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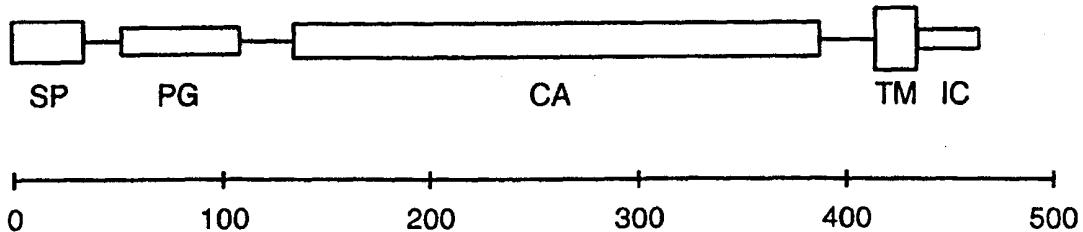
(57) Abstract

Identified herein is the location of the MN protein binding site, and MN proteins/polypeptides that compete for attachment to vertebrate cells with immobilized MN protein. Such MN proteins/polypeptides prevent cell–cell adhesion and the formation of intercellular contacts. The MN protein binding site is a therapeutic target that can be blocked by organic or inorganic molecules, preferably organic molecules, more preferably proteins/polypeptides that specifically bind to that site. Therapeutic methods for inhibiting the growth of preneoplastic/neoplastic vertebrate cells that abnormally express MN protein are disclosed. Vectors are provided that encode the variable domains of MN–specific antibodies and a flexible linker polypeptide separating those domains. Further vectors are disclosed that encode a cytotoxic protein/polypeptide operatively linked to the MN gene promoter, and which vectors preferably further encode a cytokine. The MN gene promoter is characterized, and the binding site for a repressor of MN transcription is disclosed.

MN GENE AND PROTEIN

FIELD OF THE INVENTION

5   The present invention is in the general area of medical genetics and in the fields of biochemical engineering, immunochemistry and oncology. More specifically, it relates to the MN gene – a cellular gene considered to be an oncogene, which encodes the oncoprotein now known alternatively as the MN protein, the MN/CA IX isoenzyme or the MN/G250 protein.

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BACKGROUND OF THE INVENTION

         Zavada et al., International Publication Number WO 93/18152 (published 16 September 1993) and U.S. Patent No. 5,387,676 (issued February 7,

15   1996), describe the elucidation of the biological and molecular nature of MaTu which resulted in the discovery of the MN gene and protein. The MN gene was found to be present in the chromosomal DNA of all vertebrates tested, and its expression to be strongly correlated with tumorigenicity.

         The MN protein was first identified in HeLa cells, derived from a human

20   carcinoma of cervix uteri. It is found in many types of human carcinomas (notably uterine cervical, ovarian, endometrial, renal, bladder, breast, colorectal, lung, esophageal, and prostate, among others). Very few normal tissues have been found to express MN protein to any significant degree. Those MN-expressing normal tissues include the human gastric mucosa and gallbladder epithelium, and some other normal

25   tissues of the alimentary tract. Paradoxically, MN gene expression has been found to be lost or reduced in carcinomas and other preneoplastic/neoplastic diseases in some tissues that normally express MN, e.g., gastric mucosa.

         In general, oncogenesis may be signified by the abnormal expression of MN protein. For example, oncogenesis may be signified: (1) when MN protein is

30   present in a tissue which normally does not express MN protein to any significant degree; (2) when MN protein is absent from a tissue that normally expresses it; (3) when MN gene expression is at a significantly increased level, or at a significantly reduced

level from that normally expressed in a tissue; or (4) when MN protein is expressed in an abnormal location within a cell.

Zavada et al., WO 93/18152 and Zavada et al., WO 95/34650 (published 21 December 1995) disclose how the discovery of the MN gene and protein and the

5   strong association of MN gene expression and tumorigenicity led to the creation of methods that are both diagnostic/prognostic and therapeutic for cancer and precancerous conditions. Methods and compositions were provided therein for identifying the onset and presence of neoplastic disease by detecting or detecting and quantitating abnormal MN gene expression in vertebrates. Abnormal MN gene

10 expression can be detected or detected and quantitated by a variety of conventional assays in vertebrate samples, for example, by immunoassays using MN-specific antibodies to detect or detect and quantitate MN antigen, by hybridization assays or by PCR assays, such as RT-PCR, using MN nucleic acids, such as, MN cDNA, to detect or detect and quantitate MN nucleic acids, such as, MN mRNA.

15   Zavada et al, WO 93/18152 and WO 95/34650 describe the production of MN-specific antibodies. A representative and preferred MN-specific antibody, the monoclonal antibody M75 (Mab M75), was deposited at the American Type Culture Collection (ATCC) in Manassus, VA (USA) under ATCC Number HB 11128. The M75 antibody was used to discover and identify the MN protein and can be used to identify

20 readily MN antigen in Western blots, in radioimmunoassays and immunohistochemically, for example, in tissue samples that are fresh, frozen, or formalin-, alcohol-, acetone- or otherwise fixed and/or paraffin-embedded and deparaffinized. Another representative and preferred MN-specific antibody, Mab MN12, is secreted by the hybridoma MN 12.2.2, which was deposited at the ATCC

25 under the designation HB 11647. Example 1 of Zavada et al., WO 95/34650 provides representative results from immunohistochemical staining of tissues using MAb M75, which results support the designation of the MN gene as an oncogene.

Many studies have confirmed the diagnostic/prognostic utility of MN. The following articles discuss the use of the MN-specific MAb M75 in

30 diagnosing/prognosing precancerous and cancerous cervical lesions: Leff, D. N., "Half a Century of HeLa Cells: Transatlantic Antigen Enhances Reliability of Cervical Cancer Pap Test, Clinical Trials Pending," BioWorld® Today: The Daily Biotechnology

Newspaper, 9(55) (March 24, 1998); Stanbridge, E. J., "Cervical marker can help
resolve ambigous Pap smears," Diagnostics Intelligence, 10(5): 11 (1998); Liao and
Stanbridge, "Expression of the MN Antigen in Cervical Papanicolaou Smears Is an Early
Diagnostic Biomarker of Cervical Dysplasia," Cancer Epidemiology, Biomarkers &
5   Prevention, 5: 549-557 (1996); Brewer et al., "A Study of Biomarkers in Cervical
Carcinoma and Clinical Correlation of the Novel Biomarker MN," Gynecologic
Oncology, 63: 337-344 (1996); and Liao et al., "Identification of the MN Antigen as a
Diagnostic Biomarker of Cervical Intraepithelial Squamous and Glandular Neoplasia
and Cervical Carcinomas," American Journal of Pathology, 145(3): 598-609 (1994).

10   Premalignant and Malignant Colorectal Lesions. MN has been detected
in normal gastric, intestinal, and biliary mucosa. [Pastorekova et al., Gastroenterology,
112: 398-408 (1997).] Immunohistochemical analysis of the normal large intestine
revealed moderate staining in the proximal colon, with the reaction becoming weaker
distally. The staining was confined to the basolateral surfaces of the cryptal epithelial
15 cells, the area of greatest proliferative capacity. As MN is much more abundant in the
proliferating cryptal epithelium than in the upper part of the mucosa, it may play a role
in control of the proliferation and differentiation of intestinal epithelial cells. Cell
proliferation increases abnormally in premalignant and malignant lesions of the
colorectal epithelium, and therefore, is considered an indicator of colorectal tumor
20 progression. [Risio, M., J. Cell Biochem, 16G: 79-87 (1992); and Moss et al.,
Gastroenterology, 111: 1425-1432 (1996).]

            The MN protein is now considered to be the first tumor-associated
carbonic anhydrase (CA) isoenzyme that has been described. Carbonic anhydrases
(CAs) form a large family of genes encoding zinc metalloenzymes of great physiological
25 importance. As catalysts of reversible hydration of carbon dioxide, these enzymes
participate in a variety of biological processes, including respiration, calcification, acid-
base balance, bone resorption, formation of aqueous humor, cerebrospinal fluid, saliva
and gastric acid [reviewed in Dodgson et al., The Carbonic Anhydrases, Plenum Press,
New York-London, pp. 398 (1991)]. CAs are widely distributed in different living
30 organisms.

            In mammals, at least seven isoenzymes (CA I-VII) and a few CA-related
proteins (CARP/CA VIII, RPTP-β, RPTP-τ) had been identified [Hewett-Emmett and

Tashian, Mol. Phyl. Evol., 5: 50-77 (1996)], when analysis of the MN deduced amino acid sequence revealed a striking homology between the central part of the MN protein and carbonic anhydrases, with the conserved zinc-binding site as well as the enzyme's active center. Then MN protein was found to bind zinc and to have CA activity. Based

5   on that data, the MN protein is now considered to be the ninth carbonic anhydrase isoenzyme -- MN/CA IX. [Opavsky et al., Genomics, 33: 480-487 (May 1996)]. [See also, Hewett-Emmett, supra, wherein CA IX is suggested as a nomenclatural designation.]

          CAs and CA-related proteins show extensive diversity in both their tissue

10   distribution and in their putative or established biological functions [Tashian, R. E., Adv. in Genetics, 30: 321-356 (1992)]. Some of the CAs are expressed in almost all tissues (CA II), while the expression of others appears to be more restricted (CA VI and CA VII in salivary glands). In cells, they may reside in the cytoplasm (CA I, CA II, CA III, and CA VII), in mitochondria (CA V), in secretory granules (CA VI), or they may associate

15   with membrane (CA IV). Occasionally, nuclear localization of some isoenzymes has been noted [Parkkila et al., Gut, 35: 646-650 (1994); Parkkilla et al., Histochem. J., 27: 133-138 (1995); Mori et al., Gastroenterol., 105: 820-826 (1993)].

          The CAs and CA-related proteins also differ in kinetic properties and susceptibility to inhibitors [Sly and Hu, Annu. Rev. Biochem., 64: 375-401 (1995)]. In

20   the alimentary tract, carbonic anhydrase activity is involved in many important functions, such as saliva secretion, production of gastric acid, pancreatic juice and bile, intestinal water and ion transport, fatty acid uptake and biogenesis in the liver. At least seven CA isoenzymes have been demonstrated in different regions of the alimentary tract. However, biochemical, histochemical and immunocytochemical studies have

25   revealed a considerable heterogeneity in their levels and distribution [Swensen, E. R., "Distribution and functions of carbonic anhydrase in the gastrointestinal tract," In: The Carbonic Anhydrases. Cellular Physiology and Molecular Genetics, (Dodgson et al. eds.) Plenum Press, New York, pages 265-287 (1991); and Parkkila and Parkkila, Scan J. Gastroenterol., 31: 305-317 (1996)]. While CA II is found along the entire

30   alimentary canal, CA IV is linked to the lower gastrointestinal tract, CA I, III and V are present in only a few tissues, and the expression of CA VI and VII is restricted to

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salivary glands [Parkkila et al., Gut, 35: 646-650 (1994); Fleming et al., J. Clin. Invest.,
96: 2907-2913 (1995); Parkkila et al., Hepatology, 24: 104 (1996)].

MN/CA IX has a number of properties that distinguish it from other
known CA isoenzymes and evince its relevance to oncogenesis. Those properties

5   include its density dependent expression in cell culture (e.g., HeLa cells), its correlation
with the tumorigenic phenotype of somatic cell hybrids between HeLa and normal
human fibroblasts, its close association with several human carcinomas and its absence
from corresponding normal tissues [e.g., Zavada et al., Int. J. Cancer, 54: 268-274
(1993); Pastorekova et al., Virology, 187: 620-626 (1992); Liao et al., Am. J. Pathol.,

10   145: 598-609 (1994); Pastorek et al., Oncogene, 9: 2788-2888 (1994); Cote, Women's
Health Weekly: News Section, p. 7 (March 30, 1998); Liao et al., Cancer Res., 57: 2827
(1997); Vermylen et al., "Expression of the MN antigen as a biomarker of lung
carcinoma and associated precancerous conditions," Proceedings AACR, 39: 334
(1998); McKiernan et al., Cancer Res., 57: 2362 (1997); and Turner et al., Hum. Pathol.,

15   28(6): 740 (1997)]. In addition, the in vitro transformation potential of MN/CA IX
cDNA has been demonstrated in NIH 3T3 fibroblasts [Pastorek et al., id.].

The MN protein has also been identified with the G250 antigen. Uemura
et al., "Expression of Tumor-Associated Antigen MN/G250 in Urologic Carcinoma:
Potential Therapeutic Target, " J. Urol., 154 (4 Suppl.): 377 (Abstract 1475; 1997) states:

20   "Sequence analysis and database searching revealed that G250 antigen is identicial to
MN, a human tumor-associated antigen identified in cervical carcinoma (Pastorek et al.,
1994)."

SUMMARY OF THE INVENTION

25

Identified herein is the location of the MN protein binding site. Of
particular importance is the region within the proteoglycan-like domain, aa 61-96 (SEQ
ID NO: 97) which contains a 6-fold tandem repeat of 6 amino acids, and within which
the epitope for the M75 MAb resides in at least two copies, and within which the MN

30   binding site is considered to be located. An alternative MN binding site may be located
in the CA domain.

Also identified are MN proteins and MN polypeptides that compete for attachment to cells with immobilized MN protein. Such MN proteins/polypeptides prevent cell-cell adhesion and the formation of intercellular contacts.

Disclosed herein are cell adhesion assay methods that are used to identify
5   binding site(s) on the MN protein to which vertebrate cells, preferably mammalian cells, more preferably human cells, bind. Such a MN binding site is then identified as a therapeutic target which can be blocked with MN-specific antibodies, or inorganic or organic molecules, preferably organic molecules, more perferably proteins/polypeptides that specifically bind to said site.

10   Further disclosed are therapeutic methods to treat patients with preneoplastic/neoplastic disease associated with or characterized by abnormal MN expression, which methods are based on blocking said MN binding site with molecules, inorganic or organic, but preferably organic molecules, more preferably proteins/polypeptides, that bind specifically to said binding site. The growth of a
15   vertebrate preneoplastic/neoplastic cell that abnormally expresses MN protein can be inhibited by administering such organic or inorganic molecules, preferably organic molecules, more preferably proteins/polypeptides in a therapeutically effective amount in a physiologically acceptable formulation. Such a preferred therapeutic protein/polypeptide is herein considered to comprise an amino acid sequence selected
20   from the group consisting of SEQ ID NOS: 107-109. Such heptapeptides are considered to be comprised by MN protein partner(s). Blocking the interaction between MN protein and its binding partner(s), is expected to lead to a decrease of tumor growth.

