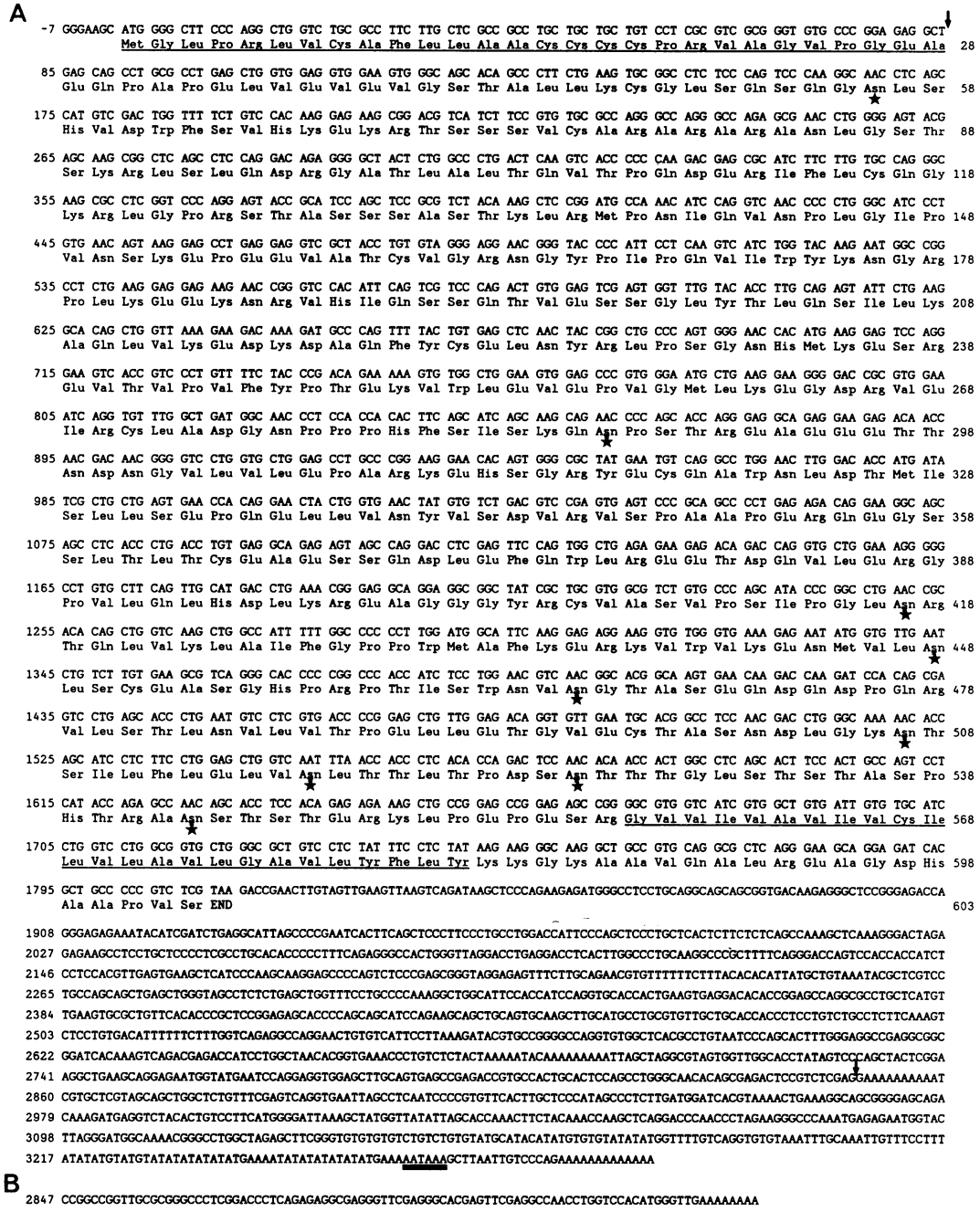


FIG. 6. Nucleotide and deduced amino acid sequence of the MUC18 glycoprotein. (*A*) Nucleotides are numbered at left and amino acids are at right of the sequence, starting with the initiation codon and the corresponding methionine, respectively. The hydrophobic putative signal peptide and transmembrane sequences are underlined. The potential signal peptide cleavage site between Ala-28 and Glu-29 is marked with an arrow. Nine potential asparagine-linked glycosylation sites are indicated by asterisks. The consensus polyadenylylation site AATAAA at position +3264 is marked with a bold line. (*B*) Divergent 3′ nucleotide sequence of cDNA clone drop4.7, which begins at position +2847 (marked with an arrow in *A*).

residues with a calculated molecular weight of 63,195. This is in excellent agreement with the observed size of 65 kDa for the deglycosylated MUC18 (glycopeptidase F digestion, data not shown). The deduced amino acid sequence includes a second stretch of 24 hydrophobic residues characteristic for a membrane-spanning region and eight sites for putative asparagine-linked glycosylation (Asn-Xaa-Ser/Thr) that are located in the extracellular region of the molecule. Due to proline in the Asn-Pro-Ser sequence at positions 286–288, this site will not be used for glycosylation (23).

