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A Human Gene Coding for a Membrane-associated Nucleic Acid-binding Protein*

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Studies to clone a cell-surface DNA-binding protein involved in the binding and internalization of extracellular DNA have led to the isolation of a gene for a membrane-associated nucleic acid-binding protein (MNAB). The full-length cDNA is 4.3 kilobases with an open reading frame of 3576 base pairs encoding a protein of ~130 kDa (GenBank accession numbers AF255303 and AF255304). The MNAB gene is on human chromosome 9 with wide expression in normal tissues and tumor cells. A C3HC4 RING finger and a CCCH zinc finger have been identified in the amino-terminal half of the protein. MNAB bound DNA (K_D ~4 nM) and mutagenesis of a single conserved amino acid in the zinc finger reduced DNA binding by 50%. A potential transmembrane domain exists near the carboxyl terminus. Antibodies against the amino-terminal half of the protein immunoprecipitated a protein of molecular mass ~150 kDa and reacted with cell surfaces. The MNAB protein is membrane-associated and primarily localized to the perinuclear space, probably to the endoplasmic reticulum or trans-Golgi network. Characterization of the MNAB protein as a cell-surface DNA-binding protein, critical in binding and internalization of extracellular DNA, awaits confirmation of its localization to cell surfaces.

The interaction of extracellular DNA with the plasma membrane, and its subsequent internalization into the cell, can trigger specific cellular events. For instance, gene therapy involves the cellular binding and uptake of DNA as an intact molecule, with subsequent overexpression of the gene product(s). In some cases this overexpression can result in the stimulation of host immune responses as seen with DNA vaccines (1–3). Recently, there has been evidence demonstrating the immunostimulatory activity of bacterial DNA. The binding and internalization of bacterial DNA can initiate a variety of responses, both harmful as well as beneficial. Bacterial DNA has been reported to induce potentially harmful inflammation in the lung (4–8) and to activate macrophages with the subsequent release of tumor necrosis factor-α and interleukin-1α (9). In contrast, the immunostimulatory motif found in bacterial DNA (CpG) has been shown to mediate a number of beneficial effects. Hartman and colleagues (10) have shown that CpG motifs can activate human dendritic cells, implying a possible role in dendritic cell-mediated immune responses. In addition, the role of CpG motifs as adjuvants has recently been reported (11, 12). Binding and internalization of exogenous DNA are both required for initiation of these events.

The mechanism(s) that mediate the cellular binding and internalization of exogenous DNA remains, for the most part, undefined at the molecular level. There are numerous reports of the association of different forms of nucleic acids, such as DNA, RNA, and oligonucleotides, with cell surfaces (13–19) as well as reports of cellular binding and internalization of nucleic acids (20–26) to a variety of cell types. Oligonucleotides are bound and internalized by cells (19, 27). Whether oligonucleotides are internalized via a receptor-mediated endocytic pathway, a pinocytic mechanism, or a combination of both has not been resolved. Loke *et al.* (28) reported the uptake of unmodified oligonucleotides was a saturable phenomenon that was competitively inhibited by unlabeled oligonucleotide, double or single stranded DNA, unrelated oligonucleotides, or RNA, suggesting a receptor-mediated endocytic pathway. A possible endocytic pathway for oligonucleotide uptake was described by Yakubov *et al.* (29) but it appeared that pinocytosis was the preferred pathway at a high concentration of oligonucleotides. Recently, the involvement of the macrophage-1 cell-surface molecule as a receptor for oligonucleotide binding has been reported (30). However, whether binding of oligonucleotides to macrophage-1 is the relevant mechanism by which DNA activates cells is unknown. In addition, the mechanism(s) for oligo and/or plasmid DNA binding and internalization by cells lacking macrophage-1 has yet to be established. Other studies substantiate the notion that cells possess specific cell-surface proteins that mediate nucleic acid binding and internalization. Bennett *et al.* (31) provided evidence that cell surface binding of high molecular weight DNA by human leukocytes was mediated by membrane-associated proteins with subsequent uptake and degradation of internalized DNA. Emlen and colleagues (32), working with hepatic cells, have demonstrated saturable DNA binding, suggesting a receptor-mediated process and Kawabata and colleagues (33) showed rapid uptake of plasmid DNA by the liver. Takagi and colleagues (34) demonstrated plasmid DNA binding and internalization by murine peritoneal

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AF255303 and AF255304.

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macrophages. However, characterization of the receptor(s) which mediate the binding and internalization of plasmid DNA remains undefined at the molecular level.

The molecular characterization of the cell-surface receptor(s) involved in the binding and internalization of DNA will provide the basis for the potential regulation of DNA binding to cells and the selective enhancement or diminishment of the biological effects of DNA. To facilitate the identification of this cell-surface receptor(s), we have demonstrated that DNA binding to cell surfaces could be inhibited by pretreatment of cells with serum from patients with SLE[1] (35–37). Inhibition of DNA binding to cells was shown to be antibody-mediated and to be distinct from anti-DNA antibodies. We used a serum that inhibited DNA binding to cell surfaces and reacted to cell-surface DNA-binding proteins (21) to screen a λgt11 cDNA expression library derived from human monocytes. A novel membrane-associated protein of molecular mass ~130 kDa that binds DNA was identified. The molecular characterization and DNA binding properties of this molecule are detailed in this report.

MATERIALS AND METHODS

Cloning of MNAB—Serum from a patient with SLE was used to screen a λgt11 cDNA expression library from lipopolysaccharide stimulated human monocytes (CLONTECH). This serum was anti-nuclear antigen positive, had been depleted of anti-DNA antibodies by multiple (×6) passages over a DNA-Sepharose column, and had previously been shown to inhibit DNA binding to cell surfaces (21). Approximately 10^6 plaques were screened and 10 positive clones were identified and isolated according to the technique of Young and Davis (38). Two clones were sequenced by dideoxy chain termination with 7-deaza-2′-deoxyquanosine-5′-triphosphate (39). Clone 88 (1.4 kilobases) was investigated further. A 731-bp DNA probe (probe 11) from clone 88 was used to screen a λgt11 phage library from a Raji cell line (CLONTECH Labs). A 2409-bp clone termed 97d42 was purified. It contained 1197 bp of clone 88 and 1212 bp of a new open reading frame sequence. Additional 5′ sequence was obtained using PCR. RNA from HeLa cells was reverse transcribed into single stranded DNA and a poly(A) tail added to the 5′ ends. A PCR product (clone 30F18) was synthesized with gene specific and poly(T) primers. It had a 77-bp overlap with the 5′ end of clone 97d42 and 753 bp of new 5′ sequence containing a contiguous open reading frame with clone 97d42. The remainder of the 5′ sequence was obtained from MOLT-4 cDNA by Marathon cDNA amplification (CLONTECH) according to the manufacture's instructions. This generated the final 1290-bp clone 141F13 containing 340 bp of new 5′ open reading frame and 950 bp of 5′-untranslated region. The full-length coding sequence (3576 bp) was cloned as a single PCR product from MOLT-4 cDNA. The PCR primers for this reaction (5′-ACCCGAGCATGGATCCGCCACC**ATG**GCTGTGCAGGCAGC-3′ and 5′-GGTATCTAGATCCATGGTGTGGTCAC-3′) introduced a *Bam*HI site (underlined) into the 5′ end and an *Xba*I site (underlined) at the 3′ end. In addition, three base changes were introduced to create a more frequently observed Kozak sequence around the start codon: TCCACA**ATG**C to GCCACC**ATG**G. This also resulted in changing the second codon in the gene from CCT (coding for proline) to GCT (coding for alanine) (40).