Further provided are other therapeutic methods wherein the growth of a
25   vertebrate, preferably mammalian, more preferably human, preneoplastic or neoplastic cell that abnormally expresses MN protein is inhibited. Said methods comprise transfecting said cell with a vector comprising an expression control sequence operatively linked to a nucleic acid encoding the variable domains of an MN-specific antibody, wherein said domains are separated by a flexible linker peptide, preferably
30   SEQ ID NO: 116. Preferably said expression control sequence comprises the MN gene promoter.

Still further therapeutic methods comprise transfecting said cell with a vector comprising a nucleic acid that encodes a cytotoxic protein/polypeptide, such as HSVtk, operatively linked to the MN gene promoter. Such a therapeutic vector may also comprise a nucleic acid encoding a cytokine, such as, IL-2 or IFN.

5   Aspects of the instant invention disclosed herein are described in more detail as follows. The therapeutic use of organic or inorganic molecules, preferably organic molecules, is disclosed. Preferred such molecules bind specifically to a site on MN protein to which vertebrate cells adhere in a cell adhesion assay, wherein said molecule when tested in vitro inhibits the adhesion of cells to MN protein. Further

10   preferred are such molecules, which when in contact with a vertebrate preneoplastic or neoplastic cell that abnormally expresses MN protein, inhibit the growth of said cell. Said vertebrate cells are preferably mammalian and more preferably human.

Preferably such a molecule is organic, and more preferably such a organic molecule is a protein or a polypeptide. Still further preferably, said protein or

15   polypeptide comprises an amino acid sequence selected from the group consisting of SEQ ID NOS: 107, 108, 109, 137 and 138. Even more preferably, said polypeptide is selected from the group consisting of SEQ ID NOS: 107, 108, 109, 137 and 138.

The site on MN proteins to which vertebrate cells adhere in said cell adhesion assay is preferably within the proteoglycan-like domain [SEQ ID NO: 50] or

20   within the carbonic anhydrase domain [SEQ ID NO: 51] of the MN protein. Preferably that site comprises an amino acid sequence selected from the group consisting of SEQ ID NOS: 10 and 97-106. Still further preferably, that site has an amino acid sequence selected from the group consisting of SEQ ID NOS: 10 and 97-106.

Another aspect of this invention concerns MN proteins and MN

25   polypeptides which mediate attachment of vertebrate cells in a cell adhesion assay, wherein said MN protein or MN polypeptide when introduced into the extracellular fluid environment of vertebrate cells prevents the formation of intercellular contacts and the adhesion of said vertebrate cells to each other. Such MN proteins and MN polypeptides may be useful to inhibit the growth of vertebrate preneoplastic or

30   neoplastic cells that abnormally express MN protein, when such MN proteins or MN polypeptides are introduced into the extracellular fluid environment of such vertebrate cells. Said vertebrate cells are preferably mammalian, and more preferably human.

Said MN proteins or MN polypeptides which mediate attachment of vertebrate cells in a cell adhesion assay, preferably have amino acid sequences from SEQ ID NO: 97, from SEQ ID NO: 50, or from SEQ ID NO: 51, more preferably from SEQ ID NO: 50. Still more preferably such MN proteins or MN polypeptides comprise

5   amino acid sequences selected from the group consisting of SEQ ID NOS: 10 and 97-106. Alternatively, said MN polypeptides are selected from the group consisting of SEQ ID NOS: 10 and 97-106.

Representative MN proteins and MN polypeptides which mediate attachment of vertebrate cells in a cell adhesion assay, are specifically bound by either

10   the M75 monoclonal antibody that is secreted from the hybridoma VU-M75, which was deposited at the American Type Culture Collection under ATCC No. HB 11128, or by the MN12 monoclonal antibody that is secreted from the hybridoma MN 12.2.2, which was deposited at the American Type Culture Collection under ATCC No. HB 11647, or by both said monoclonal antibodies.

15   Another aspect of the instant invention is a method of identifying a site on an MN protein to which vertebrate cells adhere by testing a series of overlapping polypeptides from said MN protein in a cell adhesion assay with vertebrate cells, and determining that if cells adhere to a polypeptide from said series, that said polypeptide comprises a site on said MN protein to which vertebrate cells adhere.

20   Still another aspect of the instant invention is a vector comprising an expression control sequence operatively linked to a nucleic acid encoding the variable domains of a MN-specific antibody, wherein said domains are separated by a flexible linker polypeptide, and wherein said vector, when transfected into a vertebrate preneoplastic or neoplastic cell that abnormally expresses MN protein, inhibits the

25   growth of said cell. Preferably said expression control sequence comprises the MN gene promoter operatively linked to said nucleic acid. Further preferably, said flexible linker polypeptide has the amino acid sequence of SEQ ID NO: 116, and even further preferably, said MN gene promoter has the nucleotide sequence of SEQ ID NO: 27.

Another further aspect of the instant invention concerns a vector

30   comprising a nucleic acid that encodes a cytotoxic protein or cytotoxic polypeptide operatively linked to the MN gene promoter, wherein said vector, when transfected into a vertebrate preneoplastic or neoplastic cell that abnormally expresses MN protein,

inhibits the growth of said cell. In one preferred embodiment said cytotoxic protein is HSV thymidine kinase. Preferably, said vector further comprises a nucleic acid encoding a cytokine operatively linked to said MN gene promoter. In alternative and preferred embodiments, said cytokine is interferon or interleukin-2.

5   The MN gene promoter is characterized herein. The identification of the binding site for a repressor of MN transcription is disclosed. Mutational analysis indicated that the direct repeat AGGGCacAGGGC [SEQ ID NO: 143] is required for efficient repressor binding.

           Identification of the protein that binds to the repressor and modification
10   of its binding properties is another route to modulate MN expression leading to cancer therapies. Suppression of MN expression in tumor cells by over expression of a negative regulator is expected to lead to a decrease of tumor growth. A repressor complex comprising at least two subunits was found to bind to SEQ ID NO: 115 of the MN gene promoter. A repressor complex, found to be in direct contact with SEQ ID
15   NO: 115 by UV crosslinking, comprised two proteins having molecular weights of 35 and 42 kilodaltons, respectively.

Abbreviations

The following abbreviations are used herein:

20   aa   -   amino acid

     ATCC   -   American Type Culture Collection

     bp   -   base pairs

     BLV   -   bovine leukemia virus

     BSA   -   bovine serum albumin

25   BRL   -   Bethesda Research Laboratories

     CA   -   carbonic anhydrase

     CAM   -   cell adhesion molecule

     CARP   -   carbonic anhydrase related protein

     CAT   -   chloramphenicol acetyltransferase

30   Ci   -   curie

     cm   -   centimeter

     CMV   -   cytomegalovirus

cpm	-	counts per minute
C-terminus	-	carboxyl-terminus
CTL	-	cytotoxic T lymphocytes
°C	-	degrees centigrade
5 DEAE	-	diethylaminoethyl
DMEM	-	Dulbecco modified Eagle medium
ds	-	double-stranded
EDTA	-	ethylenediaminetetraacetate
EGF	-	epidermal growth factor
10 EIA	-	enzyme immunoassay
ELISA	-	enzyme-linked immunosorbent assay
EMSA	-	electrophoretic mobility shift assay
F	-	fibroblasts
FACS	-	cytofluorometric study
15 FCS	-	fetal calf serum
FITC	-	fluorescein isothiocyanate
FTP	-	DNase 1 footprinting analysis
GST-MN	-	fusion protein MN glutathione S-transferase
GVC	-	ganciclovir
20 H	-	HeLa cells
H-E	-	haematoxylin-eosin
HEF	-	human embryo fibroblasts
HeLa K	-	standard type of HeLa cells
HeLa S	-	Stanbridge's mutant HeLa D98/AH.2
25 H/F-T	-	hybrid HeLa fibroblast cells that are tumorigenic; derived from HeLa D98/AH.2
H/F-N	-	hybrid HeLa fibroblast cells that are nontumorigenic; derived from HeLa D98/AH.2
HPV	-	Human papilloma virus
30 HRP	-	horseradish peroxidase
HSV	-	Herpes simplex virus
IC	-	intracellular

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	IFN	-	interferon
	IL-2	-	interleukin-2
	Inr	-	initiator
	IPTG	-	isopropyl-Beta-D-thiogalacto-pyranoside
5	kb	-	kilobase
	kbp	-	kilobase pairs
	kd or kDa	-	kilodaltons
	KS	-	keratan sulphate
	LCMV	-	lymphocytic choriomeningitis virus
10	LTR	-	long terminal repeat
	M	-	molar
	mA	-	milliampere
	MAb	-	monoclonal antibody
	MCSF	-	macrophage colony stimulating factor
15	ME	-	mercaptoethanol
	MEM	-	minimal essential medium
	min.	-	minute(s)
	mg	-	milligram
	ml	-	milliliter
20	mM	-	millimolar
	MMC	-	mitomycin C
	mmol	-	millimole
	MLV	-	murine leukemia virus
	N	-	normal concentration
25	NEG	-	negative
	ng	-	nanogram
	nm	-	nanometer
	nt	-	nucleotide
	N-terminus	-	amino-terminus
30	ODN	-	oligodeoxynucleotide
	ORF	-	open reading frame
	PA	-	Protein A

PBS	-	phosphate buffered saline
PCR	-	polymerase chain reaction
PEST	-	combination of one-letter abbreviations for proline, glutamic acid, serine, threonine
PG	-	proteoglycan
pI	-	isoelectric point
PMA	-	phorbol 12-myristate 13-acetate
POS	-	positive
Py	-	pyrimidine
RACE	-	rapid amplification of cDNA ends
RCC	-	renal cell carcinoma
RIA	-	radioimmunoassay
RIP	-	radioimmunoprecipitation
RIPA	-	radioimmunoprecipitation assay
RNP	-	RNase protection assay
RT-PCT	-	reverse transcription polymerase chain reaction
SAC	-	Staphylococcus aureus cells
S. aureus	-	Staphylococcus aureus
sc	-	subcutaneous
SDRE	-	serum dose response element
SDS	-	sodium dodecyl sulfate
SDS-PAGE	-	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SINE	-	short interspersed repeated sequence
SP	-	signal peptide
SP-RIA	-	solid-phase radioimmunoassay
SSDS	-	synthetic splice donor site
SSH	-	subtractive suppressive PCR
SSPE	-	NaCl (0.18 M), sodium phosphate (0.01 M), EDTA (0.001 M)
TBE	-	Tris-borate/EDTA electrophoresis buffer
TC	-	tissue culture
TCA	-	trichloroacetic acid
TC media	-	tissue culture media

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TC   -   tissue culture

tk   -   thymidine kinase

TM   -   transmembrane

TMB   -   tetramethylbenzidine

5   Tris   -   tris (hydroxymethyl) aminomethane

μCi   -   microcurie

μg   -   microgram

μl   -   microliter

μM   -   micromolar

10 VSV   -   vesicular stomatitis virus

VV   -   vaccinia virus

X-MLV   -   xenotropic murine leukemia virus

Cell Lines

15 AGS   –   cell line derived from a primary adenogastric carcinoma
                    [Barranco and Townsend, Cancer Res., 43: 1703 (1983) and
                    Invest. New Drugs, 1: 117 (1983)]; available from the ATCC
                    under CRL-1739;

20 BL-3   –   bovine B lymphocytes [ATCC CRL-8037; leukemia cell
                    suspension; J. Natl. Cancer Inst. (Bethesda) 40: 737 (1968)];

C33   –   a cell line derived from a human cervical carcinoma biopsy
                    [Auersperg, N., J. Nat'l. Cancer Inst. (Bethesda), 32: 135-148
25   (1964)]; available from the ATCC under HTB-31;

C33A   –   human cervical carcinoma cells [ATCC HTB-31; J. Natl. Cancer
                    Inst. (Bethesda) 32: 135 (1964)];

30 COS   –   simian cell line [Gluzman, Y., Cell, 23: 175 (1981)];

HeLa K	–	standard type of HeLa cells; aneuploid, epithelial-like cell line isolated from a human cervical adenocarcinoma [Gey et al., Cancer Res., 12: 264 (1952); Jones et al., Obstet. Gynecol., 38: 945-949 (1971)] obtained from Professor B. Korych, [Institute of Medical Microbiology and Immunology, Charles University; Prague, Czech Republic];

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HeLa D98/AH.2 (also HeLa s)	–	Mutant HeLa clone that is hypoxanthine guanine phosphoribosyl transferase-deficient (HGPRT⁻) kindly provided by Eric J. Stanbridge [Department of Microbiology, College of Medicine, University of California, Irvine, CA (USA)] and reported in Stanbridge et al., Science, 215: 252-259 (15 Jan. 1982); parent of hybrid cells H/F-N and H/F-T, also obtained from E.J. Stanbridge;

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KATO III	–	cell line prepared from a metastatic form of a gastric carcinoma [Sekiguichi et al., Japan J. Exp. Med., 48: 61 (1978)]; available from the ATCC under HTB-103;

20 NIH-3T3	–	murine fibroblast cell line reported in Aaronson, Science, 237: 178 (1987);

QT35	–	quail fibrosarcoma cells [ECACC: 93120832; Cell, 11: 95 (1977)];

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Raj	–	human Burkitt's lymphoma cell line [ATCC CCL-86; Lancet, 1: 238 (1964)];

Rat2TK⁻	–	cell line (rat embryo, thymidine kinase mutant) was derived from a subclone of a 5'-bromo-deoxyuridine resistant strain of the Fischer rat fibroblast 3T3-like cell line Rat1; the cells lack

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appreciable levels of nuclear thymidine kinase [Ahrens, B.,

Virology, 113: 408 (1981)];

5   SiHa   –   human cervical squamous carcinoma cell line [ATCC HTB-35;

Friedl et al., Proc. Soc. Exp. Biol. Med., 135: 543 (1990)];

XC   –   cells derived from a rat rhabdomyosarcoma induced with Rous

sarcoma virus-induced rat sarcoma [Svoboda, J., Natl. Cancer

Center Institute Monograph No. 17, IN: "International

10   Conference on Avian Tumor Viruses" (J.W. Beard ed.), pp. 277-

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Molecular Genetics, Czechoslovak Academy of Sciences;

Prague, Czech Republic]; and

15 CGL1   –   H/F-N hybrid cells (HeLa D98/AH.2 derivative);

CGL2   –   H/F-N hybrid cells (HeLa D98/AH.2 derivative);

CGL3   –   H/F-T hybrid cells (HeLa D98/AH.2 derivative);

20
CGL4   –   H/F-T hybrid cells (HeLa D98/Ah.2 derivative).

Nucleotide and Amino Acid Sequence Symbols

The following symbols are used to represent nucleotides herein:

25   Base
     Symbol   Meaning
      A   adenine
      C   cytosine
      G   guanine
30   T   thymine
      U   uracil
      I   inosine

15

M	A or C
R	A or G
W	A or T/U
S	C or G
Y	C or T/U
K	G or T/U
V	A or C or G
H	A or C or T/U
D	A or G or T/U
B	C or G or T/U
N/X	A or C or G or T/U

There are twenty main amino acids, each of which is specified by a different arrangement of three adjacent nucleotides (triplet code or codon), and which are linked together in a specific order to form a characteristic protein. A three-letter or one-letter convention is used herein to identify said amino acids, as, for example, in Figure 1 as follows:

Amino acid name	3 Ltr. Abbrev.	1 Ltr. Abbrev.
Alanine	Ala	A
Arginine	Arg	R
Asparagine	Asn	N
Aspartic Acid	Asp	D
Cysteine	Cys	C
Glutamic Acid	Glu	E
Glutamine	Gln	Q
Glycine	Gly	G
Histidine	His	H
Isoleucine	Ile	I
Leucine	Leu	L
Lysine	Lys	K

Methionine	Met	M
Phenylalanine	Phe	F
Proline	Pro	P
Serine	Ser	S
Threonine	Thr	T
Tryptophan	Trp	W
Tyrosine	Tyr	Y
Valine	Val	V
Unknown or other		X

BRIEF DESCRIPTION OF THE FIGURES

Figure 1A-C provides the nucleotide sequence for a MN cDNA [SEQ ID NO: 1] clone isolated as described herein. Figure 1 A-C also sets forth the predicted amino acid sequence [SEQ ID NO: 2] encoded by the cDNA.