MUC18 Is a Member of the Immunoglobulin Superfamily. The MUC18 molecule fulfills all criteria proposed for membership in the immunoglobulin superfamily (24). The entire extracellular part of MUC18 is constructed from five domains, all homologous to each other and to immunoglobulin-like domains (Fig. 7). Domains I and II of the MUC18 molecule belong to the V-set sequences and the domains III–V belong to the C2-set sequences. Each of the domains is approximately 100 amino acids long and contains two appropriately spaced cysteines as well as conserved residues flanking the cysteines. The observed change in the apparent molecular mass from 113 kDa under reducing conditions to 100 kDa under nonreducing conditions in SDS/PAGE (7) strongly suggests the presence of intrachain disulfide bonds in the MUC18 molecule. The algorithm of Chou and Fasman (25) to predict protein secondary structure indicates predom-

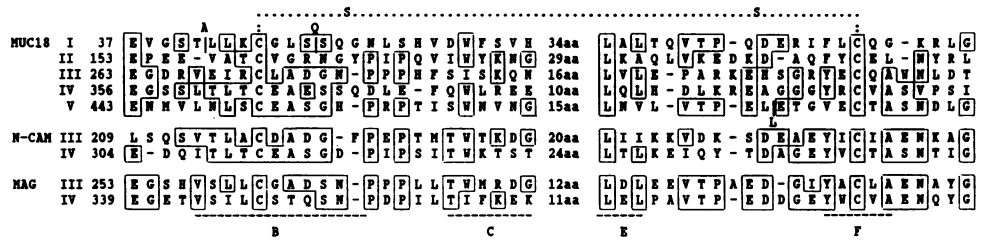


FIG. 7. Sequence homology between five repeated domains in the MUC18 molecule and relationship to two immunoglobulin-like domains of neural cell adhesion molecule (N-CAM) and myelin-associated glycoprotein (MAG). Sequences were visually aligned to show maximum internal homology. Boxed amino acids correspond to residues shared by three or more sequences. The middle portion of each domain was omitted for simplicity, and the number of amino acids not shown is indicated. The position of the putative disulfide bond is indicated by S . . . S. The location of the known β-strands in immunoglobulin domains is marked below the aligned sequences with dashed lines. The single-letter amino acid sequence is used.

inantly β-strands in each of the MUC18 domains, fulfilling the prediction for an immunoglobulin domain.

A search of the Max Planck and Martinsried Institute Protein Sequence Data Bank (MIPSZ; release 9) using the FASTP program (26) detected no significant similarities outside the immunoglobulin superfamily. Among the immunoglobulin-related molecules, MUC18 shows the greatest similarity to the cell adhesion molecules of the nervous system neural cell adhesion molecule (N-CAM) (27), L1 (28), amalgam (29), fasciclin II (30), contactin (31), and myelin-associated glycoprotein (MAG) (32) and to human carcinoembryonic antigen (CEA) (33), which has also been shown to mediate cell adhesion (34) (Table 1). The immunoglobulin-related molecules are thought to have been derived by gene duplication and divergence from an archetypal gene. Within the superfamily, some molecules show greater than average similarity suggesting a more recent common origin. One such group consists of the neural cell adhesion molecules that share functional as well as structural properties. The close structural relationship of MUC18 to these adhesion molecules predicts that MUC18, like these molecules, may also be developmentally regulated and involved in intercellular adhesion. Melanocytes are derivatives of the neural crest and MUC18 expression by melanomas may reflect a stage in their ontogeny. If the MUC18 molecule is indeed involved in adhesive interactions, its expression may allow melanoma cells to interact with cellular elements of the vascular system (where MUC18 is also expressed) thereby enhancing hematogeneous tumor spread.

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Table 1. Proteins with sequence similarity to MUC18 glycoprotein

Molecule	Identity, %	Overlap amino acids, no.	Optimized score	SD value
N-CAM	25.5	133	133	22.1
CEA	20.2	297	160	19.7
L1	22.6	288	172	17.2
Amalgam	26.0	169	133	15.4
Fasciclin II	16.2	229	132	14.7
Contactin	22.2	117	79	13.3
MAG	21.8	362	130	11.9

Comparisons were performed using the FASTP and RELATE programs (26). The optimized score is obtained when one takes deletions and insertions into account (that is, gaps). It is the usual output from the FASTP program and is discussed in ref. 26. SD values greater than 10 are considered to indicate significant sequence homology. N-CAM, neural cell adhesion molecule; CEA, carcinoembryonic antigen; MAG, myelin-associated glycoprotein.

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