Northern Analysis—Human MTN Blots (CLONTECH) were probed with a ^{32}P-labeled 442-bp *Eco*RV-*Nco*I restriction fragment from the extreme 3′ end of the coding sequence for the MNAB gene. The blots were prehybridized for 6 h at 42 °C in 5 × SSPE (20 × SSPE contains 3 M NaCl, 0.2 M $NaH_2PO_4·H_2O$, 0.02 M Na_2EDTA), 10 × Denhardt's solution (50 × contains 1% Ficoll 400, 1% polyvinylpyrrolidone, 1% bovine serum albumin fraction V), 100 μg/ml denatured salmon sperm DNA, 50% deionized formamide, and 2% SDS. The random primed ^{32}P-labeled probe (1–2 × 10^6 cpm/ml) was denatured for 4 min at 95 °C and added to fresh prewarmed (42 °C) prehybridization buffer. Hybridization was carried out for 18 h at 42 °C with gentle shaking. The blots were washed under low stringency conditions in 0.1 × SSC (20 × contains 3 M NaCl, 0.3 M Na_3 citrate·$2H_2O$), 0.1% SDS and exposed to x-ray film.

Production of a Soluble MNAB Fusion Protein—A *Bam*HI-*Eco*RI fragment of the cDNA coding for amino acids 1–575 (including the ring and zinc finger motifs) of MNAB was cloned in-frame into pGEX-6-P (Amersham Pharmacia Biotech, Piscataway, NJ). The resulting vector was named pGEX-MNAB and the fusion protein expressed from this vector was named GST/MNAB. Expression of GST fusion protein in *Escherichia coli* DH5α was induced with 1 mM isopropyl-β-D-thiogalactopyrannoside for 2–4 h at 37 °C. The fusion protein was purified from lysates by chromatography on glutathione-Sepharose (Amersham Pharmacia Biotech) and eluted from the column with 15 mM reduced glutathione (Sigma), 50 mM Tris-HCl, pH 8.0. Alternatively, the fusion protein was cleaved *in situ* with Prescission protease (Amersham Pharmacia Biotech) to release the MNAB peptide from the GST peptide essentially according to the manufacturer's instructions. The released peptide was desalted and concentrated (Centricon 30; Amicon, Beverly, MA), then incubated with glutathione-Sepharose to remove traces of GST or GST fusion proteins. GST fusion proteins used as controls in DNA binding assays (GST/CBD, GST/HST.1) were provided by Dr. Rowland Kwok, University of Michigan, Ann Arbor, MI. Proteins were stored at −80 °C.

Expression Vector for an Epitope-tagged MNAB—A *Bam*HI-*Hpa*I cDNA fragment containing the coding sequence for amino acids 1–1190 (missing the two most carboxyl-terminal amino acids) of the MNAB was subcloned into pTriplFlu (constructed by Dr. John Epstein, University of Pennsylvania, Philadelphia, PA). The vector contains the sequence coding for an epitope tag from the influenza virus HA in triplicate inserted immediately 3′ of the multicloning site in pcDNA3 which are in-frame with the inserted MNAB cDNA sequence.

Production of Antibodies to MNAB—Polyclonal antibodies were produced to the purified GST/MNAB peptide from which the GST fusion partner had been removed by site-specific proteolysis. Female New Zealand White rabbits (2.3–3.0 kg) were injected subcutaneously with 60 μg of purified MNAB-(1–575) peptide emulsified 1:1 with TitreMax (CytRx Corp., Norcross, GA) in a final volume of 0.5 ml. The rabbits were boosted 4 weeks later with 15 μg of antigen/Titer-Max mixture, again 2 weeks later, and were maintained on a monthly intramuscular injection schedule thereafter. The final 2 injections consisted of a 3–5-μg antigen challenge. The rabbits were bled 7–10 days after each immunization and the sera analyzed for reactivity to the immunizing antigen. Sera were collected from each of the rabbits prior to immunization with the MNAB peptide and stored at −20 °C. All immune sera reacted against the immunizing peptide as assessed by ELISA and Western blot analysis.[2] This sera is referred to as anti-MNAB.

Other Antibodies—Antibody to the human transferrin receptor was provided by Dr. Caroline Enns, OHSU, Portland, OR. Monoclonal anti-HA (12CA5) was purchased from Roche Molecular Biochemicals (Indianapolis, IN). Monoclonal anti-γ-tubulin and rabbit anti-GST were from Sigma.

Cell Culture and Transfections—Cells were routinely cultured in Dulbecco's modified Eagle's medium (Life Technologies, Gaithersburg, MD) supplemented with 10% fetal bovine serum (HyClone, Logan, UT), 2 mM L-glutamine, 100 units/ml penicillin G, 100 μg/ml streptomycin. Clones of 293 cells stably expressing HA-tagged MNAB were established by transfection with pTriplFlu-MNAB using LipofectAMINE (Life Technologies) and were selected with 500 μg/ml G418 (Life Technologies). G418-resistant cells were cloned by limiting dilution and tested for expression of HA-tagged MNAB by Western blotting.

Western Analysis—Lysates of cells were prepared on ice in lysis buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100) plus a protease inhibitor mixture (Sigma P8340, used at 1:100). Lysates were cleared by centrifugation at 20,000 × *g*, and the protein contents were estimated by Coomassie dye binding (Bio-Rad). Lysates were stored at −80 °C. Cells were fractionated into cytoplasmic and crude membrane fractions by Dounce homogenation on ice in hypotonic buffer (10 mM HEPES, pH 7.5, 5 mM KCl, 1.5 mM $MgCl_2$, protease inhibitor mixture), followed by sequential centrifugation at 10,000 × *g* for 20 min then 100,000 × *g* for 60 min at 4 °C. The supernatant and pellet from the latter centrifugation were considered cytoplasmic and membrane fractions, respectively. Lysates, fractionations (typically 100 μg), and immunoprecipitates were heated at 85 °C in 2 × sample buffer (50 mM Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, 5% 2-mercaptoethanol)

[1] The abbreviations used are: SLE, systemic lupus erythematosus; MNAB, membrane-associated nucleic acid-binding protein; PCR, polymerase chain reaction; PBS, phosphate-buffered saline; GST, glutathione *S*-transferase; HA, hemagglutinin; ELISA, enzyme-linked immunosorbent assay; PAGE, polyacrylamide gel electrophoresis; bp, base pair(s); EST, expressed tag sequence; TRAF, tumor necrosis factor receptor-associated factor.

[2] D. Siess, C. T. Vedder, L. S. Merkens, T. Tanaka, A. C. Freed, S. L. McCoy, M. C. Heinrich, M. E. Deffebach, R. M. Bennett, and S. H. Hefeneider, unpublished observations.

for 10 min, then separated by 7% SDS-polyacrylamide gel electrophoresis and electrophoretically transferred to nitrocellulose (Hybond ECL, Amersham Pharmacia Biotech). The membranes were stained with Ponceau S to confirm transfer and identify standards. Membranes were blocked for 1 h at room temperature in 5% nonfat dry milk in TNT (50 mM Tris-HCl, pH 8.0, 100 mM NaCl, 0.01% Tween 20). They were then incubated sequentially for 1–2 h in primary antibody diluted in 5% nonfat dry milk in TNT followed by horseradish peroxidase-conjugated secondary antibody (Roche Molecular Biochemicals) in TNT. They were washed extensively in TNT in between each reagent. Antibody binding was detected with ECL and exposure of the membrane to Hyperfilm ECL (Amersham).