Figure 2A-F provides a 10,898 bp complete genomic sequence of MN [SEQ ID NO: 5]. The base count is as follows: 2654 A; 2739 C; 2645 G; and 2859 T. The 11 exons are in general shown in capital letters, but exon 1 is considered to begin at position 3507 as determined by RNase protection assay.

Figure 3 is a restriction map of the full-length MN cDNA. The open reading frame is shown as an open box. The thick lines below the restriction map illustrate the sizes and positions of two overlapping cDNA clones. The horizontal arrows indicate the positions of primers R1 [SEQ ID NO: 7] and R2 [SEQ ID NO: 8] used for the 5' end RACE. Relevant restriction sites are BamHI (B), EcoRV (V), EcoRI (E), PstI (Ps), PvuII (Pv).

Figure 4 schematically represents the 5' MN genomic region of a MN genomic clone wherein the numbering corresponds to transcription initiation sites estimated by RACE.

Figure 5 provides an exon-intron map of the human MN/CA IX gene. The positions and sizes of the exons (numbered, cross-hatched boxes), Alu repeat elements (open boxes) and an LTR-related sequence (first unnumbered stippled box) are adjusted to the indicated scale. The exons corresponding to individual MN/CA IX protein domains are enclosed in dashed frames designated PG (proteoglycan-like domain), CA

17

(carbonic anhydrase domain), TM (transmembrane anchor) and IC (intracytoplasmic

tail). Below the map, the alignment of amino acid sequences illustrates the extent of

homology between the MN/CA IX protein PG region (aa 53-111) [SEQ ID NO: 50] and

the human aggrecan (aa 781-839) [SEQ ID NO: 54].

5   Figure 6 is a nucleotide sequence for the proposed promoter of the

human MN gene [SEQ ID NO: 27]. The nucleotides are numbered from the

transcription initiation site according to RNase protection assay. Potential regulatory

elements are overlined. Transcription start sites are indicated by asterisks (RNase

protection) and dots (RACE) above the corresponding nucleotides. The sequence of the

10   1st exon begins under the asterisks. FTP analysis of the MN4 promoter fragment

revealed 5 regions (I-V) protected at both the coding and noncoding strands, and two

regions (VI and VII) protected at the coding strand but not at the noncoding strand.

          Figure 7 provides a schematic of the alignment of MN genomic clones

according to their position related to the transcription initiation site. All the genomic

15   fragments except Bd3 were isolated from a lambda FIX II genomic library derived from

HeLa cells. Clone Bd3 was derived from a human fetal brain library.

          Figure 8 schematically represents the MN protein structure. The

abbreviations are the same as used in Figure 5. The scale indicates the number of

amino acids.

20

DETAILED DESCRIPTION

          The terms "MN/CA IX" and "MN/CA9" are herein considered to be

synonyms for MN. Also, the G250 antigen is considered to refer to MN

25   protein/polypeptide. [Uemura et al., J. Urol., 154 (4 Suppl.): 377 (Abstract 1475;

1997).]

          MN/CA IX was first identified in HeLa cells, derived from human

carcinoma of cervix uteri, as both a plasma membrane and nuclear protein with an

apparent molecular weight of 58 and 54 kilodaltons (kDA) as estimated by Western

30   blotting. It is N-glycosylated with a single 3kDa carbohydrate chain and under non-

reducing conditions forms S-S-linked oligomers [Pastorekova et al., Virology, 187: 620-

626 (1992); Pastorek et al., Oncogene, 9: 2788-2888 (1994)]. MN/CA IX is a

transmembrane protein located at the cell surface, although in some cases it has been detected in the nucleus [Zavada et al., Int. J. Cancer, 54: 268-274 (1993); Pastorekova et al., supra].

MN is manifested in HeLa cells by a twin protein, p54/58N. 5 Immunoblots using a monoclonal antibody reactive with p54/58N (MAb M75) revealed two bands at 54 kd and 58 kd. Those two bands may correspond to one type of protein that most probably differs by post-translational processing. Herein, the phrase "twin protein" indicates p54/58N.

Zavada et al., WO 93/18152 and/or WO 95/34650 disclose the MN 10 cDNA sequence (SEQ ID NO: 1) shown herein in Figure 1A-1C, the MN amino acid sequence (SEQ ID NO: 2) also shown in Figure 1A-1C, and the MN genomic sequence (SEQ ID NO: 5) shown herein in Figure 2A-2F. The MN gene is organized into 11 exons and 10 introns.

The first thirty seven amino acids of the MN protein shown in Figure 1A-15 1C is the putative MN signal peptide [SEQ ID NO: 6]. The MN protein has an extracellular domain [amino acids (aa) 38-414 of Figure 1A-1C (SEQ ID NO: 87)], a transmembrane domain [aa 415-434 (SEQ ID NO: 52)] and an intracellular domain [aa 435-459 (SEQ ID NO: 53)]. The extracellular domain contains the proteoglycan-like domain [aa 53-111 (SEQ ID NO: 50)] and the carbonic anhydrase (CA) domain [aa 135-20 391 (SEQ ID NO: 51].

Anticancer Drugs and Antibodies that Block
Interaction of MN Protein and Receptor Molecules

25   MN protein is considered to be a uniquely suitable target for cancer therapy for a number of reasons including the following. (1) It is localized on the cell surface, rendering it accessible. (2) It is expressed in a high percentage of human carcinomas (e.g., uterine cervical, renal, colon, breast, esophageal, lung, head and neck carcinomas, among others), but is not normally expressed to any significant extent in 30 the normal tissues from which such carcinomas originate.

(3) It is normally expressed only in the stomach mucosa and in some epithelia of the digestive tract (epithelium of gallbladder and small intestine). An anatomic barrier thereby exists between the MN-expressing preneoplastic/neoplastic

and MN-expressing normal tissues. Drugs, including antibodies, can thus be administered which can reach tumors without interfering with MN-expressing normal tissues.

5   (4) MAb M75 has a high affinity and specificity to MN protein. (5) MN cDNA and MN genomic clones which encompass the protein-coding and gene regulatory sequences have been isolated. (6) MN-specific antibodies have been shown to have among the highest tumor uptakes reported in clinical studies with antitumor antibodies in solid tumors, as shown for the MN-specific chimeric antibody G250 in animal studies and in phase I clinical trials with renal carcinoma patients. [Steffens et

10 al., J. Clin. Oncol., 15: 1529 (1997).] Also, MN-specific antibodies have low uptake in normal tissues.

          Data, e.g. as presented herein, are consistent with the following theory concerning how MN protein acts in normal tissues and in preneoplastic/neoplastic tissues. In normal tissues (e.g., in stomach mucosa), MN protein is considered to be a

15 differentiation factor. It binds with its normal receptor S (for stomach). Stomach carcinomas have been shown not to contain MN protein.

          Ectopic expression of MN protein in other tissues causes malignant conversion of cells. Such ectopic expression is considered to be caused by the binding of MN protein with an alternative receptor H (for HeLa cells), coupled to a signal

20 transduction pathway leading to malignancy. Drugs or antibodies which block the binding site of MN protein for receptor H would be expected to cause reversion of prenoplastic/neoplastic cells to normal or induce their death.

Design and Development of MN-Blocking Drugs or Antibodies

25   A process to design and develop MN-blocking drugs, e.g., peptides with high affinity to MN protein, or antibodies, has several steps. First, is to test for the binding of MN protein to receptors based on the cell adhesion assay described infra. That same procedure would also be used to assay for drugs blocking the MN protein binding site. In view of the alternative receptors S and H, stomach epithelial cells or

30 revertants (containing preferentially S receptors), HeLa cells (containing the H receptor and lacking the S receptor) would be used in the cell adhesion assay.

To identify the receptor binding site of MN protein, deletion variants of
MN protein lacking different domains can be used to identify region(s) responsible for
interaction of MN protein with a receptor. Example 2 identifies and illustrates how to
detect other binding sites on MN protein. A preferred MN binding site is considered to

5   be closely related or identical to the epitope for MAb M75, which is located in at least
2 copies within the 6-fold tandem repeat of 6 amino acids [aa 61-96 (SEQ ID NO: 97)]
in the proteoglycan-like domain of the MN protein. Smaller deletion variants can be
prepared within that relevant domain, e.g., fusion proteins with only small segments of
MN protein can be prepared. Also, controlled digestion of MN protein with specific

10   proteases followed by separation of the products can be performed.

Further, peptides comprising the expected binding site can be
synthesized. All of those products can be tested in cell adhesion assays, as exemplified
below. [See, e.g., Pierschbacher and Ruoslahti, PNAS, 81:5985 (1984); Ruoslahti and
Pierschbacher, Science, 238: 491.]

15   Molecules can be constructed to block the MN receptor binding site. For
example, use of a phage display peptide library kit [as Ph.D®-7 Peptide 7-Mer Library
Kit from New England Biolabs; Beverly, MA (USA)] as exemplified in Examples 2 and 3,
can be used to find peptides with high affinity to the target molecules. Biologic activity
of the identified peptides will be tested in vitro by inhibition of cell adhesion to MN

20   protein, by effects on cell morphology and growth characteristics of MN-related tumor
cells (HeLa) and of control cells. [Symington, J. Biol. Chem., 267: 25744 (1992).] In
vivo screening will be carried out in nude mice that have been injected with HeLa
cells.

Peptides containing the binding site of the MN protein will be prepared

25   [e.g. MAPs (multiple antigen peptides); Tam, J.P., PNAS (USA) 85: 5409 (1988); Butz et
al., Peptide Res., 7: 20 (1994)]. The MAPs will be used to immunize animals to obtain
antibodies (polyclonal and/or monoclonal) that recognize and block the binding site.
[See, e.g., Brooks et al., Cell, 79: 1157 (1994).] "Vaccination" would then be used to
test for protection in animals. Antibodies to the MN binding site could potentially be

30   used to block MN protein's interaction(s) with other molecules.

Computer modeling can also be used to design molecules with specific
affinity to MN protein that would mediate steric inhibition between MN protein and its

21

receptor. A computer model of the MN binding site for the receptor will contain spatial, electrostatic, hydrophobic and other characteristics of this structure. Organic molecules complementary to the structure, that best fit into the binding site, will be designed. Inorganic molecules can also be similarly tested that could block the MN

5   binding site.

The use of oncoproteins as targets for developing new cancer therapeutics is considered conventional by those of skill in the art. [See, e.g., Mendelsohn and Lippman, "Growth Factors," pp. 114-133, IN: DeVita et al. (eds.), Cancer: Principles and Practice of Oncology (4th Ed.; Lippincott; Philadelphia, 1993).] In

10   its broadest sense, the design of blocking drugs can be based in competitive inhibition experiments. Such experiments have been used to invent drugs since the discovery of sulfonamides (competitive inhibitors of para-aminobenzoic acid, a precursor of folic acid). Also, some cytostatics are competitive inhibitors (e.g., halogenated pyrimidines, among others).

15   However, the application of such approaches to MN is new. In comparison to other tumor-related molecules (e.g. growth factors and their receptors), MN has the unique property of being differentially expressed in preneoplastic/neoplastic and normal tissues, which are separated by an anatomic barrier.

20

MN Gene – Cloning and Sequencing

Figure 1A-C provides the nucleotide sequence for a full-length MN cDNA clone isolated as described below [SEQ ID NO: 1]. Figure 2A-F provides a complete MN genomic sequence [SEQ ID NO: 5]. Figure 6 shows the nucleotide sequence for a

25   proposed MN promoter [SEQ ID NO: 27].

It is understood that because of the degeneracy of the genetic code, that is, that more than one codon will code for one amino acid [for example, the codons TTA, TTG, CTT, CTC, CTA and CTG each code for the amino acid leucine (leu)], that variations of the nucleotide sequences in, for example, SEQ ID NOS: 1 and 5 wherein

30   one codon is substituted for another, would produce a substantially equivalent protein or polypeptide according to this invention. All such variations in the nucleotide

22

sequences of the MN cDNA and complementary nucleic acid sequences are included within the scope of this invention.

It is further understood that the nucleotide sequences herein described and shown in Figures 1, 2 and 6, represent only the precise structures of the cDNA,

5   genomic and promoter nucleotide sequences isolated and described herein. It is expected that slightly modified nucleotide sequences will be found or can be modified by techniques known in the art to code for substantially similar or homologous MN proteins and polypeptides, for example, those having similar epitopes, and such nucleotide sequences and proteins/ polypeptides are considered to be equivalents for

10 the purpose of this invention. DNA or RNA having equivalent codons is considered within the scope of the invention, as are synthetic nucleic acid sequences that encode proteins/polypeptides homologous or substantially homologous to MN proteins/polypeptides, as well as those nucleic acid sequences that would hybridize to said exemplary sequences [SEQ. ID. NOS. 1, 5 and 27] under stringent conditions, or

15 that, but for the degeneracy of the genetic code would hybridize to said cDNA nucleotide sequences under stringent hybridization conditions. Modifications and variations of nucleic acid sequences as indicated herein are considered to result in sequences that are substantially the same as the exemplary MN sequences and fragments thereof.

20   Stringent hybridization conditions are considered herein to conform to standard hybridization conditions understood in the art to be stringent. For example, it is generally understood that stringent conditions encompass relatively low salt and/or high temperature conditions, such as provided by 0.02 M to 0.15 M NaCl at temperatures of 50°C to 70°C. Less stringent conditions, such as, 0.15 M to 0.9 M salt

25 at temperatures ranging from 20°C to 55°C can be made more stringent by adding increasing amounts of formamide, which serves to destabilize hybrid duplexes as does increased temperature.

Exemplary stringent hybridization conditions are described in Sambrook et al., Molecular Cloning: A Laboratory Manual, pages 1.91 and 9.47-9.51 (Second

30 Edition, Cold Spring Harbor Laboratory Press; Cold Spring Harbor, NY; 1989); Maniatis et al., Molecular Cloning: A Laboratory Manual, pages 387-389 (Cold Spring Harbor

Laboratory; Cold Spring Harbor, NY; 1982); Tsuchiya et al., Oral Surgery, Oral

Medicine, Oral Pathology, 71(6): 721-725 (June 1991).

       Zavada et al., WO 95/34650 described how a partial MN cDNA clone, a

full-length MN cDNA clone and MN genomic clones were isolated and sequenced.

5   Also, Zavada et al., Int. J. Cancer, 54: 268 (1993) describes the isolation and

sequencing of a partial MN cDNA of 1397 bp in length. Briefly attempts to isolate a

full-length clone from the original cDNA library failed. Therefore, the inventors

performed a rapid amplification of cDNA ends (RACE) using MN-specific primers, R1

and R2 [SEQ ID NOS: 7 and 8], derived from the 5' region of the original cDNA clone.

10   The RACE product was inserted into pBluescript, and the entire population of

recombinant plasmids was sequenced with an MN-specific primer ODN1 [SEQ ID NO:

3]. In that way, a reliable sequence at the very 5' end of the MN cDNA as shown in

Figure 1 [SEQ ID NO: 1] was obtained.

       Specifically, RACE was performed using 5' RACE System [GIBCO BRL;

15   Gaithersburg, MD (USA)] as follows. 1 μg of mRNA (the same as above) was used as a

template for the first strand cDNA synthesis which was primed by the MN-specific

antisense oligonucleotide, R1 (5'-TGGGGTTCTTGAGGATCTCCAGGAG-3') [SEQ ID

NO: 7]. The first strand product was precipitated twice in the presence of ammonium

acetate and a homopolymeric C tail was attached to its 3' end by TdT. Tailed cDNA

20   was then amplified by PCR using a nested primer, R2 (5'-

CTCTAACTTCAGGGAGCCCTCTTCTT-3') [SEQ ID NO: 8] and an anchor primer that

anneals to the homopolymeric tail (5'-CUACUACUACUAGGCCACGCGTCGAC

TAGTACGGGI IGGGIIGGGIIG-3') [SEQ ID NO: 9]. The amplified product was

digested with BamHI and SalI restriction enzymes and cloned into pBluescript II KS

25   plasmid. After transformation, plasmid DNA was purified from the whole population of

transformed cells and used as a template for sequencing with the MN-specific primer

ODN1 [SEQ ID NO: 3; a 29-mer 5' CGCCCAGTGGGTCATCTTCCCCAGAAGAG 3'].

       To study MN regulation, MN genomic clones were isolated. One MN

genomic clone (Bd3) was isolated from a human cosmid library prepared from fetal

30   brain using both MN cDNA as a probe and the MN-specific primers derived from the 5'

end of the cDNA ODN1 [SEQ ID NO: 3, supra] and ODN2 [SEQ. ID NO.: 4; 19-mer

(5' GGAATCCTCCTGCATCCGG 3')]. Sequence analysis revealed that that genomic

clone covered a region upstream from a MN transcription start site and ending with the BamHI restriction site localized inside the MN cDNA. Other MN genomic clones can be similarly isolated.

Figure 7 provides a schematic of the alignment of MN genomic clones
5   according to the transcription initiation site. Plasmids containing the A4a clone and the XE1 and XE3 subclones were deposited at the American Type Culture Collection (ATCC) on June 6, 1995, respectively under ATCC Deposit Nos. 97199, 97200, and 97198.

10 Exon-Intron Structure of Complete MN Genomic Region

The complete sequence of the overlapping clones contains 10,898 bp (SEQ ID NO: 5). Figure 5 depicts the organization of the human MN gene, showing the location of all 11 exons as well as the 2 upstream and 6 intronic Alu repeat elements. All the exons are small, ranging from 27 to 191 bp, with the exception of the
15 first exon which is 445 bp. The intron sizes range from 89 to 1400 bp. The CA domain is encoded by exons 2-8, while the exons 1, 10 and 11 correspond respectively to the proteoglycan-like domain, the transmembrane anchor and cytoplasmic tail of the MN/CA IX protein. Table 1 below lists the splice donor and acceptor sequences that conform to consensus splice sequences including the AG-GT motif [Mount, Nucleic
20 Acids Res. 10: 459-472 (1982)].

TABLE 1

Exon-Intron Structure of the Human MN Gene

Intron	Size	Genomic Position**	SEQ ID NO	5'splice acceptor	SEQ ID NO
1	445	*3507-3951	28	AGAAG gtaagt	67
2	30	5126-5155	29	TGGAG gtgaga	68
3	171	5349-5519	30	CAGTC gtgagg	69
4	143	5651-5793	31	CCGAG gtgagc	70
5	93	5883-5975	32	TGGAG gtacca	71
6	67	7376-7442	33	GGAAG gtcagt	72
7	158	8777-8934	34	AGCAG gtgggc	73
8	145	9447-9591	35	GCCAG gtacag	74
9	27	9706-9732	36	TGCTG gtgagt	75
10	82	10350-70431	37	CACAG gtatta	76
11	191	10562-10752	38	ATAAT end	

Intron	Size	Genomic Position **	SEQ ID NO	3'splice acceptor	SEQ ID NO
1	1174	3952-5125	39	atacag GGGAT	77
2	193	5156-5348	40	ccccag GCGAC	78
3	131	5520-5650	41	acgcag TGCAA	79
4	89	5794-5882	42	tttcag ATCCA	80
5	1400	5976-7375	43	ccccag GAGGG	81
6	1334	7443-8776	44	tcacag GCTCA	82
7	512	8935-9446	45	ccctag CTCCA	83
8	114	9592-9705	46	ctccag TCCAG	84
9	617	9733-10349	47	tcgcag GTGACA	85
10	130	10432-10561	48	acacag AAGGG	86

** positions are related to nt numbering in whole genomic sequence including the 5' flanking region [Figure 2A-F]

* number corresponds to transcription initiation site determined below by RNase protection assay

Mapping of MN Gene Transcription Initiation and Termination Sites

Zavada et al., WO 95/34650 describes the process of mapping the MN gene transcription initiation and termination sites. A RNase protection assay was used for fine mapping of the 5' end of the MN gene. The probe was a uniformly labeled 470

5   nucleotide copy RNA (nt -205 to +265) [SEQ ID NO: 55], which was hybridized to total RNA from MN-expressing HeLa and CGL3 cells and analyzed on a sequencing gel. That analysis has shown that the MN gene transcription initiates at multiple sites, the 5' end of the longest MN transcript being 30 nt longer than that previously characterized by RACE.

10

Characterization of the 5' Flanking Region

The Bd3 genomic clone isolated from human fetal brain cosmid library was found to cover a region of 3.5 kb upstream from the transcription start site of the MN gene. It contains no significant coding region. Two Alu repeats are situated at

15   positions -2587 to -2296 [SEQ ID NO: 56] and -1138 to -877 [SEQ ID NO: 57] (with respect to the transcription start determined by RNP).

Nucleotide sequence analysis of the DNA 5' to the transcription start (from nt -507) revealed no recognizable TATA box within the expected distance from the beginning of the first exon. However, the presence of potential binding sites for

20   transcription factors suggests that this region might contain a promoter for the MN gene. There are several consensus sequences for transcription factors AP1 and AP2 as well as for other regulatory elements, including a p53 binding site [Locker and Buzard, J., DNA Sequencing and Mapping, 1: 3-11 (1990); Imagawa et al. Cell, 51: 251-260 (1987); El Deiry et al., Nat. Genet., 1: 44-49 (1992)]. Although the putative promoter region

25   contains 59.3% C+G, it does not have additional attributes of CpG-rich islands that are typical for TATA-less promoters of housekeeping genes [Bird, Nature, 321: 209-213 (1986)]. Another class of genes lacking TATA box utilizes the initiator (Inr) element as a promoter. Many of these genes are not constitutively active, but they are rather regulated during differentiation or development. The Inr has a consensus sequence of

30   PyPyPyCAPyPyPyPy [SEQ ID NO: 23] and encompasses the transcription start site [Smale and Baltimore, Cell, 57: 103-113 (1989)]. There are two such consensus

sequences in the MN putative promoter; however, they do not overlap the transcription start (Figure 6).

An interesting region was found in the middle of the MN gene. The region is about 1.4 kb in length [nt 4,600-6,000 of the genomic sequence; SEQ ID NO:

5   49] and spans from the 3' part of the 1st intron to the end of the 5th exon. The region has the character of a typical CpG-rich island, with 62.8% C+G content and 82 CpG: 131 GpC dinucleotides. Moreover, there are multiple putative binding sites for transcription factors AP2 and Sp1 [Locker and Buzard, supra; Briggs et al., Science, 234: 47-52 (1986)] concentrated in the center of this area. Particularly the 3rd intron of 131

10 bp in length contains three Sp1 and three AP2 consensus sequences. That data indicates the possible involvement of that region in the regulation of MN gene expression. However, functionality of that region, as well as other regulatory elements found in the proposed 5' MN promoter, remains to be determined.

15 MN Promoter

Study of the MN promoter has shown that it is TATA-less and contains regulatory sequences for AP-1, AP-2, as well as two p53 binding sites. The sequence of the 5' end of the 3.5 kb flanking region upstream of the MN gene has shown extensive homology to LTR of HERV-K endogenous retroviruses. Basal transcription activity of

20 the promoter is very weak as proven by analyses using CAT and neo reporter genes. However, expression of the reporter genes is severalfold increased when driven from the 3.5 kb flanking region, indicating involvement of putative enhancers.

Functional characterization of the 3.5 kb MN 5' upstream region by deletion analysis lead to the identification of the [-173, +31] fragment [SEQ ID NO: 21]

25 (also alternatively, but less preferably, the nearly identical -172, +31 fragment [SEQ ID NO: 91]) as the MN promoter. In vitro DNase I footprinting revealed the presence of five protected regions (PR) within the MN promoter. Detailed deletion analysis of the promoter identified PR 1 and 2 (numbered from the transcription start) as the most critical for transcriptional activity. PR4 [SEQ ID NO: 115] negatively affected

30 transcription as its deletion led to increased promoter activity and was confirmed to function as a promoter-, position- and orientation-independent silencer element. Mutational analysis indicated that the direct repeat AGGGCacAGGGC [SEQ ID NO:

143] is required for efficient repressor binding. Two components of the repressor complex (35 and 42 kDa) were found to be in direct contact with PR4 by UV crosslinking. Increased cell density, known to induce MN expression, did not affect levels of PR4 binding in HeLa cells. Significantly reduced repressor level seems to be

5   responsible for MN up-regulation in the case of tumorigenic CGL3 as compared to non-tumorigenic CGL1 HeLa x normal fibroblast hybrid cells.

Utility of MN Promoter as a Tumor-Specific
Promoter for Gene Therapy

10   Being investigated is whether the MN gene promoter can be used as a tumor-specific promoter to drive the expression of a suicide gene [thymidine kinase (tk) of HSV)] and mediate the direct and bystander killing of tumor cells. HSVtk gene transferred to tumor cells converts nucleoside analogue ganciclovir (GCV) to toxic triphosphates and mediates the death of transduced and also neighboring tumor cells.

15 The control of HSVtk by the MN gene promoter would allow its expression only in tumor cells, which are permissive for the biosynthesis of MN protein, and selectively kill such tumor cells, but not normal cells in which MN expression is repressed.

            A plasmid construct in which HSVtk was cloned downstream of the MN promoter region Bd3, containing both proximal and distant regulatory elements of MN,

20 was prepared. That plasmid pMN-HSVtk was transfected to Rat2TK- cells and C33 human cervical carcinoma cells using calcium phosphate precipitation and lipofection, respectively. Transfectants were tested for expression of HSVtk and GVC sensitivity. Analysis of the transfectants has shown the remarkable cytotoxic in vitro effect of GVC even in low concentrations (up to 95% of cells killed).

25   Polyclonal rabbit antiserum against HSVtk, using fusion protein with GST in pGEX-3X, has been prepared to immunodetect HSVtk synthesized in transfected cells. This model system is being studied to estimate the bystander effect, the inhibition of cloning efficiency and invasiveness of transduced and GVC-treated cells to collagen matrices. A recombinant retroviral vector with the MN promoter-driven HSVtk is to be

30 prepared to test its in vivo efficacy using an animal model (e.g., SCID-mouse).

29

MN Promoter Analysis

Since the MN promoter is weak, a classical approach to study it would be limited due to the relatively low efficiency of transient transfections (up to 10%). Therefore, stable clonal cell lines expressing constructs containing the MN promoter

5   fused to the CAT gene were prepared. In such clonal lines, 100% of the cells express the CAT gene driven from the MN promoter, and thus, the activity of the promoter is detectable easier than in transient experiments. Also, the promoter activity can be analysed repeatedly in the same cells under different conditions or treated by different factors and drugs. This approach allows for the study of the mechanisms underlying

10 MN regulation at the level of transcription initiation.

Several types of transfections with promoter constructs linked to a reporter CAT gene (calcium precipitation, DEAE dextran combined with DMSO shock and/or chloroquine, as well as electroporation), different methods of CAT activity assay (scintillation method, thin layer chromatography) and several recipient cell lines

15 differing in the level of MN expression and in transfection efficiency (HeLa, SiHa, CGL3, KATO III, Rat2TK⁻ and C33 cells). Activity of the MN promoter was detected preferably by the electroporation of CGL3 cells and thin layer chromatography. Further preferably, C33 cells cotransfected with MN promoter-CAT constructs and pSV2neo were used.

20   1. To detect basal activity of the MN promoter and to estimate the position of the core promoter, expression of the CAT gene from constructs pMN1 to pMN7 after transfection to CGL3 cells was analyzed. Plasmids with progressive 5' deletions were transfected into CGL3 cells and activity was analyzed by CAT assay. [8 μg of DNA was used for transfection in all cases except pBLV-LTR (2 μg).]