Immunoprecipitations—Lysates were precleared by incubating with protein A-agarose (Roche Molecular Biochemicals) for 3 h at 4 °C with rotation. Immunoprecipitations were carried out by incubating the precleared lysates with either rabbit anti-MNAB or preimmune serum at 1:500, or mouse monoclonal anti-HA (12CA5, Roche Molecular Biochemicals) or control mouse monoclonal antibody (MOPC141, Sigma) at 5 μg/reaction for 2 h at 4 °C with rotation. After incubation with protein A-agarose for 1 h with rotation at 4 °C, the immune complexes were collected by brief centrifugation then washed 3–4 times with lysis buffer. The immune complexes were then heated at 85 °C for 10 min in 25 μl of 2 × sample buffer.

Immunofluorescence—Cells (2×10^4) were seeded into the wells of poly-L-lysine (1 mg/ml, Sigma) coated 8-well Permanox chamber slides (Nunc, Naperville, IL) and incubated at 37 °C in 5% CO_2 for 2 days at which time the cells were typically 70–80% confluent. Cells were fixed in 4% paraformaldehyde in PBS containing 1 mM each $CaCl_2$ and $MgCl_2$ (Ca,Mg-PBS) for 30 min at room temperature, washed twice with Ca,Mg-PBS, then permeablized by incubation in permeablization buffer (Ca,Mg-PBS, 10% fetal bovine serum, 0.05% sodium azide, and 0.1% Triton X-100) for 30 min at room temperature. The cells were incubated sequentially for 1 h at room temperature with primary antibody (1:100 rabbit anti-MNAB, 1:100 sheep anti-transferrin receptor, 1:200 mouse anti-γ-tubulin) then with the appropriate fluorophore-conjugated secondary antibody (1:200 Cy3 anti-rabbit IgG, 1:50 DTAF anti-mouse IgG (Accurate Chemical, Westbury, NY), or 1:100 fluorescein isothiocyanate anti-goat IgG (Tago, BIOSOURCE International, Camarillo, CA), diluted in permeablization buffer, with extensive washing in permeablization buffer following each antibody incubation. Slides were mounted in Antifade Light (Molecular Probes, Eugene, OR) and viewed with epifluorescence microscopy. Digital images were obtained with a Zeiss Axialphot Photo 3 fluorescence microscope equipped with a Hamamatsu digital charged coupled device camera and processed with QED imaging software. Non-immune sera from the appropriate species were used as controls for the primary antibodies. For doubly stained cells, control staining showed that there was no reactivity between the secondary antibody and the inappropriate primary antibody.[2]

Flow Cytometric Analysis—Human 293 cells were lifted with 8 mM EDTA in PBS and washed once in PBS. Cells (5×10^5) were incubated at 4 °C for 30 min with preimmune serum or anti-MNAB diluted in assay medium (1% fetal calf serum, 1 mM $MgCl_2$, 1 mM $CaCl_2$, and 0.1% sodium azide in PBS). After washing, cells were incubated at 4 °C for 30 min with fluorescein isothiocyanate-conjugated goat anti-rabbit IgG (Caltag, Burlingame, CA) diluted 1:400 in assay medium. Viable cells were identified by incubation at 4 °C for 20 min with 2.5 μg/ml 7-aminoactinomycin D (Molecular Probes) in assay medium. The fluorescence intensity of the cells was analyzed by FACScan (Becton Dickinson, Mountain View, CA) with CellQuest software. Ten thousand viable cells were counted in each analysis to determine the geometric mean fluorescence intensity. Means were compared with ANOVA and Tukey's post-hoc multiple comparisons test.

Site-directed Mutagenesis—Mutagenesis of the zinc finger motif in MNAB was done in pGEX-MNAB using the QuickChange site-directed mutagenesis kit (Stratagene, La Jolla, CA) according to the manufacturer's instructions. The coding sequences of the complimentary mutagenic primer pairs (Operon Technologies, Alameda, CA) used were: C416A, 5′-AAATACAAGACTAGCATG**GC**CCGAGATTTGCGACAGC-3′; C416S, 5′-AAATACAAGACTAGCATG**A**GCCGAGATTTGCGACAGC-3′; C431A, 5′-GTCCACGAGGAACAAAT**GC**TACATTTGCCCATTCTCAGG-3′; C431S, 5′-GTCCACGAGGAACAAATT**C**TACATTTGCCCATTCTCAGG-3′. Altered nucleotides are in bold and underlined. All mutations were verified by DNA sequencing.

ELISA for DNA Binding—Purified GST or GST fusion proteins were diluted in ELISA buffer (PBS, 1% bovine serum albumin) to 1 μg/ml and 100 μl of diluted proteins were added to wells of a microtiter plate containing immobilized calf thymus DNA (United Biotech, Mountainview, CA). After 1 h at room temperature the wells were washed with wash buffer (PBS containing 0.1% bovine serum albumin) and 100 μl of a 1:1000 dilution of rabbit anti-GST (Sigma) in ELISA buffer were added. After 1 h at room temperature the wells were washed and 100 μl of a 1:1000 dilution of horseradish peroxidase-conjugated goat anti-rabbit IgG (Roche Molecular Biochemicals) in ELISA buffer were added. After 1 h at room temperature the wells were washed and 100 μl of chromogenic substrate (3,3′,5,5′-tetramethyl benzidine, H_2O_2; Genzyme Diagnostics, San Carlos, CA) were added. Chromogen conversion was measured by absorbance at 450 nm in a microplate reader (Bio-Tek Instruments, Winooski, VT).

Nitrocellulose Filter Assay for DNA Binding—Qiagen (Valencia, CA) column purified pGEM-4Z DNA was linearized with *Bam*HI then sequentially extracted with phenol:chloroform:isoamyl alcohol (24:24:1), chloroform:isoamyl alcohol (24:1) then ethanol precipitated. After resuspension the DNA was labeled by a fill-in reaction with the Klenow fragment of DNA polymerase I and [α-^{32}P]dATP (800 or 3000 Ci/mmol; NEN Life Sciences, Boston, MA). Unincorporated label was removed by spin chromatography through Sephadex G-50. The specific activity of the labeled DNA was determined by precipitation with 10% trichloroacetic acid and scintillation counting. For the binding assay, the GST/MNAB protein and DNA were diluted into binding buffer (20 mM HEPES, pH 7.4, 75 mM NaCl, 0.5 mM EDTA, 1 mM dithiothreitol, 5% glycerol, 100 μg/ml bovine serum albumin) to make 2 × stocks. The GST/MNAB protein and DNA were combined and incubated for 30 min at room temperature, then 50-μl aliquots were vacuum filtered (~1 ml/min) through nitrocellulose discs (0.45 μm pore size, Schleicher and Schuell, Keene, NH). The filters were washed 2 times with 0.5 ml of binding buffer then counted by scintillation counting in Ecolume (ICN, Irvine, CA).

RESULTS

Cloning Strategy—One million plaques from a λgt11 lipopolysaccharide-activated human monocyte cDNA library were screened with an SLE serum that was previously shown to inhibit DNA binding to cell surfaces (21). Ten clones were identified and plaque purified. Sequence analysis of the 1.4-kb clone 88, which was highly reactive on Western blots with the SLE serum, revealed an open reading frame coding for a partial protein.[2] The reading frame remained open at the 5′ end and a stop codon was found near the 3′ end of the clone. Thus, clone 88 coded for the 3′ end of a larger protein.

Biotinylated DNA was used to isolate DNA-binding proteins from the S49 murine cell line. A 150-kDa protein was obtained that reacted by Western blot with both the SLE serum used to screen the library and with a rabbit polyclonal antibody that was raised against the fusion protein encoded by clone 88.[2] Thus, clone 88 appeared to be part of a gene that encoded a DNA-binding protein. The remainder of the gene was cloned and sequenced from several sources as described under "Materials and Methods." A full-length coding sequence was cloned as a single PCR product from MOLT-4 cDNA. The completed sequence was 4351 nucleotides in length with a predicted open reading frame of 3576 nucleotides encoding a protein of 1192 amino acids. The sequence obtained from MOLT-4 cells matched the sequences obtained from other sources (human monocytes, Raji, and HeLa cell lines). The gene coding for this protein has been termed MNAB, for membrane-associated nucleic acid-binding protein.