25   Only very weak CAT activity was detected in cells transfected by pMN1 and pMN2 (containing respectively 933 bp and 600 bp of the promoter sequence). A little higher activity was exhibited with the constructs pMN3, pMN4 and pMN6 (containing respectively 446 bp, 243 bp and 58 bp of the promoter). A slight peak of activity was obtained with pMN5 (starting at position -172 with respect to the

30 transcription start.) Thus, the function of the MN core promoter can be assigned to a region of approximately 500 bp immediately upstream from the MN transcription initiation site.

Interestingly, the activity of the large Bd3 region (covering 3.5 kbp upstream of the transcription start) was severalfold higher than the activity of the core promoter. However, its level was still much lower than that exhibited by a positive control, i.e., BLV-LTR transactivated by Tax, and even lower than the activity of BLV-

5 LTR without transactivation. That the activity of Bd3 was elevated in comparison to the core promoter suggests the presence of some regulatory elements. Such elements are most probably situated in the sequence between pMN1 and Bd3 (i.e. from -1 kbp to -3.5 kbp) [SEQ ID NO: 58]. The cloning and transfection of several deletion versions of Bd3 covering the indicated region can be used to determine the location of the putative

10 regulatory elements.

Similar results were obtained from transfecting KATO III cells with Bd3 and pMN4. The transfected cells expressed a lower level of MN than the CGL3 cells. Accordingly, the activity of the MN promoter was found to be lower than in CGL3 cells.

15 2. In a parallel approach to study the MN promoter, an analysis based on G418 selection of cells transfected by plasmids containing the promoter of interest cloned upstream from the neo gene was made. This approach is suitable to study weak promoters, since its sensitivity is much higher than that of a standard CAT assay. The principle underlying the method is as follows: an active promoter drives expression of

20 the neo gene which protects transfected cells from the toxic effect of G418, whereas an inactive promoter results in no neo product being made and the cells transfected thereby die upon the action of G418. Therefore, the activity of the promoter can be estimated according to the number of cell colonies obtained after two weeks of selection with G418. Three constructs were used in the initial experiments –

25 pMN1neo, pMN4neo and pMN7neo. As pMN7neo contains only 30 bp upstream of the transcription start site, it was considered a negative control. As a positive control, pSV2neo with a promoter derived from SV40 was used. Rat2TK⁻ cells were chosen as the recipient cells, since they are transfectable with high efficiency by the calcium precipitation method.

30 After transfection, the cells were subjected to two weeks of selection. Then the medium was removed, the cells were rinsed with PBS, and the colonies were rendered visible by staining with methylene blue. The results obtained from three

31

independent experiments corroborated the data from the CAT assays. The promoter

construct pMN4neo exhibited higher transcriptional activity than pMN1neo. However,

the difference between the positive control and pMN4neo was not so striking as in the

CAT assay. That may have been due to both lower promoter activity of pSV2neo

5   compared to Tax-transactivated pBLV-LTR and to different conditions for cell growth

after transfection. From that point of view, stable transfection is probably more

advantageous for MN expression, since the cells grow in colonies with close cell to cell

contact, and the experiment lasts much longer, providing a better opportunity to detect

promoter activity.

10   3. Stable transfectants expressing MN promoter-CAT chimeric genes

were prepared by the cotransfection of relevant plasmids with pSV2neo. As recipient

cells, HeLa cells were used first. However, no clones expressing the promoter-CAT

constructs were obtained. That negative result was probably caused by homologic

recombination of the transfected genomic region of MN (e.g. the promoter) with the

15   corresponding endogenous sequence. On the basis of that experience, C33 cells

derived from a HPV-negative cervical carcinoma were used. C33 cells do not express

MN, since during the process of tumorigenesis, they lost genetic material including

chromosomal region 9p which contains the MN gene. In these experiments, the

absence of the MN gene may represent an advantage as the possibility of homologic

20   recombinations is avoided.

C33 Cells Transfected with MN Promoter-CAT Constructs

             C33 cells expressing the CAT gene under MN promoter regions Bd3 (-

3500/+31) [SEQ ID NO: 90] and MN5 (-172/+31) [SEQ ID NO: 91] were used for

25   initial experiments to analyze the influence of cell density on the transcriptional activity

of the MN promoter. The results indicated that signals generated after cells come into

close contact activate transcription of the CAT protein from the MN promoter in

proportion to the density of the cell culture. Interestingly, the data indicated that the

MN protein is not required for this phase of signal transduction, since the influence of

30   density is clearly demonstrated in MN-negative C33 cells. Rather, it appears that MN

protein acts as an effector molecule produced in dense cells in order to perform a

certain biological function (i.e., to perturb contact inhibition). Also interestingly, the

MN promoter activity is detectable even in very sparse cell cultures suggesting that MN is expressed at a very low level also is sparse subconfluent culture.

Deletion Variants. Deletion variants of the Bd3-CAT promoter construct were then prepared. The constructs were cotransfected with pSV2neo into C33 cervical

5   cells. After selection with G418, the whole population of stably transfected cells were subjected to CAT ELISA analysis. Expression of the deletion constructs resulted in the synthesis of similar levels of CAT protein to that obtained with the Bd3-CAT construct. On the basis of that preliminary data, the inventors proposed that sequences stimulating transcription of MN are located between -3506 and -3375 bp [SEQ ID NO: 92]

10   upstream from the transcription start. That is the sequence exhibiting homology to HERV-K LTR.

However, transient transfection studies in CGL3 cells repeatedly revealed that the LTR region is not required for the enhancement of basal MN promoter activity. Further, results obtained in CGL3 cells indicate that the activating element is localized

15   in the region from -933 to -2179 [SEQ ID NO: 110] with respect to transcription initiation site (the position of the region having been deduced from overlapping sequences in the Bd3 deletion mutants).

Interaction of Nuclear Proteins with MN Promoter Sequences

20   In order to identify transcription factors binding to the MN promoter and potentially regulating its activity, a series of analyses using an electrophoretic mobility shift assay (EMSA) and DNase I footprinting analysis (FTP) were performed.

EMSA

25   In the EMSA, purified promoter fragments MN4 (-243/+31) [SEQ ID NO: 93], MN5 (-172/+31) [SEQ ID NO: 91], MN6 (-58/+31) [SEQ ID NO: 94] and pMN7 (-30/+31) [SEQ ID NO: 95], labeled at the 3' ends by Klenow enzyme, were allowed to interact with proteins in nuclear extracts prepared from CGL1 and CGL3 cells. [40 μg of nuclear proteins were incubated with 30,000 cpm end-labeled DNA fragments in

30   the presence of 2 μg poly(dldC).] DNA-protein complexes were analysed by PAGE (native 6%), where the complexes created extra bands that migrated more slowly than

the free DNA fragments, due to the shift in mobility which is dependent on the moiety
of bound protein.

The EMSA of the MN4 and MN5 promoter fragments revealed several
DNA-protein complexes; however, the binding patterns obtained respectively with

5   CGL1 and CGL3 nuclear extracts were not identical. There is a single CGL-1 specific
complex.

The EMSA of the MN6 promoter fragment resulted in the formation of
three identical complexes with both CGL1 and CGL3 nuclear extracts, whereas the
MN7 promoter fragment did not bind any nuclear proteins.

10   The EMSA results indicated that the CGL1 nuclear extract contains a
specific factor, which could participate in the negative regulation of MN expression in
CGL1 cells. Since the specific DNA-protein complex is formed with MN4
(-243/+31) [SEQ. ID NO.: 93] and MN5 (-172/+31) [SEQ. ID NO.: 91] promoter
fragments, but not with MN6 (-58/+31) [SEQ ID NO: 94], it appears that the binding

15   site of the protein component of that specific complex is located between -173 and -58
bp [SEQ. ID NO.: 96] with respect to transcription initiation.

The next step was a series of EMSA analyses using double stranded (ds)
oligonucleotides designed according to the protected regions in FTP analysis. A ds
oligonucleotide derived from the protected region PR2 [covering the sequence from -72

20   to -56 bp (SEQ ID NO: 111)] of the MN promoter provided confirmation of the binding
of the AP-1 transcription factor in competitive EMSA using commercial ds
olignucleotides representing the binding site for AP-1.

EMSA of ds oligonucleotides derived from the protected regions of PR1 [-
46 to -24 bp (SEQ ID NO: 112)], PR2 [-72 to -56 bp (SEQ ID NO: 111)], PR3 [-102 to -

25   85 (SEQ ID NO: 113)] and PR5 [-163 to -144 (SEQ ID NO: 114)] did not reveal any
differences in the binding pattern of nuclear proteins extracted from CGL1 and CGL3
cells, indicating that those regions do not bind crucial transcription factors which
control activation of the MN gene in CGL3, or its negative regulation in CGL1.
However, EMSA of ds oligonucleotides from the protected region PR4 [-133 to -108;

30   SEQ ID NO: 115] repeatedly showed remarkable quantitative differences between
binding of CGL1 and CGL3 nuclear proteins. CGL1 nuclear proteins formed a
substantially higher amount of DNA-protein complexes, indicating that the PR4 region

34

contains a binding site for specific transcription factor(s) that may represent a negative

regulator of MN gene transcription in CGL1 cells. That fact is in accord with the

previous EMSA data which showed CGL-1 specific DNA-protein complex with the

promoter fragments pMN4 (-243/+31; SEQ ID NO: 93) and pMN5 (-172/+31; SEQ ID

5   NO: 91), but not with pMN6 (-58/+31; SEQ ID NO: 94).

To identify the protein involved or the formation of a specific complex

with the MN promoter in the PR4 region, relevant ds oligonucleotides covalently

bound to magnetic beads will be used to purify the corresponding transcription factor.

Alternatively the ONE Hybrid System® [Clontech (Palo Alto, CA (USA)] will be used to

10   search for and clone transcription factors involved in regulation of the analysed

promoter region. A cDNA library from HeLa cells will be used for that investigation.

FTP

To determine the precise location of cis regulatory elements that

15   participate in the transcriptional regulation of the MN gene, FTP was used. Proteins in

nuclear extracts prepared respectively from CGL1 and CGL3 cells were allowed to

interact with a purified ds DNA fragment of the MN promoter (MN4, -243/+31) [SEQ

ID NO: 93] which was labeled at the 5' end of one strand. [MN4 fragments were

labeled either at Xho1 site (-243/+31*) or at Xba1 site (*-243/+31).] The DNA-protein

20   complex was then subjected to DNase I attack, which causes the DNA chain to break at

certain bases if they are not in contact with proteins. [A control used BSA instead of

DNase.] Examination of the band pattern of the denatured DNA after gel

electrophoresis [8% denaturing gel] indicates which of the bases on the labeled strand

were protected by protein.

25   FTP analysis of the MN4 promoter fragment revealed 5 regions (I-V)

protected at both the coding and noncoding strand, as well as two regions (VI and VII)

protected at the coding strand but not at the noncoding strand. Figure 6 indicates the

general regions on the MN promoter that were protected.

The sequences of the identified protected regions (PR) were subjected to

30   computer analysis using the SIGNALSCAN program to see if they corresponded to

known consensus sequences for transcription factors. The data obtained by that

computer analyses are as follows:

PR I   -   coding strand - AP-2, p53, GAL4 noncoding strand - JCV-repeated

PR II   -   coding strand - AP-1, CGN4 noncoding strand - TCF-1, dFRA, CGN4

PR III -   coding strand - no known consensus sequence, only partial overlap of

AP1 noncoding strand - 2 TCF-1 sites

5   PR IV   -   coding strand - TCF-1, ADR-1 noncoding strand - CTCF, LF-A1, LBP-1

PR V   -   coding strand - no known consensus motif noncoding strand - JCV

repeated

PR VI   -   coding strand - no known consensus motif noncoding strand - T antigen

of SV 40, GAL4

10   PR VII -   coding strand - NF-uE4, U2snRNA.2 noncoding strand - AP-2, IgHC.12,

MyoD.

In contrast to EMSA, the FTP analysis did not find any differences

between CGL1 and CGL3 nuclear extracts. However, the presence of specific DNA-

15   protein interactions detected in the CGL1 nuclear extracts by EMSA could have resulted

from the binding of additional protein to form DNA protein-protein complex. If that

specific protein did not contact the DNA sequence directly, its presence would not be

detectable by FTP.

20   EMSA Supershift Analysis

The results of the FTP suggests that transcription factors AP-1, AP-2 as

well as tumor suppressor protein p53 are potentially involved in the regulation of MN

expression. To confirm binding of those particular proteins to the MN promoter, a

supershift analysis using antibodies specific for those proteins was performed. For this

25   analysis, DNA-protein complexes prepared as described for EMSA were allowed to

interact with MAbs or polyclonal antibodies specific for proteins potentially included in

the complex. The binding of antibody to the corresponding protein results in an

additional shift (supershift) in mobility of the DNA-protein-antibody complex which is

PAGE visualized as an additional, more slowly migrating band.

30   By this method, the binding of AP-2 to the MN promoter was confirmed.

However, this method did not evidence binding of the AP-1 transcription factor. It is

possible that MN protein binds AP-1-related protein, which is antigenically different from the AP-1 recognized by the antibodies used in this assay.

Also of high interest is the possible binding of the p53 tumor suppressor protein to the MN promoter. It is well known that wt p53 functions as a transcription

5   factor, which activates expression of growth-restricting genes and down-modulates, directly or indirectly, the expression of genes that are required for ongoing cell proliferation. Transient co-transfection experiments using the pMN4-CAT promoter construct in combination with wt p53 cDNA and mut p53 cDNA, respectively, suggested that wt p53, but not mut p53, negatively regulates expression of MN. In

10   addition, one of two p53-binding sites in the MN promoter is protected in FTP analysis (Figure 6), indicating that it binds to the corresponding protein. Therefore, supershift analysis to prove that p53 binds to the MN promoter with two p53-specific antibodies, e.g. Mabs 421 and DO-1 [the latter kindly provided by Dr. Vojtesek from Masaryk Memorial Cancer Institute in Brno, Czech Republic] are to be performed with

15   appropriate nuclear extracts, e.g. from MCF-7 breast carcinoma cells which express wt p53 at a sufficient level.

Regulation of MN Expression and MN Promoter

MN appears to be a novel regulatory protein that is directly involved in

20   the control of cell proliferation and in cellular transformation. In HeLa cells, the expression of MN is positively regulated by cell density. Its level is increased by persistent infection with LCMV. In hybrid cells between HeLa and normal fibroblasts, MN expression correlates with tumorigenicity. The fact that MN is not present in nontumorigenic hybrid cells (CGL1), but is expressed in a tumorigenic segregant

25   lacking chromosome 11, indicates that MN is negatively regulated by a putative suppressor in chromosome 11.

Evidence supporting the regulatory role of MN protein was found in the generation of stable transfectants of NIH 3T3 cells that constitutively express MN protein. As a consequence of MN expression, the NIH 3T3 cells acquired features

30   associated with a transformed phenotype: altered morphology, increased saturation density, proliferative advantage in serum-reduced media, enhanced DNA synthesis and capacity for anchorage-independent growth. Further, flow cytometric analyses of

asynchronous cell populations indicated that the expression of MN protein leads to accelerated progression of cells through G1 phase, reduction of cell size and the loss of capacity for growth arrest under inappropriate conditions. Also, MN expressing cells display a decreased sensitivity to the DNA damaging drug mitomycin C.

5   Nontumorigenic human cells, CGL1 cells, were also transfected with the full-length MN cDNA. The same pSG5C-MN construct in combination with pSV2neo plasmid as used to transfect the NIH 3T3 cells was used. Out of 15 MN-positive clones (tested by SP-RIA and Western blotting), 3 were chosen for further analysis. Two MN-negative clones isolated from CGL1 cells transfected with empty plasmid were added as controls. Initial analysis indicates that the morphology and growth habits of MN-transfected CGL1 cells are not changed dramatically, but their proliferation rate and plating efficiency is increased.

MN Promoter – Sense/Antisense Constructs

15   When the promoter region from the MN genomic clone, isolated as described above, was linked to MN cDNA and transfected into CGL1 hybrid cells, expression of MN protein was detectable immediately after selection. However, then it gradually ceased, indicating thus an action of a feedback regulator. The putative regulatory element appeared to be acting via the MN promoter, because when the full-length cDNA (not containing the promoter) was used for transfection, no similar effect was observed.

An "antisense" MN cDNA/MN promoter construct was used to transfect CGL3 cells. The effect was the opposite of that of the CGL1 cells transfected with the "sense" construct. Whereas the transfected CGL1 cells formed colonies several times larger than the control CGL1, the transfected CGL3 cells formed colonies much smaller than the control CGL3 cells. The same result was obtained by antisense MN cDNA transfection in SiHa and HeLa cells.

For those experiments, the part of the promoter region that was linked to the MN cDNA through a BamHI site was derived from a NcoI - BamHI fragment of the MN genomic clone [Bd3] and represents a region a few hundred bp upstream from the transcription initiation site. After the ligation, the joint DNA was inserted into a pBK-CMV expression vector [Stratagene]. The required orientation of the inserted sequence

was ensured by directional cloning and subsequently verified by restriction analysis. The tranfection procedure was the same as used in transfecting the NIH 3T3 cells, but co-transfection with the pSV2neo plasmid was not necessary since the neo selection marker was already included in the pBK-CMV vector.

5   After two weeks of selection in a medium containing G418, remarkable differences between the numbers and sizes of the colonies grown were evident as noted above. Immediately following the selection and cloning, the MN-transfected CGL1 and CGL3 cells were tested by SP-RIA for expression and repression of MN, respectively. The isolated transfected CGL1 clones were MN positive (although the

10   level was lower than obtained with the full-length cDNA), whereas MN protein was almost absent from the transfected CGL3 clones. However, in subsequent passages, the expression of MN in transfected CGL1 cells started to cease, and was then blocked perhaps evidencing a control feedback mechanism.

          As a result of the very much lowered proliferation of the transfected

15   CGL3 cells, it was difficult to expand the majority of cloned cells (according to SP-RIA, those with the lowest levels of MN), and they were lost during passaging. However, some clones overcame that problem and again expressed MN. It is possible that once those cells reached a higher quantity, that the level of endogenously produced MN mRNA increased over the amount of ectopically expressed antisense mRNA.

20

Identification of Specific Transcription
Factors Involved in Control of MN Expression

          Control of MN expression at the transcription level involves regulatory

25   elements of the MN promoter. Those elements bind transcription factors that are responsible for MN activation in tumor cells and/or repression in normal cells. The identification and isolation of those specific transcription factors and an understanding of how they regulate MN expression could result in their therapeutic utility in modulating MN expression.

30   EMSA experiments indicate the existence of an MN gene repressor. Using the One Hybrid System® [Clontech (Palo Alto, CA); an in vivo yeast genetic assay for isolating genes encoding proteins that bind to a target, cis-acting regulatory element or any other short DNA-binding sequence; Fields and Song, Nature, 340: 245 (1989);

Wu et al., EMBO J., 13: 4823 (1994)] and subtractive suppressive PCR (SSH). SSH

allows the cloning of genes that are differentially expressed under conditions which are

known to up or down regulate MN expression such as density versus sparsity of HeLa

cells, and suspension versus adherent HeLa cells.

5   In experiments with HPV immobilized cervical cells (HCE 16/3), it was

found that the regulation of MN expression differs from that in fully transformed

carcinoma cells. For example, glucocorticoid hormones, which activate HPV

transcription, negatively regulate MN expression in HCE, but stimulate MN in HeLa and

SiHa. Further keratinocyte growth factors, which down regulates transcription of HPV

10   oncogenes, stimulates MN expression in suspension HCE but not in adherent cells.

EGF and insulin are involved in the activation of MN expression in both

immortalized and carcinoma cells. All the noted facts can be used in the search for

MN-specific transcription factors and in the modulation of MN expression for

therapeutic purposes.

15

Deduced Amino Acid Sequence

The ORF of the MN cDNA shown in Figure 1 has the coding capacity for

a 459 amino acid protein with a calculated molecular weight of 49.7 kd. The overall

amino acid composition of the MN/CA IX protein is rather acidic, and predicted to have

20   a pi of 4.3. Analysis of native MN/CA IX protein from CGL3 cells by two-dimensional

electrophoresis followed by immunoblotting has shown that in agreement with

computer prediction, the MN/CA IX is an acidic protein existing in several isoelectric

forms with pIs ranging from 4.7 to 6.3.

As assessed by amino acid sequence analysis, the deduced primary

25   structure of the MN protein can be divided into four distinct regions. The initial

hydrophobic region of 37 amino acids (aa) corresponds to a signal peptide. The mature

protein has an N-terminal or extracellular part of 377 amino acids [aa 38-414 (SEQ ID

NO: 87], a hydrophobic transmembrane segment of 20 amino acids [aa 415-434 (SEQ

ID NO: 52)] and a C-terminal region of 25 amino acids [aa 435-459 (SEQ ID NO: 53)].

30   The extracellular part is composed of two distinct domains: (1) a

proteoglycan-like domain [aa 53-111 (SEQ ID NO: 50)]; and (2) a CA domain, located

close to the plasma membrane [aa 135-391 (SEQ ID NO: 51)]. [The amino acid

numbers are keyed to those of Figure 1.]

More detailed insight into MN protein primary structure disclosed the

presence of several consensus sequences. One potential N-glycosylation site was

5   found at position 346 of Figure 1. That feature, together with a predicted membrane-

spanning region are consistent with the results, in which MN was shown to be an N-

glycosylated protein localized in the plasma membrane. MN protein sequence

deduced from cDNA was also found to contain seven S/TPXX sequence elements [SEQ

ID NOS: 25 AND 26] (one of them is in the signal peptide) defined by Suzuki, J. Mol.

10   Biol., 207: 61-84 (1989) as motifs frequently found in gene regulatory proteins.

However, only two of them are composed of the suggested consensus amino acids.

Experiments have shown that the MN protein is able to bind zinc cations,

as shown by affinity chromatography using Zn-charged chelating sepharose. MN

protein immunoprecipitated from HeLa cells by Mab M75 was found to have weak

15   catalytic activity of CA. The CA-like domain of MN has a structural predisposition to

serve as a binding site for small soluble domains. Thus, MN protein could mediate

some kind of signal transduction.

MN protein from LCMV-infected HeLA cells was shown by using DNA

cellulose affinity chromatography to bind to immobilized double-stranded salmon

20   sperm DNA. The binding activity required both the presence of zinc cations and the

absence of a reducing agent in the binding buffer.

CA Domain Required for Anchorage
Independence But for Increased
25   ### Proliferation of Transfected NIH 3T3 Fibroblasts

In transfected NIH 3T3 fibroblasts, MN protein induces morphologic

transformation, increased proliferation and anchorage independence. The

consequences of constitutive expression of two MN-truncated variants in NIH 3T3 cells

30   were studied. It was found that the proteoglycan-like region is sufficient for the

morphological alteration of transfected cells and displays the growth-promoting activity

presumably related to perturbation of contact inhibition.

The CA domain is essential for induction of anchorage independence, whereas the TM anchor and IC tail are dispensable for that biological effect. The MN protein is also capable of causing plasma membrane ruffling in the transfected cells and appears to participate in their attachment to the solid support. The data evince the

5   involvement of MN in the regulation of cell proliferation, adhesion and intercellular communication.

Sequence Similarities

Computer analysis of the MN cDNA sequence was carried out using

10   DNASIS and PROSIS (Pharmacia Software packages). GenBank, EMBL, Protein Identification Resource and SWISS-PROT databases were searched for all possible sequence similarities. In addition, a search for proteins sharing sequence similarities with MN was performed in the MIPS databank with the FastA program [Pearson and Lipman, PNAS (USA), 85: 2444 (1988)].

15   The proteoglycan-like domain [aa 53-111 (SEQ ID NO: 50)], which is between the signal peptide and the CA domain, shows significant homology (38% identity and 44% positivity) with a keratan sulphate attachment domain of a human large aggregating proteoglycan aggrecan [Doege et al., J. Biol. Chem., 266: 894-902 (1991)].

20   The CA domain [aa 135-391 (SEQ ID NO: 51)] is spread over 265 aa and shows 38.9% amino acid identity with the human CA VI isoenzyme [Aldred et al., Biochemistry, 30: 569-575 (1991)]. The homology between MN/CA IX and other isoenzymes is as follows: 35.2% with CA II in a 261 aa overlap [Montgomery et al., Nucl. Acids. Res., 15: 4687 (1987)], 31.8% with CA I in a 261 aa overlap [Barlow et

25   al., Nucl. Acids Res., 15: 2386 (1987)], 31.6% with CA IV in a 266 aa overlap [Okuyama et al., PNAS (USA) 89: 1315-1319 (1992)], and 30.5% with CA III in a 259 aa overlap (Lloyd et al., Genes. Dev., 1: 594-602 (1987)].

In addition to the CA domain, MN/CA IX has acquired both N-terminal and C-terminal extensions that are unrelated to the other CA isoenzymes. The amino

30   acid sequence of the C-terminal part, consisting of the transmembrane anchor and the intracytoplasmic tail, shows no significant homology to any known protein sequence.

42

The MN gene was clearly found to be a novel sequence derived from the human genome. The overall sequence homology between the cDNA MN sequence and cDNA sequences encoding different CA isoenzymes is in a homology range of 48-50% which is considered by ones in the art to be low. Therefore, the MN cDNA

5   sequence is not closely related to any CA cDNA sequences.

Only very closely related nt sequences having a homology of at least 80-90% would hybridize to each other under stringent conditions. A sequence comparison of the MN cDNA sequence shown in Figure 1 and a corresponding cDNA of the human carbonic anhydrase II (CA II) showed that there are no stretches of identity

10   between the two sequences that would be long enough to allow for a segment of the CA II cDNA sequence having 25 or more nucleotides to hybridize under stringent hybridization conditions to the MN cDNA or vice versa.

A search for nt sequences related to MN gene in the EMBL Data Library did not reveal any specific homology except for 6 complete and 2 partial Alu-type

15   repeats with homology to Alu sequences ranging from 69.8% to 91% [Jurka and Milosavljevic, J. Mol. Evol. 32: 105-121 (1991)]. Also a 222 bp sequence proximal to the 5' end of the genomic region is shown to be closely homologous to a region of the HERV-K LTR.

In general, nucleotide sequences that are not in the Alu or LTR-like

20   regions, of preferably 25 bases or more, or still more preferably of 50 bases or more, can be routinely tested and screened and found to hybridize under stringent conditions to only MN nucleotide sequences. Further, not all homologies within the Alu-like MN genomic sequences are so close to Alu repeats as to give a hybridization signal under stringent hybridization conditions. The percent of homology between MN Alu-like

25   regions and a standard Alu-J sequence are indicated as follows:

Region of Homology within MN Genomic Sequence [SEQ ID NO: 5; Figure 2A-F]	SEQ. ID. NOS.	% Homology to Entire Alu-J Sequence
921-1212	59	89.1%
2370-2631	60	78.6%
4587-4880	61	90.1%

30

43

6463-6738	62	85.4%
7651-7939	63	91.0%
9020-9317	64	69.8%

		% Homology to One Half of Alu-J Sequence
8301-8405	65	88.8%
10040-10122	66	73.2%.

MN Proteins and/or Polypeptides

The phrase "MN proteins and/or polypeptides" (MN proteins/polypeptides) is herein defined to mean proteins and/or polypeptides encoded by an MN gene or fragments thereof. An exemplary and preferred MN protein according to this invention has the deduced amino acid sequence shown in Figure 1. Preferred MN proteins/polypeptides are those proteins and/or polypeptides that have substantial homology with the MN protein shown in Figure 1. For example, such substantially homologous MN proteins/ polypeptides are those that are reactive with the MN-specific antibodies of this invention, preferably the Mabs M75, MN12, MN9 and MN7 or their equivalents.

A "polypeptide" or "peptide" is a chain of amino acids covalently bound by peptide linkages and is herein considered to be composed of 50 or less amino acids. A "protein" is herein defined to be a polypeptide composed of more than 50 amino acids. The term polypeptide encompasses the terms peptide and oligopeptide.

MN proteins exhibit several interesting features: cell membrane localization, cell density dependent expression in HeLa cells, correlation with the tumorigenic phenotype of HeLa x fibroblast somatic cell hybrids, and expression in several human carcinomas among other tissues. MN protein can be found directly in tumor tissue sections but not in general in counterpart normal tissues (exceptions noted infra as in normal gastric mucosa and gallbladder tissues). MN is also expressed sometimes in morphologically normal appearing areas of tissue specimens exhibiting dysplasia and/or malignancy. Taken together, these features suggest a possible

involvement of MN in the regulation of cell proliferation, differentiation and/or transformation.

It can be appreciated that a protein or polypeptide produced by a neoplastic cell in vivo could be altered in sequence from that produced by a tumor cell

5   in cell culture or by a transformed cell. Thus, MN proteins and/or polypeptides which have varying amino acid sequences including without limitation, amino acid substitutions, extensions, deletions, truncations and combinations thereof, fall within the scope of this invention. It can also be appreciated that a protein extant within body fluids is subject to degradative processes, such as, proteolytic processes; thus, MN

10 proteins that are significantly truncated and MN polypeptides may be found in body fluids, such as, sera. The phrase "MN antigen" is used herein to encompass MN proteins and/or polypeptides.