Gene Expression and Protein Sequence Analysis—A survey of MNAB gene expression by Northern blot analysis revealed two major transcripts of 9.5 and 6.8 kb in all human tissues and cancer cell lines examined (Fig. 1). Several smaller transcript sizes were also observed in some of those tissues and cell lines. Expression was most abundant in spleen, testis, ovary, and small intestine. An homology search using MNAB sequence identified the genomic sequence which was known to be located at q34 on chromosome 9 (GenBank accession number 007066, marker HIM9.89 on Contig CHR9.SL.27). The genomic clone sequence, which covered 85% of the cDNA starting from the 5′ end, revealed the location of 16 complete exons. A Blast search of the expressed tag sequence (EST) data base indicated wide

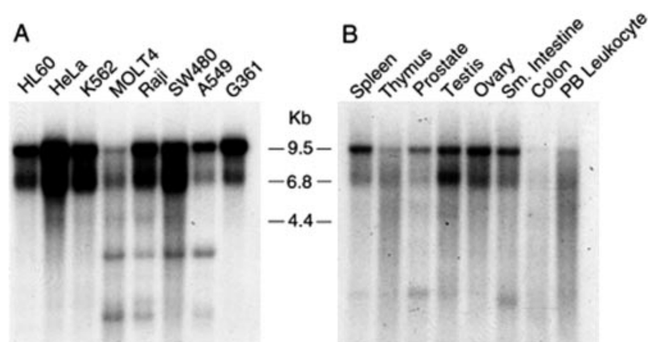

FIG. 1. **Northern blot analysis on blots of human cancer cell lines (*A*) and human tissues (*B*).** A 442-bp DNA fragment (probe 11) from the 3′ end of the gene coding for MNAB was used as the radiolabeled probe for each blot.

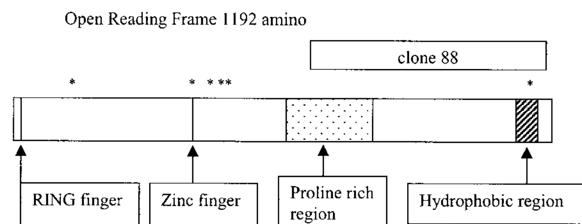

FIG. 2. **Schematic diagram of MNAB showing the location of clone 88, the RING finger, zinc finger, proline-rich, and hydrophobic regions.** An *asterisk* (*) denotes the consensus potential *N*-linked glycosylation sites at amino acid positions 122, 394, 430, 451, 466, 468, and 1150.

expression of this gene in normal human tissue (liver/spleen, prostate epithelial, germinal B cell, white adipose, pregnant uterus, fetal heart/liver, and spleen) and in tumor and transformed cells (Jurkat, HL60, 293, G361, B-cell lymphotic leukemia, colon tumor, melanoma, and parathyroid tumor).

Hydropathy analysis (41) identified a 38-amino acid hydrophobic region near the carboxyl terminus of the protein (amino acids 1133–1171) which is a potential transmembrane domain. No consensus amino-terminal signal sequence was identified. A proline-rich region (20%) spanning amino acids 549–809 and 7 consensus sites for *N*-linked glycosylation at amino acid positions 122, 394, 430, 451, 466, 468, and 1150, have been identified (Fig. 2). The calculated isoelectric point of the MNAB protein is 6.4.

Blast search has also identified two motifs: a C3HC3D RING finger subtype located near the amino terminus (amino acids 14–50) and a CCCH zinc finger located near the center of the MNAB protein sequence (amino acids 416–435). An alignment of several RING finger motifs is shown in Fig. 3*A*. MNAB differs from the originally identified C3HC4 RING finger motif by the replacement of the last cysteine with an aspartic acid. The alignment of the conserved cysteines and histidines of the CCCH zinc finger motif is shown in Fig. 3*B*.

Characterization of MNAB Protein Expression in Mammalian Cells—Immunoprecipitation and Western blotting experiments with anti-MNAB antibodies identified a protein of M_r $\sim 1.5 \times 10^5$ in most cells (293, COS7, G361, Hela, HRE605, Molt-4, Raji, A549, and B16).[2] A protein with this same (or slightly slower) mobility was also detected by immunoprecipitation and/or Western blotting with either rabbit anti-MNAB or mouse monoclonal anti-HA from lysates of cells (293-MNAB/flu) stably transfected with an expression vector for a carboxyl-terminal HA-tagged MNAB (pMNAB/flu, Fig. 4).

Subcellular Protein Localization—Fractionation by centrifugation of Dounce-homogenized 293-MNAB/flu cells into soluble and crude membrane fractions followed by Western blotting with either anti-MNAB or anti-HA showed that essentially all the MNAB protein in those cells was associated with the membrane fraction (Fig. 5*A*). Indirect immunofluorescence on fixed, permeablized cells (A549, Fig. 5*B*; COS7, HeLa[2]) showed that anti-MNAB staining was predominantly localized to the perinuclear region of the cell. No nuclear staining was observed. Double staining with anti-MNAB and anti-transferrin receptor antibodies showed partial co-localization of the MNAB and transferrin receptor. The MNAB did not co-localize with the transferrin receptor in peripheral endosomes and neither MNAB nor transferrin receptor were visualized on cell surfaces suggesting the number of receptors on the cell surface are below the limits of detection in this assay. Double staining with anti-MNAB and anti-γ-tubulin showed no co-localization,[2] indicating that MNAB does not localize to the centrosome. The observed staining pattern is consistent with the results obtained by cell fractionation and with the protein being located primarily in the ER or trans-Golgi network. However, these results do not exclude the possibility that the MNAB protein is expressed on cell surfaces.

To determine if MNAB is located on the cell surface, human kidney cell line 293 was incubated with anti-MNAB. Antibody binding was detected by flow cytometry with fluorescein isothiocyanate-labeled secondary antibodies to rabbit IgG. At all serum dilutions the fluorescence intensity of the cells incubated with anti-MNAB was significantly higher than that of cells incubated with preimmune serum ($p < 0.003$) (Fig. 6) suggesting that MNAB is expressed on the cell surface. However, attempts to overexpress the MNAB protein on the cell surface, as assessed by an increase in DNA or anti-MNAB antibody binding to the surface of MNAB-transfected cells, have not been successful.[2]

Soluble MNAB Fusion Protein Binds DNA—Purified GST/MNAB fusion protein bound to ELISA plates coated with either calf thymus DNA (Fig. 7) or plasmid DNA.[2] Negative controls using GST peptide alone or two irrelevant GST fusion proteins (GST/CBD, GST/HST.1)[2] showed no binding. To demonstrate that the binding of DNA by the soluble form of the MNAB was not simply due to a charge related interaction, the role in DNA binding of the zinc finger domain at amino acids 416–435 was examined (see Fig. 2). Using site-directed mutagenesis, the codons for the conserved zinc finger cysteines at either amino acids 416 or 431 were altered to a codon for either alanine or serine. The mutagenized GST/MNAB fusion proteins were expressed in *E. coli* and affinity purified on glutathione-Sepharose, then tested for their ability to bind to immobilized DNA by ELISA. Mutagenesis of either cysteine 416 or 431 reduced DNA binding to approximately 50% of the level observed for wild type GST/MNAB fusion protein (Fig. 7). The affinity of GST/MNAB for DNA was estimated using a nitrocellulose filter binding assay (Fig. 8). GST/MNAB (2 nM) and labeled DNA (200 pM) were titrated with increasing amounts of unlabeled DNA (0–48 nM). A Scatchard transformation of the data yields a $K_D \sim 4$ nM.

DISCUSSION

Evidence for high molecular weight DNA binding to cell surfaces, with subsequent internalization via a receptor-mediated mechanism, was first reported in 1985 by Bennett *et al.* (31). In an attempt to identify, at the molecular level, a cell-surface receptor(s) that binds nucleic acids we have cloned a novel human gene coding for a membrane-associated protein (MNAB) that binds DNA. The deduced amino acid sequence of this novel DNA-binding protein has several distinctive features, including a RING finger near the amino-terminal, an internal zinc finger, multiple possible glycosylation sites and a

A C3HC3D RING Finger Homologies

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MNAB                         TEFLSCPIC---YNEFDENVHKPISLGCSHTVCKTCLNKLHRKA------CPFDQTAIN  58
H.sapiens ARD1               VKVLECGVCEDVFSL--QGDKVPRLLLCGHTVCHDCLTRLPLHGR--AIRCPFDRQVTD  80
H.sapiens CART1              KRRLLCPLCGKPMREPVQVST------CGHRFCDTCLQEFLSEG---VFKCPEDQLPLD  62
H.sapiens SBBIO3             DEDLICPICSGVLEEPVQAPH------CEHAFCNACITQWFSQQ---Q-TCPVDRSVVT  61
C.elegans cDNA EST 3879246   --YSECLVC---YQKFDENTRIPRVMDCGHTLCDFCINQIVKMAGCYSATCPFDRVRI- 182
C.elegans 25.8KD protein     -EWRSCFICTMEYSRTDKNLH-PIILNCGHNLCRSCINKL---TGNGIVKCPFDR---- 199
C.elegans cDNA EST 3878739   QEVLCCSICNRHFNE----TFLPVSLICGHVICRKCAEKPENQTK----PCPHDDWKTT  61
C3HC3D motif                 -----C--C------------------C-H--C--C-------------C--D-----
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B C3H Zinc Finger Homologies