It will further be appreciated that the amino acid sequence of MN proteins and polypeptides can be modified by genetic techniques. One or more amino

15 acids can be deleted or substituted. Such amino acid changes may not cause any measurable change in the biological activity of the protein or polypeptide and result in proteins or polypeptides which are within the scope of this invention, as well as, MN muteins.

The MN proteins and polypeptides of this invention can be prepared in a

20 variety of ways according to this invention, for example, recombinantly, synthetically or otherwise biologically, that is, by cleaving longer proteins and polypeptides enzymatically and/or chemically. A preferred method to prepare MN proteins is by a recombinant means. Particularly preferred methods of recombinantly producing MN proteins are described below for the GST-MN, MN 20-19, MN-Fc and MN-PA proteins.

25

Recombinant Production of MN Proteins and Polypeptides

A representative method to prepare the MN proteins shown in Figure 1 or fragments thereof would be to insert the full-length or an appropriate fragment of MN cDNA into an appropriate expression vector as exemplified below. In Zavada et al.,

30 WO 93/18152, supra, production of a fusion protein GEX-3X-MN (now termed GST-MN) using the partial cDNA clone (described above) in the vector pGEX-3X (Pharmacia)

is described. Nonglycosylated GST-MN (the MN fusion protein MN glutathione S-transferase) from XL1-Blue cells.

Zavada et al., WO 95/34650 describes the recombinant production of both a glycosylated MN protein expressed from insect cells and a nonglycosylated MN

5   protein expressed from E. coli using the expression plasmid pEt-22b [Novagen Inc.; Madison, WI (USA)]. Recombinant baculovirus express vectors were used to infect insect cells. The glycosylated MN 20-19 protein was recombinantly produced in baculovirus-infected sf9 cells [Clontech; Palo Alto, CA (USA)]. The MN 20-19 protein misses the putative signal peptide (aas 1-37) of SEQ ID NO: 6 (Figure 1), has a

10   methionine (Met) at the N-terminus for expression, and a Leu-Glu-His-His-His-His-His-His [SEQ. ID NO.: 22] added to the C-terminus for purification.

In order to insert the portion of the MN coding sequence for the GST-MN fusion protein into alternate expression systems, a set of primers for PCR was designed. The primers were constructed to provide restriction sites at each end of the coding

15   sequence, as well as in-frame start and stop codons. The sequences of the primers, indicating restriction enzyme cleavage sites and expression landmarks, are shown below.

Primer #20:N-terminus

20   ┌Translation start

5'GTCGCTAGCTCCATGGGTCATATGCAGAGGTTGCCCCGGATGCAG 3'

         Nhel   Ncol   Ndel   └MN cDNA #1   [SEQ. ID. NO. 17]

Primer #19:C-terminus

25   ┌Translation stop

5'GAAGATCTCTTACTCGAGCATTCTCCAAGATCCAGCCTCTAGG 3'

         BglII   Xhol   └MN cDNA   [SEQ. ID. NO. 18]

The SEQ ID NOS: 17 and 18 primers were used to amplify the MN coding sequence

30   present in the GEX-3X-MN vector using standard PCR techniques. The resulting PCR product (termed MN 20-19) was electrophoresed on a 0.5% agarose/1X TBE gel; the 1.3

kb band was excised; and the DNA recovered using the Gene Clean II kit according to the manufacturer's instructions [Bio101; LaJolla, CA (USA)].

<div align="center">

Identification of MN Protein Partner(s)

</div>

5   A search for protein(s) interacting with MN was initiated using expression cloning of the corresponding cDNA(s) and a MN-Fc fusion protein as a probe. The chimerical MN-Fc cDNA was constructed in pSG5C vector by substitution of MN cDNA sequences encoding both the transmembrane anchor and the intracellular tail of MN protein with the cDNA encoding Fc fragment of the mouse IgG. The Fc fragment

10 cDNA was prepared by RT-PCR from the mouse hybridoma producing IgG2a antibody.

The chimerical MN-Fc cDNA was expressed by transient transfection in COS cells. COS cells were transfected using leptofection. Recombinant MN-Fc protein was released to TC medium of the transfected cells (due to the lack of the transmembrane region), purified by affinity chromatography on a Protein A Sepharose

15 and used for further experiments.

Protein extracts from mock-transfected cells and the cells transfected with pSG5C-MN-Fc were analysed by immunoblotting using the M75 MAb, SwαM-Px and ECL detection® [ECL® -- enhanced chemoluminescent system to detect phosphorylated tyrosine residues; Amersham; Arlington, Hts., IL (USA)]. The size of MN-Fc protein

20 expressed from the pSG5C vector corresponds to its computer predicted molecular weight.

^{35}S-labeled MN-Fc protein was employed in cell surface binding assay. It was found to bind to several mammalian cells, e.g., HeLa, Raji, COS, QT35, BL3. Similar results were obtained in cell adhesion assay using MN-Fc protein dropped on

25 bacterial Petri dishes. These assays revealed that KATO III human stomach adenocarcinoma cell line is lacking an ability to interact with MN-Fc protein. This finding allowed us to use KATO III cells for expression cloning and screening of the cDNA coding for MN-binding protein.

The cDNA expression library in pBK-CMV vector was prepared from

30 dense HeLa cells and used for transfection of KATO III cells. For the first round of screening, KATO III cells were transfected by electroporation. After two days of incubation, the ligand-expressing cells were allowed to bind to MN-Fc protein, then to

<div align="center">

47

</div>

Protein A conjugated with biotin and finally selected by pulling down with streptavidin-coated magnetic beads. Plasmid DNA was extracted from the selected cells and transformed to E. coli. Individual E. coli colonies were picked and pools of 8-10 clones were prepared. Plasmid DNA from the pools was isolated and used in the second

5   round of screening.

In the second round of screening, KATO III cells were transfected by DEAE dextran method. To identify the pool containing the cDNA for MN-binding protein, an ELISA method based on the binding of MN-Fc to the transfected cells, and detection using peroxidase labelled Protein A were used. Pools are selected by ability

10   to bind MN-Fc.

In the third round of screening, plasmid DNAs isolated from individual bacterial colonies of selected pools are transfected to KATO III cells. The transfected cells are subjected to binding with MN-Fc and detection with Protein A as before. Such exemplary screening is expected to identify a clone containing the cDNA which codes

15   for the putative MN protein partner. That clone would then be sequenced and the expression product confirmed as binding to MN protein by cell adhesion assay. (Far-Western blotting, co-precipitation etc.) Hybridomas producing Mabs to the expression product would then be prepared which would allow the analysis of the biological characteristics of the protein partner of MN.

20

Preparation of MN-Specific Antibodies

The term "antibodies" is defined herein to include not only whole antibodies but also biologically active fragments of antibodies, preferably fragments containing the antigen binding regions. Further included in the definition of antibodies

25   are bispecific antibodies that are specific for MN protein and to another tissue-specific antigen.

Zavada et al., WO 93/18152 and WO 95/34650 describe in detail methods to produce MN-specific antibodies, and detail steps of preparing representative MN-specific antibodies as the M75, MN7, MN9, and MN12 monoclonal

30   antibodies. Preferred MN antigen epitopes comprise: aa 62-67 (SEQ ID NO: 10); aa 61-66, aa 79-84, aa 85-90 and aa 91-96 (SEQ ID NO: 98); aa 62-65, aa 80-83, aa 86-89 and aa 92-95 (SEQ ID NO: 99); aa 62-66, aa 80-84, aa 86-90 and aa 92-96 (SEQ ID

NO: 100); aa 63-68 (SEQ ID NO: 101); aa 62-68 (SEQ ID NO: 102); aa 82-87 and aa

88-93 (SEQ ID NO: 103); aa 55-60 (SEQ ID NO: 11); aa 127-147 (SEQ ID NO: 12); aa

36-51 (SEQ ID NO: 13); aa 68-91 (SEQ ID NO: 14); aa 279-291 (SEQ ID NO: 15);

and aa 435-450 (SEQ ID NO: 16). Example 2 provides further description concerning

5   preferred MN antigen epitopes.

Bispecific Antibodies. Bispecific antibodies can be produced by

chemically coupling two antibodies of the desired specificity. Bispecific MAbs can

preferably be developed by somatic hybridization of 2 hybridomas. Bispecific MAbs

for targeting MN protein and another antigen can be produced by fusing a hybridoma

10   that produces MN-specific MAbs with a hybridoma producing MAbs specific to another

antigen. For example, a cell (a quadroma), formed by fusion of a hybridoma producing

a MN-specific MAb and a hybridoma producing an anti-cytotoxic cell antibody, will

produce hybrid antibody having specificity of the parent antibodies. [See, e.g.,

Immunol. Rev. (1979); Cold Spring Harbor Symposium Quant. Biol., 41: 793 (1977);

15   van Dijk et al., Int. J. Cancer, 43: 344-349 (1989).] Thus, a hybridoma producing a

MN-specific MAb can be fused with a hybridoma producing, for example, an anti-T3

antibody to yield a cell line which produces a MN/T3 bispecific antibody which can

target cytotoxic T cells to MN-expressing tumor cells.

It may be preferred for therapeutic and/or imaging uses that the

20   antibodies be biologically active antibody fragments, preferably genetically engineered

fragments, more preferably genetically engineered fragments from the V_H and/or V_L

regions, and still more preferably comprising the hypervariable regions thereof.

However, for some therapeutic uses bispecific antibodies targeting MN protein and

cytotoxic cells would be preferred.

25

Epitopes

The affinity of a MAb to peptides containing an epitope depends on the

context, e.g. on whether the peptide is a short sequence (4-6 aa), or whether such a

short peptide is flanked by longer aa sequences on one or both sides, or whether in

30   testing for an epitope, the peptides are in solution or immobilized on a surface.

Therefore, it would be expected by ones of skill in the art that the representative

epitopes described herein for the MN-specific MAbs would vary in the context of the use of those MAbs.

The term "corresponding to an epitope of an MN protein/polypeptide"
will be understood to include the practical possibility that, in some instances, amino
5   acid sequence variations of a naturally occurring protein or polypeptide may be
antigenic and confer protective immunity against neoplastic disease and/or anti-
tumorigenic effects. Possible sequence variations include, without limitation, amino
acid substitutions, extensions, deletions, truncations, interpolations and combinations
thereof. Such variations fall within the contemplated scope of the invention provided
10   the protein or polypeptide containing them is immunogenic and antibodies elicited by
such a polypeptide or protein cross-react with naturally occurring MN proteins and
polypeptides to a sufficient extent to provide protective immunity and/or anti-
tumorigenic activity when administered as a vaccine.

15   Epitope for M75 MAb

The M75 epitope is considered to be present in at least two copies within
the 6X tandem repeat of 6 amino acids [aa 61-96 (SEQ ID NO: 97)] in the proteglycan
domain of the MN protein. Exemplary peptides representing that epitope depending on
the context may include the following peptides from that tandem repeat: EEDLPS (SEQ
20   ID NO: 10; aa 62-67); GEEDLP (SEQ ID NO: 98; aa 61-66; aa 79-84; aa 85-90; aa 91-
96); EEDL (SEQ ID NO: 99; aa 62-65; aa 80-83; aa 86-89; aa 92-95); EEDLP (SEQ ID
NO. 100; aa 62-66; aa 80-84; aa 86-90; aa 92-96); EDLPSE (SEQ ID NO: 101; aa 63-
68); EEDLPSE (SEQ ID NO: 102; aa 62-68); and DLPGEE (SEQ ID NO: 103; aa 82-87,
aa 88-93).

25   Three synthetic peptides from the deduced aa sequence for the EC
domain of the MN protein shown in Figure 1 were prepared. Those synthetic peptides
are represented by aa 51-72 (SEQ ID NO: 104), aa 61-85 (SEQ ID NO: 105) and aa 75-
98 (SEQ ID NO.: 106). Each of those synthetic peptides contains the motif EEDLP (SEQ
ID NO: 100) and were shown to be reactive with the M75 MAb.

30

Other Epitopes

Mab MN9. Monoclonal antibody MN9 (Mab MN9) reacts to the same epitope as Mab M75, as described above. As Mab M75, Mab MN9 recognizes both the GST-MN fusion protein and native MN protein equally well.

5   Mabs corresponding to Mab MN9 can be prepared reproducibly by screening a series of mabs prepared against an MN protein/polypeptide, such as, the GST-MN fusion protein, against the peptides representing the epitope for Mabs M75 and MN9. Alternatively, the Novatope system [Novagen] or competition with the deposited Mab M75 could be used to select mabs comparable to Mabs M75 and MN9.

10   Mab MN12. Monoclonal antibody MN12 (Mab MN12) is produced by the mouse lymphocytic hybridoma MN 12.2.2 which was deposited under ATCC HB 11647. Antibodies corresponding to Mab MN12 can also be made, analogously to the method outlined above for Mab MN9, by screening a series of antibodies prepared against an MN protein/polypeptide, against the peptide representing the epitope for

15 Mab MN12. That peptide is aa 55 - aa 60 of Figure 1 [SEQ ID NO: 11]. The Novatope system could also be used to find antibodies specific for said epitope.

Mab MN7. Monoclonal antibody MN7 (Mab MN7) was selected from mabs prepared against nonglycosylated GST-MN as described above. It recognizes the epitope represented by the amino acid sequence from aa 127 to aa 147 [SEQ ID NO:

20 12] of the Figure 1 MN protein. Analogously to methods described above for Mabs MN9 and MN12, mabs corresponding to Mab MN7 can be prepared by selecting mabs prepared against an MN protein/polypeptide that are reactive with the peptide having SEQ ID NO: 12, or by the stated alternative means.