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MNAB                            ETPQPQPNSKYKTSMCRDLRQQGGCPRGTNCTFAHSQEELEKYRLRNK  448
C.elegans        PIE-1          -------HTEYKTRLCDAFRREGYCPYNDNCTYAHGQDELRVPRRRQE  136
D.melanogaster   DTIS 11        ---QPMNTSRYKTELCRPFEEAGECKYGEKCQFAHGSHEL--------  166
H.sapiens        TIS11B         -------SSRYKTELCRPFEENGACKYGDKCQFAHGIHEL--------  181
S.cerevisiae     CTH1           QLPQLVNKTLYKTELCESFTIKGYCKYGNKCQFAHGLNELK-------  235
CCCH motif                      ----------YKTELC--------C-----C---H-------------
```

FIG. 3. *A,* alignment of conserved cysteines, histidines, and aspartic acids of the C3HC3D RING finger. *Homo sapiens* ARD1 GTP-binding protein (GenBank A46054) (66), *H. sapiens* CART1 protein (GenBank X80200 (67), *H. sapiens* SBBI03 hypothetical protein (GenBank 5032071), *C. elegans* cDNA EST (GenBank 3879246), *C. elegans* hypothetical 25.8 kDa protein (GenBank 2496825), *C. elegans* cDNA EST (GenBank 3878739). *B,* alignment of conserved cysteines and histidines of the CCCH type zinc finger. *C. elegans* PIE-1 (GenBank U62896), *Drosophila melanogaster* DTIS 11 (GenBank U13397), *H. sapiens* TIS11B Buryrate response factors (EFT Response factor) (GenBank X79066), *Saccharomyces cerevisiae* CTH1 zinc finger protein (GenBank L42133).

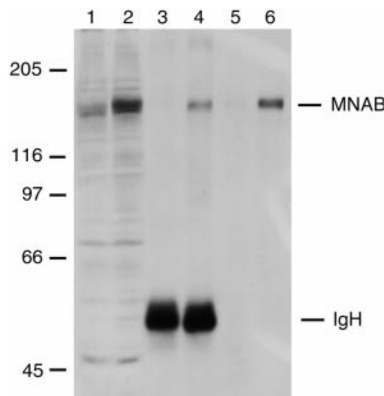


FIG. 4. **Analysis of MNAB protein expression in mammalian cells.** Immunoprecipitation of MNAB and HA-tagged MNAB proteins in stably transfected 293-MNAB/flu cells. *Lane 1*, lysate of 293 cells; *lane 2*, lysate of 293-MNAB/flu cells; *lanes 3–6*, immunoprecipitation of 293-MNAB/flu cell lysates with: rabbit preimmune serum (*lane 3*) or anti-MNAB (*lane 4*), mouse control monoclonal antibody (*lane 5*), or anti-HA (*lane 6*). Detected by Western blotting with rabbit anti-MNAB. *IgH*, immunoprecipitating rabbit IgG heavy chain.

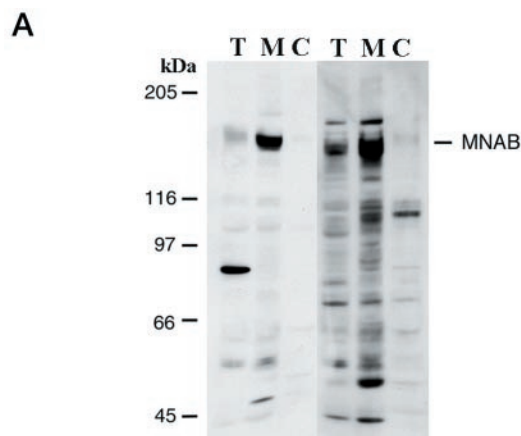
potential transmembrane domain near the carboxyl-terminal of the protein.

The MNAB gene was found to be widely expressed in human tissues and is conserved across several species. The most abundant transcripts were shown by Northern blot analysis to be in the spleen, testis, ovary, and small intestines. MNAB gene expression was also observed in all tumor and transformed cell lines examined. A search of the mouse EST data base indicated conservation of the sequence in the mouse homologue as well. Several mouse clones were identified that have 76–95% identical DNA sequence with the MNAB gene. These clones together spanned 30% of the MNAB gene. A homologous gene was also identified in *Caenorhabditis elegans* (GenBank accession number 3878739) which codes for a protein with 42% identity and 57% similarity with the amino-terminal portion of the MNAB protein. No function has yet been assigned to any of these encoded proteins.

Antibodies to MNAB reacted with a 150-kDa protein and bound to the surface of intact cells as well as to the perinuclear region of permeablized cells. When these antibodies were tested for their ability to inhibit DNA binding to cell surfaces, no inhibition was observed, a phenomena that could be due to the antibodies having been directed toward epitopes other than the DNA-binding domain of the MNAB protein. Overexpression of MNAB protein in several cell lines produced an increase of MNAB protein within the cell but not on the cell surface. We were unable to detect an increase in the binding of DNA or anti-MNAB antibodies to the cell surface of cells transfected with the MNAB gene.[2] It is possible that when we altered the Kozak sequence to more closely agree with the consensus sequence, the subsequent change of the second codon from a proline to an alanine may have affected the integrity of the MNAB protein in such a way that transport and/or insertion into the membrane was compromised.

It is increasingly evident from the work of several laboratories, that there are probably more than one DNA cell surface-binding molecule. Five cell-surface proteins ranging in size from 20 to 143 kDa were found in K562 cells (42) and multiple membrane proteins ranging from 30 to 110 kDa were found in Caco-2 cells (43). It may be that the DNA receptor is composed of several proteins complexed together to form a functional receptor, and the overexpression of one subunit may not effect a cell-surface change due to limiting numbers of its co-subunits. These proteins may be related by alternative splicing or post-translational protein processing as is found with the peptidylglycine α-amidating monooxygenase enzyme of pro-opiomelanocortin (44). Not only does the gene for peptidylglycine α-amidating monooxygenase generate multiple RNAs by alternative splicing, but the protein products themselves undergo tissue-specific endoproteolysis such that at least 7 functional forms of the enzyme exist ranging in size from 35 to 108 kDa. These products have various combinations of domains which generate independent soluble or membrane bound forms of the bifunctional enzyme. The soluble forms lack the transmembrane domain. DNA sequence analysis of an homologous MNAB gene clone from HL60 cells revealed a divergent sequence at its 3′ end lacking the entire putative transmembrane domain.[2] Since HL60 cells have been found to have a low DNA binding capacity (<5% by FACS)[2] (45), it may be possible that the MNAB gene variant found in HL60 cells, which lacks the putative transmembrane domain, codes for a secreted soluble form of the receptor which is not found on cell surfaces, as has been observed for other receptors (44, 46–49).

Although, the function of the RING finger domain in the MNAB protein is not yet characterized, it could be involved in nucleic acid binding. Recently it was shown that the RING



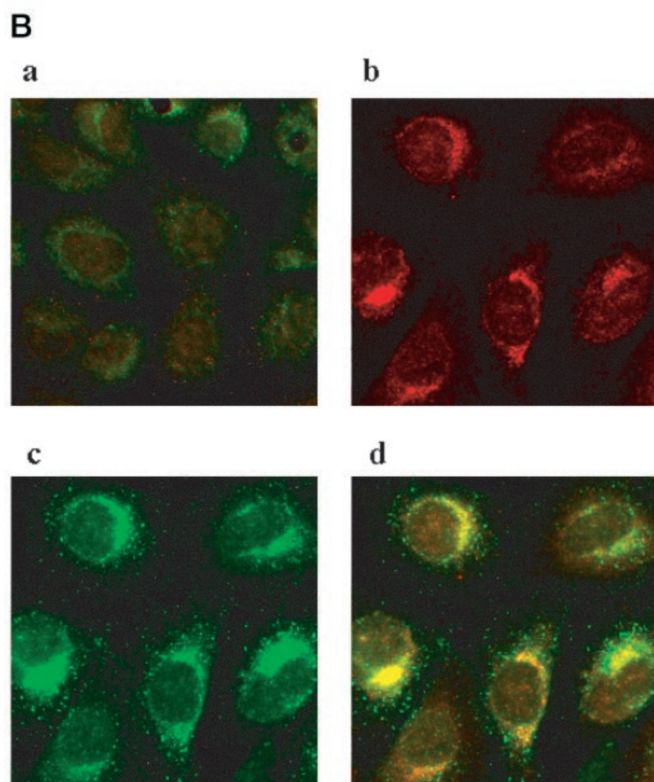


FIG. 5. **Intracellular location of the MNAB protein.** *A,* MNAB protein is associated with cell membranes in 293-MNAB/flu cells. *T*, Triton X-100 whole cell lysate; *M*, crude membrane fraction; *C*, cytosolic fraction. Detected by Western blotting with anti-HA (*left half*) or anti-MNAB (*right half*). *B,* immunofluorescence staining for anti-MNAB and anti-transferrin receptor on fixed, permeablized A549 cells. *a*, double stained with rabbit and sheep non-immune sera. *b*, anti-MNAB. *c*, anti-transferrin receptor. *d*, double stained for anti-MNAB (*red*) and anti-transferrin receptor (*green*); co-localized staining appears yellow.

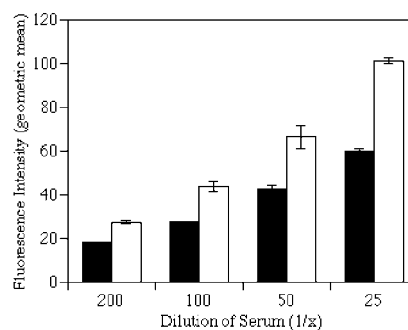


FIG. 6. **Anti-MNAB immune rabbit serum binds to cell surface.** 293 cells were incubated with preimmune serum (*black bars*) or immune serum (*white bars*). Antibody binding was detected with fluorescein isothiocyanate-conjugated goat anti-rabbit IgG by flow cytometry. Each *bar* represents the geometric mean fluorescence intensity ± S.D. (n = 3, 10,000 viable cells in each analysis). The geometric mean fluorescence of the secondary antibody alone was 7.6 ± .08 (n = 3)

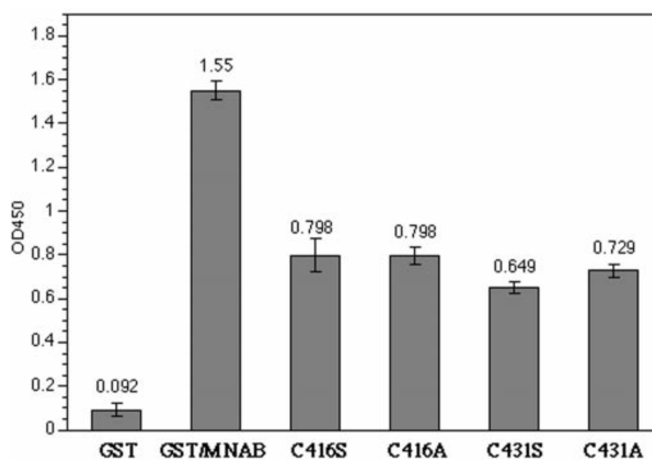


FIG. 7. **The MNAB zinc finger domain at amino acids 416–435 participates in DNA binding by GST/MNAB.** Altering the zinc finger cysteines at either 416 or 431 to either a serine (C416S, C431S) or an alanine (C416A, C431A) reduces their DNA binding by approximately 50% compared with wild-type GST/MNAB. Nonspecific binding was estimated with GST alone. Two irrelevant non-DNA binding GST fusion proteins were also used to determine nonspecific binding.[2] Data are the mean ± S.D. of triplicate determinations.

finger motif in the proto-oncogene MDM2 specifically binds RNA (50). The consensus RING finger motif is described by the sequence $C\text{-}x_2\text{-}C\text{-}x_{(9-39)}\text{-}C\text{-}x_{(1-3)}\text{-}H\text{-}x_{(2-3)}\text{-}C\text{-}x_2\text{-}C\text{-}x_{(4-48)}\text{-}C\text{-}x_2\text{-}C$, termed C3HC4, but several variations have been described (51). The MNAB motif is of the subtype C3HC3D RING finger where the last cysteine is replaced by an aspartic acid. Other members of this subtype include CART1 (a member of the TRAF family, see below), ARD1 GPT-binding protein, various uncharacterized proteins from *C. elegans*, and SSBI03 an uncharacterized hypothetical human protein (Fig. 3*A*). No specific function has yet been attributed to this subtype of the motif.

There are several hundred cDNAs encoding RING finger proteins in GenBank (52), and several RING finger subfamilies have been identified based on the presence of multiple common motifs. For example, the RING-B box coiled-coil family consists of proteins with a RING finger and one or two zinc-binding domains (B-box motif) followed by a leucine coiled-coil domain (53–55). A different arrangement of motifs describes the TRAF family members which contain a RING finger domain, a large cysteine/histidine-rich region, a coiled-coil domain, and a tumor necrosis factor receptor-associated factor (TRAF) domain (56). A common functionality among subfamily members based on these groupings of motifs has been reported (51). The RING-B box coiled-coil family members are proto-oncogenes, whereas the TRAF family members are involved in signal transduction pathways. It is interesting to note that the TRAF-1 gene maps to q33–34 in close proximity to MNAB on chromosome 9 (57). Another family of RING fingers has been linked to vacuolar/peroxisome biogenesis (58). In some cases the RING finger-containing protein is part of a multiprotein complex (58, 59). Recently the RING finger motifs of eight otherwise unrelated proteins have been shown to have ubiquitin ligase activity (52, 60). Modification with chains of ubiquitin constitutes the primary mechanism by which proteins are targeted for proteasomal degradation (61). These findings have led to the hypothesis that RING finger containing proteins may

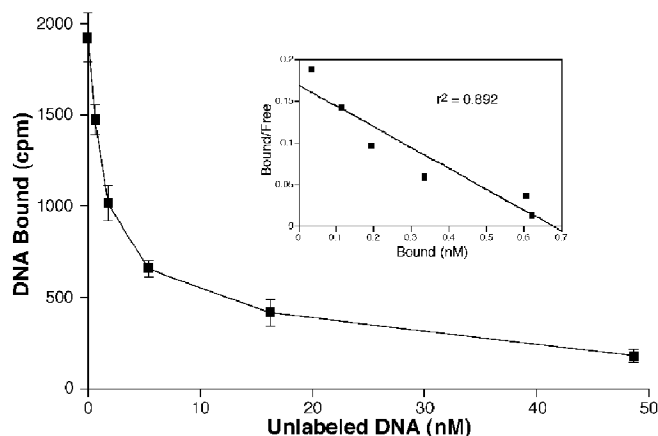


FIG. 8. **GST/MNAB binds DNA.** Nitrocellulose filter binding assay with the concentration of GST/MNAB (2 nM) and labeled DNA (200 pM, 1×10^6 cpm/pmol) held constant, incubated with increasing concentrations of unlabeled DNA. Cpm of DNA bound in the absence of GST/MNAB (~50 cpm) were subtracted from the total cpm bound at each data point. Data are the mean ± S.D. of triplicate determinations. Control assays using GST alone showed no specific binding.[2] *Inset*, Scatchard transformation of the binding data.

regulate a wide variety of cellular activities, such as growth factor receptor signaling, cell cycle progression, and DNA repair by facilitating ubiquitin-dependent degradation of key components of these processes. Whether the RING finger motif in the MNAB protein mediates DNA binding and/or ubiquitination remains to be established.

The zinc finger in the MNAB protein may participate in DNA binding because mutagenesis of a single conserved amino acid in this domain significantly reduced DNA binding. However, it is possible that mutagenesis of a single amino acid resulted in a change in the confirmation of the MNAB protein, limiting its ability to effectively bind DNA. The MNAB motif is of the CCCH subfamily of zinc finger motifs which is comprised of the consensus sequence YKTELC-x_8-C-x_5-C-x_3-H usually found in tandem. The MNAB protein contains a single motif in which three (YKT) of the 5 amino acids (YKTEL) leading into the first cysteine are conserved (see Fig. 3). The function of this motif in the majority of subfamily members is largely uncharacterized, however, one other member of this subfamily, the zfs+ protein, has been reported to bind DNA (62). Recently, tristetraprolin, the prototype of the CCCH subfamily, has been shown to bind RNA via its zinc finger. Tristetraprolin regulates expression of tumor necrosis factor-α by binding to the AU-rich element in the tumor necrosis factor-α mRNA targeting it for degradation (63, 64). Tristetraprolin also appears to regulate granulocyte macrophage-colony stimulating factor mRNA stability by a similar mechanism (65).

In summary, we have cloned a novel human gene coding for a membrane-associated protein. Given the wide expression of the MNAB gene, both within and between species, this protein most likely has a role in non-organ specific cellular processes. The MNAB protein binds DNA with relatively high affinity, suggesting that this may be an important function for this molecule. The demonstration that this membrane-associated DNA-binding protein is a cell-surface receptor critical in the binding and internalization of extracellular DNA awaits definitive confirmation of its location on the cell surface.

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REFERENCES

1. Donnelly, J. J., Ulmer, J. B., Shiver, J. W., and Liu, M. A. (1997) *Annu. Rev. Immunol.* **15,** 617–648
2. Robinson, H. L. (1999) *Int. J. Mol. Med.* **4,** 549–555
3. Feltquate, D. M. (1998) *J. Cell. Biochem. Suppl.* **30–31,** 304–311
4. Freimark, B. D., Blezinger, H. P., Florack, V. J., Nordstrom, J. L., Long, S. D., Deshpande, D. S., Nochumson, S., and Petrak, K. L. (1998) *J. Immunol.* **160,** 4580–4586
5. Manzel, L., and Macfarlane, D. E. (1999) *Antisense and Nucleic Acid Drug Dev.* **9,** 459–464
6. Schwartz, D. A., Quinn, T. J., and Krieg, A. M. (1997) *Am. J. Respir. Crit. Care Med.* **144,** A501
7. Tsan, M. F., Tacy, N. J., Lindau, S.-B. A., and White, J. E. (1997) *Proc. Assoc. Am. Phys.* **109,** P409–419
8. Yew, N. S., Wang, K. X., Przybylska, M., Bagley, R. G., Stedman, M., Marshall, J., Schenle, R. K., and Cheng, Y. C. (1999) *Human Gene Ther.* **10,** 223–234
9. Sparwasser, T., Miethke, T., Lipford, G. B., Erdmann, A., Häcker, H., Heeg, K., and Wagner, H. (1997) *Eur. J. Immunol.* **27,** 1671–1679
10. Hartmann, G., Weiner, G. J., and Krieg, A. M. (1999) *Proc. Natl. Acad. Sci. U. S. A.* **96,** 9305–9310
11. Deml, L., Schirmbeck, R., Reimann, J., Wolf, H., and Wagner, R. (1999) *Clin. Chem. Lab. Med.* **37,** 199–204
12. Bohle, B., Jahn-Schmid, B., Maurer, D., Kraft, D., and Ebner, C. (1999) *Eur. J. Immunol.* **29,** 2344–2353
13. Gasparro, F. P., DallAmico, R., O'Malley, M., Heald, P. W., and Edelson, R. (1990) *Photochem. Photobiol.* **52,** 315–321
14. Ehrlich, M., Sarafyan, L. P., and Myers, D. J. (1976) *Biochim. Biophys. Acta* **454,** 397–409
15. Russell, J. L., and Golub, E. S. (1980) *J. Immunol.* **125,** 2328–2333
16. Bennett, R. M., Davis, J., Campbell, S., and Portnoff, S. (1983) *J. Clin. Invest.* **71,** 611–618
17. Rieber, M., Urbina, C., and Rieber, M. S. (1989) *Biochem. Biophys. Res. Commun.* **159,** 1441–1447
18. Raz, E., Ben-Bassat, H., Davidi, T., Shlomai, Z., and Eilat, D. (1993) *Eur. J. Immunol.* **23,** 383–390
19. Hawley, P., and Gibson, I. (1996) *Antisense Nucleic Acid Drug Dev.* **6,** 185–195
20. Hefeneider, S. H., McCoy, S. L., Morton, J. I., Bakke, A. C., Cornell, K. A., Brown, L. E., and Bennett, R. M. (1992) *Lupus* **1,** 167–173
21. Bennett, R. M., Cornell, K. A., Merritt, M. J., Bakke, A. C., Hsu, P. H., and Hefeneider, S. H. (1991) *Clin. Exp. Immunol.* **86,** 374–379
22. Hefeneider, S. H., Cornell, K. A., Brown, L. E., Bakke, A. C., McCoy, S. L., Bennett, R. M., and Cornell, K. A. (1992) *Clin. Immunol. Immunopathol.* **63,** 245–251
23. Prabhakar, B. S., Allaway, G. P., Srinivasappa, J, and Notkins, A. L. (1990) *J. Clin. Invest.* **86,** 1301–1305
24. Kabakov, A. E., Saenko, V. A., and Poverenny, A. M. (1991) *Clin. Exp. Immunol.* **83,** 359–363
25. Bennett, R. M. (1993) *Antisense Res. Dev.* **3,** 235–241
26. Stein, C. A., Tonkinson, J. L., Zhang, L. M., Yakubov, L., Gervasoni, J., Taub, R., and Rotenberg, S. A. (1993) *Biochemistry* **32,** 4855–4861
27. Yakubov, L., Yurchenko, L., Nechaeva, M., Rykova, E., Karaymshev, V, Tonkinson, J., Vlassov, V., and Stein, C. A. (1991) *Nucleic Acids Symp. Ser.* **24,** 311–311
28. Loke, S. L., Stein, C. A., Zhang, X. H., Mori, K., Nakanishi, M., Subasinghe, C., Cohen, J. S., and Neckers, L. M. (1989) *Proc. Natl. Acad. Sci. U. S. A.* **86,** 3474–3478
29. Yakubov, L. A., Deeva, E. A., Zarytova, V. F., Ivanova, E. M., Ryte, A. S., Yurchenko, L. V., and Vlassov, V. V. (1989) *Proc. Natl. Acad. Sci. U. S.A.* **86,** 6454–6458
30. Benimetskaya, L., Loike, J. D., Khaled, Z., Loike, G., Silverstein, S. C., Cao, L., el Khoury, J., Cai, T. Q., and Stein, C. A. (1997) *Nature Med.* **3,** 414–420
31. Bennett, R. M., Gabor, G. T., and Merritt, M. J. (1985) *J. Clin. Invest.* **76,** 2182–2190
32. Emlen, W., Rifai, A., Magilavy, D., and Mannik, M. (1988) *Am. J. Pathol.* **133,** 54–60
33. Kawabata, K., Takakura, Y., and Hashida, M. (1995) *Pharmac. Res.* **12,** 825–830
34. Takagi, T., Hashiguchi, M., Mahato, R. I., Tokuda, H., Takakura, Y., and Hashida, M. (1998) *Biochem. Biophys. Res. Commun.* **245,** 729–733
35. Bennett, R. M., Peller, J. S., and Merritt, M. J. (1986) *The Lancet* **i,** 186–188
36. Bennett, R. M., Kotzin, B. L., and Merritt, M. J. (1987) *J. Exp. Med.* **166,** 850–863
37. Bennett, R. M., Cornell, K. A., Merritt, M. J., Bakke, A. C., Mourich, D., and Hefeneider, S. H. (1992) *Clin. Exp. Immunol.* **90,** 428–433
38. Young, R. A., and Davis, R. W. (1983) *Proc. Natl. Acad. Sci. U. S. A.* **80,** 1194–1198
39. Maniatis, T., Fritsch, W. F., and Sambrook, J. (1989) *Molecular Cloning: A Laboratory Manual*, Second Ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY
40. Kozak, M. (1987) *Nucleic Acids Res.* **15,** 8125–8132
41. Kyte, J., and Doolittle, R. F. (1982) *J. Mol. Biol.* **157,** 105–132
42. Beltinger, C., Saragovi, H. U., Smith, R. M., LeSauteur, L., Shah, N., DeDionisio, L., Christensen, L., Raible, A., Jarett, L., and Gewirtz, A. M. (1995) *J. Clin. Invest.* **95,** 1814–1823
43. Akhtar, S., Beck, G. F., Hawley, P., Irwin, W. J., and Gibson, I. (1996) *Antisense Nucleic Acid Drug Dev.* **6,** 197–206
44. Eipper, B. A., Stoffers, D. A., and Mains, R. E. (1992) *Annu. Rev. Neurosci.* **15,** 57–85
45. McCoy, S. L., Hausman, F. A., Deffebach, M. E., Bakke, A. C., Merkens, L. S., Bennett, R. M., and Hefeneider, S. H. (2000) *J. Immunol. Methods,* **241,** 141–146
46. Johnston, G. I., Cook, R. G., and McEver, R. P. (1989) *Cell* **56,** 1033–1044
47. Mosley, B., Beckmann, M. P., March, C. J., Idzerda, R. L., Gimpel, S. D.,

VandenBos, T., Friend, D., Alpert, A., Anderson, D., and Jackson, J. (1989) *Cell* **59,** 335–348
48. Murata, Y., Takaki, S., Migita, M., Kikuchi, Y., Tominaga, A., and Takatsu, K. (1992) *J. Exp. Med.* **175,** 341–351
49. Norment, A. M., and Littman, D. R. (1988) *EMBO J.* **7,** 3433–3439
50. Elenbaas, B., Dobbelstein, M., Roth, J., Shenk, T., and Levine, A. J. (1996) *Mol. Med.* **2,** 439–451
51. Saurin, A. J., Borden, K. L., Boddy, M. N., and Freemont, P. S. (1996) *Trends Biochem. Sci.* **21,** 208–214
52. Lorick, K. L., Jensen, J. P., Fang, S., Ong, A. M., Hatakeyyama, S., and Weissman, A. M. (1999) *Proc. Natl. Acad. Sci. U. S. A.* **96,** 11364–11369
53. Reddy, B. A., and Etkin, L. D. (1991) *Nucleic Acids Res.* **19,** 6330–6330
54. Kastner, P., Perez, A., Lutz, Y., Rochette-Egly, C., Gaub, M. P., Durand, B., Lanotte, M., Berger, R., and Chambon, P. (1992) *EMBO J.* **11,** 629–642
55. Reddy, B. A., Etkin, L. D., and Freemont, P. S. (1992) *Trends Biochem. Sci.* **17,** 344–345
56. Cheng, G., Cleary, A. M., Ye, Z. S., Hong, D. I., Lederman, S., and Baltimore, D. (1995) *Science* **267,** 1494–1498
57. Siemienski, K., Peters, N., Scheurich, P., and Wajant, H. (1997) *Gene (Amst.)* **195,** 35–39
58. Freemont, P. S. (1993) *Ann. N. Y. Acad. Sci.* **684,** 174–192
59. Borden, K. L., and Freemont, P. S. (1996) *Curr. Opin. Struct. Biol.* **6,** 395–401
60. Joazeiro, C. A., Wing, S. S., Huang, L., Leverson, J. D., Hunter, T., and Liu, Y. C. (1999) *Science* **286,** 309–312
61. Hershko, A., and Ciechanover, A. (1998) *Annu. Rev. Biochem.* **67,** 425–479
62. Worthington, M. T., Amann, B. T., Nathans, D., and Berg, J. M. (1996) *Proc. Natl. Acad. Sci. U. S. A.* **93,** 13754–13759
63. Carballo, E., Lai, W. S., and Blackshear, P. J. (1998) *Science* **281,** 1001–1005
64. Lai, W. S., Carballo, E., Strum, J. R., Kennington, E. A., Phillips, R. S., and Blackshear, P. J. (1999) *Mol. Cell. Biol.* **19,** 4311–4323
65. Carballo, E., Lai, W. S., and Blackshear, P. J. (2000) *Blood* **95,** 1891–1899
66. Mishima, K., Tsuchiya, M., Nightingale, M. S., Moss, J., and Vaughan, M. (1993) *J. Biol. Chem.* **268,** 8801–8807
67. Regnier, C. H., Tomasetto, C., Moog-Lutz, C., Chenard, M. P., Wendling, C., Basset, P., and Rio, M. C. (1995) *J. Biol. Chem.* **270,** 25715–25721