25   MN-Specific Intrabodies – Targeted Tumor Killing Via Intracellular
Expression of MN-Specific Antibodies to
Block Transport of MN Protein to Cell Surface

The gene encoding antibodies can be manipulated so that the antigen-

30 binding domain can be expressed intracellularly. Such "intrabodies" that are targeted to the lumen of the endoplasmic reticulum provide a simple and effective mechanism for inhibiting the transport of plasma membrane proteins to the cell surface. [Marasco, W.A., "Review – Intrabodies: turning the humoral immune system outside in or

intracellular immunization," Gene Therapy, 4: 11-15 (1997); Chen et al., "Intracellular antibodies as a new class of therapeutic molecules for gene therapy," Hum. Gene Ther., 5(5): 595-601 (1994); Mhashilkar et al., EMBO J., 14: 1542-1551 (1995); Mhashilkar et al., J. Virol., 71: 6486-6494 (1997); Marasco (Ed.), Intrabodies: Basic Research and

5   Clinical Gene Therapy Applications, (Springer Life Sciences 1998; ISBN 3-540-64151-3) (summarizes preclinical studies from laboratories worldwide that have used intrabodies); Zanetti and Capra (Eds.), "Intrabodies: From Antibody Genes to Intracellular Communication," The Antibodies: Volume 4, [Harwood Academic Publishers; ISBN 90-5702-559-0 (Dec. 1997)]; Jones and Marasco, Advanced Drug

10   Delivery Reviews, 31 (1-2): 153-170 (1998); Pumphrey and Marasco, Biodrugs, 9(3): 179-185 (1998); Dachs et al., Oncology Res., 9(6-7); 313-325 (1997); Rondon and Marasco, Ann. Rev. Microbiol., 51: 257-283 (1997)]; Marasco, W.A., Immunotechnology, 1(1): 1-19 (1995); and Richardson and Marasco, Trends in Biotechnology, 13(8): 306-310 (1995).]

15   MN-specific intrabodies may prevent the maturation and transport of MN protein to the cell surface and thereby prevent the MN protein from functioning in an oncogenic process. Antibodies directed to MN's EC, TM or IC domains may be useful in this regard. MN protein is considered to mediate signal transduction by transferring signals from the EC domain to the IC tail and then by associating with other intracellular

20   proteins within the cell's interior. MN-specific intrabodies could disrupt that association and perturb that MN function.

         Inactivating the function of the MN protein could result in reversion of tumor cells to a non-transformed phenotype. [Marasco et al. (1997), supra.] Antisense expression of MN cDNA in cervical carcinoma cells, as demonstrated herein, has

25   shown that loss of MN protein has led to growth suppression of the transfected cells. It is similarly expected that inhibition of MN protein transport to the cell surface would have similar effects. Cloning and intracellular expression of the M75 MAb's variable region is to be studied to confirm that expectation.

         Preferably, the intracellularly produced MN-specific antibodies are single-

30   chain antibodies, specifically single-chain variable region fragments or sFv, in which the heavy- and light-chain variable domains are synthesized as a single polypeptide and are separated by a flexible linker peptide, preferably (Gly$_4$-Ser)$_3$ [SEQ ID NO: 116].

MN-specific intracellularly produced antibodies can be used therapeutically to treat preneoplastic/neoplastic disease by transfecting preneoplastic/neoplastic cells that are abnormally expressing MN protein with a vector comprising a nucleic acid encoding MN-specific antibody variable region fragments,

5   operatively linked to an expression control sequence. Preferably said expression control sequence would comprise the MN gene promoter.

Antibody-Mediated Gene Transfer Using MN-Specific Antibodies or Peptides for Targeting MN-Expressing Tumor Cells

10   An MN-specific antibody or peptide covalently linked to polylysine, a polycation able to compact DNA and neutralize its negative charges, would be expected to deliver efficiently biologically active DNA into an MN-expressing tumor cell. If the packed DNA contains the HSVtk gene under control of the MN promoter, the system would have double specificity for recognition and expression only in MN-

15   expressing tumor cells. The packed DNA could also code for cytokines to induce CTL activity, or for other biologically active molecules. The M75 MAb (or, for example, as a single chain antibody, or as its variable region) is exemplary of such a MN-specific antibody.

The following examples are for purposes of illustration only and are not

20   meant to limit the invention in any way.

Example1

Transient Transformation of Mammalian Cells by MN Protein

25
This example (1) examines the biological consequences of transfecting human or mouse cells with MN-cDNA inserted into expression vectors, mainly from the viewpoint of the involvement of MN protein in oncogenesis; (2) determines if MN protein exerts carbonic anhydrase activity, and whether such activity is relevant for

30   morphologic transformation of cells; and (3) tests whether MN protein is a cell adhesion molecule (CAM).

Synopsis

Methods: MN-cDNA was inserted into 3 expression vectors and was used for transfecting human or mouse cells. MN protein was detected by Western blotting, radioimmunoassay or immunoperoxidase staining; in all tests the MN-specific
5   monoclonal antibody M75 (MAb M75) was used. Carbonic anhydrase activity was determined by the acidification velocity of carbonate buffer in CO_2 atmosphere.

Results: (1) Cells (human CGL-1 and mouse NIH3T3 cells) transfected with MN-cDNA showed morphologic transformation, but reverted to normal phenotype after 4-5 weeks. (2) This reversion was not due to the loss, silencing or mutation of the
10   MN insert. (3) MN protein has the enzyme activity of a carbonic anhydrase, which can be inhibited with acetazolamide; however, the inhibition of the carbonic anhydrase enzyme activity did not affect transformation. (4) MN protein is an adhesion protein, involved in cell-to-cell contacts.

15   Background

This example concerns transformation of mammalian cells by MN-cDNA inserted into expression vectors derived from retroviruses. Such vectors are suitable for efficient and stable integration into cellular DNA and for continuous expression of MN protein. Cells transfected with these constructs showed morphologic transformation,
20   but after some time, they reverted to normal phenotype.

Sulfonamides, including acetazolamide, are very potent inhibitors of known carbonic anhydrases [Maren and Ellison, Mol. Pharmacol., 3: 503-508 (1967)]. Acetazolamide was tested to determine if it inhibited also the MN-carbonic anhydrase, and if so, whether inhibition of the enzyme affected cell transformation.
25   There are reasons to believe that MN protein could be involved in direct cell-to-cell interactions: A) previous observations indicated a functional resemblance of MN protein to surface glycoproteins of enveloped viruses, which mediate virus adsorption to cell surface receptors, and MN participated in the formation of phenotypically mixed virions of vesicular stomatitis virus. B) Inducibility of MN protein
30   expression by growing HeLa cells in densely packed monolayers suggests that it may be involved in direct interactions between cells. C) Finally, there is a structural similarity between the MN protein and receptor tyrosine phosphatase β, which also contains

54

proteoglycan and carbonic anhydrase domains; those domains mediate direct contacts

between cells of the developing nervous system [Peles et al., Cell, 82: 251-260 (1995)].

Therefore, MN protein was tested to see if it bound to cell surface receptors; the result

was clearly positive that it does.

5

Materials and Methods

Cell Lines

Cells used in this example were: CGL1 and CGL3 - respectively non-

tumorigenic and tumorigenic HeLa x fibroblast hybrids [Stanbridge et al., Somat. Cell

10   Genet., 7: 699-712 (1981)], mouse cell line NIH3T3, HeLa cells and monkey Vero

cells. The NIH3T3 cells were seeded at very low density to obtain colonies started

from single cells. The most normal appearing colony, designated subclone 2, was

picked for use in the experiments reported in this example.

15   Expression Vectors

Full-length MN cDNA was acquired from a pBluescript subclone

[Pastorek et al., Oncogene, 9: 2877-2888 (1994)]. To remove 5' and 3' noncoding

sequences, that might reduce subsequent gene expression, a polymerase chain reaction

(PCR) was performed. The 5' primer

20   TAGACAGATCTACGATGGCTCCCCTGTGCCCCAG [SEQ ID NO: 88] encompasses a

translation start site and Bg1II cloning site, and the 3' primer

ATTCCTCTAGACAGTTACCGGCTCCCCCTCAGAT [SEQ ID NO: 89] encompasses a

stop codon and XbaI cloning site. Full-length MN-cDNA as a template and Pfu DNA

Polymerase [Stratagene; LaJolla, CA (USA)] were used in the reaction.

25   The PCR product was sequenced and found to be identical with the

template; it carried no mutations. The PCR product harbouring solely the MN coding

sequence was inserted into three vectors: 1. pMAMneo [Clontech; Palo Alto, CA

(USA)] plasmid allowing dexamethasone-inducible expression driven by the MMTV-

Long Terminal Repeat (LTR) promoter and containing a neo gene for selection of

30   transformants in media supplemented with Geneticin (G418) antibiotics. 2. Retroviral

expression vector pGD [Daley et al., Science, 247: 824-829 (1990); kindly provided by

Prof. David Baltimore, New York-Cambridge)] containing MLV-LTR promoter and neo

gene for G418 antibiotics selection. 3. Vaccinia virus expression vector pSC11

[Chakrabarti et al., Mol. Cell. Biol., 5: 3403-3409 (1985)]. Transfection was performed

via a calcium-phosphate precipitate according to Sambrook et al. (eds.), Molecular

cloning. A laboratory manual, 2nd ed., Cold Spring Harbor Laboratory Press (1989).

5   Vaccinia virus strain Praha clone 13 was used as parental virus [Kutinova

et al., Vaccine, 13: 487-493 (1995)]. Vaccinia virus recombinant was prepared by a

standard procedure [Perkus et al., Virology, 152: 285-297 (1986)]. Recombinant

viruses were selected and plaque purified twice in rat thymidine-kinase-less RAT2 cells

[Topp, W. C., Virology, 113: 408-411 (1981)] in the presence of 5'-bromodeoxyuridine

10   (100 μg/ml). Blue plaques were identified by overlaying with agar containing 5-bromo-

4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) (200 μg/ml).

CA Assay

              Carbonic anhydrase activity was measured by a micro-method [Brion et

15   al., Anal. Biochem., 175: 289-297 (1988)]. In principle, velocity of the reaction CO_2 +

$H_2O \rightarrow H_2CO_3$ is measured by the time required for acidification of carbonate buffer,

detected with phenol red as a pH indicator. This reaction proceeds even in absence of

the enzyme, with t_0 = control time (this was set to 60 seconds). Carbonic anhydrase

reduces the time of acidification to t; one unit of the enzyme activity reduces the time

20   to one half of control time: t/t_0 = 1/2.

              For the experiment, MN protein was immunoprecipitated with Mab M75

from RIPA buffer (1% Triton X-100, 0.1% deoxycholate, 1mM phenylmethylsulfonyl-

fluoride and 200 trypsin-inhibiting units/ml of Trasylol in PBS, pH 7.2) extract of Vero

cells infected with vaccinia-MN construct, after the cells developed cytopathic effect, or

25   with "empty" vaccinia as a control. The MN + antibody complex was subsequently

adsorbed to protein A - Staphylococcus aureus cells [Kessler, S. W., J. Immunol., 115:

1617-1624 (1975)] and rinsed 2x with PBS and 2x with 1 mM carbonate buffer, pH 8.0.

The precipitate was resuspended in the same buffer and added to the reaction mixture.

Acetazolamide (Sigma) was tested for inhibition of carbonic anhydrase [Maren and

30   Ellison, supra]. In extracts of infected cells used for immunoprecipitation, the

concentration of total proteins was determined by the Lowry method [Lowry et al., J.

Biol. Chem., 193: 265-275 (1951)] and that of MN protein by a competition

radioimmunoassay as described in Zavada et al., Int. J. Cancer, 54: 268-274 (1993).

Western Blots

5   Western blotting and development of the blots using ^{125}I-labelled M75

and autoradiography was performed as before [Pastorekova et al., Virology, 187: 620-

626 (1992); and Zavada (1993), supra].

Adhesion Assay

10   For the adhesion assay [Hoffman S., "Assays of cell adhesion," IN: Cell-

cell Interactions, (Stevenson et al. eds.) pp. 1-30 (IRL Press at Oxford University Press;

Oxford, N.Y., Tokyo; 1992)], 25 μl aliquots MN protein (affinity purified pGEX-3X MN)

[Zavada et al. (1993), supra] or of control proteins were spotted on 5 cm-diameter

bacteriological Petri dishes and allowed to bind for 2 hours at room temperature. This

15   yielded circular protein-coated areas of 4-5 mm diameter. MN protein was diluted to

10 μg/ml in 50 mM carbonate buffer, pH 9.2. Patches of adsorbed control proteins

were prepared similarly. Those included collagens type I and IV, fibronectin, laminin

and gelatin (Sigma products), diluted and adsorbed according to the manufacturer's

recommendations; FCS and BSA were also included. After aspiration of the drops, the

20   dishes were rinsed 2x with PBS and saturated for 1 hour with DMEM supplied with 5%

FCS. The plates were seeded with 5×10^5 cells in 5 ml of DMEM + 5% FCS and

incubated overnight at 37°C. The plates were rinsed with PBS, and the attached cells

were fixed with formaldehyde, post-fixed with methanol and Giemsa stained.

25   ## Results

    1.   Transformation and reversion of CGL1 cells transfected with MN-cDNA

          Since the expression of MN protein correlated with the tumorigenicity of

HeLa x fibroblast hybrids [Zavada et al. (1993), supra], the non-tumorigenic hybrid

30   CGL1 cells were first tested. Those cells, transfected with the pMAM.MN construct,

after selection with Geneticin, formed colonies with varying degrees of transformation;

some of them appeared normal. While normal CGL1 cells are contact inhibited,

growing in a parallel orientation, the transformed cells formed very dense colonies,

showing the loss of contact inhibition. Such colonies grew more slowly than the original CGL 1.

After subcloning, the cells isolated from transformed colonies segregated revertants. The reversion was a gradual, step-wise process; there were colonies with

5   different degrees of reversion. After 2 passages, all the cell population became a morphologically indistinguishable from normal CGL1. This was due to the reversion of some cells and to the selective advantage of the revertants, which grew faster than the transformed cells. Despite repeated attempts, not even one single stably transformed cell clone was obtained. No transformed colonies were found in CGL1 cells

10   transfected with an "empty" pMAM control plasmid. Growth of the CGL1 + pMAM.MN revertants in media supplied with 5 μg/ml of dexamethasone for 7 days enhanced the production of MN protein, but the morphology of the cells did not return to transformed.

15   2.   Rescue of transforming MN from the revertants

The reversion of MN-transformed cells to normal phenotype could have at least 4 causes: A) loss of the MN insert; B) silencing of the MN insert, e.g., by methylation; C) mutation of the MN insert; D) activation of a suppressor gene, coding for a product which neutralizes transforming activity of MN protein; E) loss of a MN-

20   binding protein. To decide among those alternatives, the following experiment was designed.

MN-cDNA was inserted into pGD, a vector derived from mouse leukemia virus - MLV. A defective virus was thereby engineered, which contained the MN gene and the selective marker neo instead of genes coding for viral structural proteins. With

25   this construct, mouse NIH3T3 cells were transfected. In media supplied with Geneticin, the cells formed colonies with phenotypes ranging from strongly transformed to apparently normal. All of the transformed colonies and about 50% of the normal colonies expressed MN protein. Contrasting with normal NIH3T3 cells, the transformants were also able to form colonies in soft agar, reflective of the loss of

30   anchorage dependence, characteristic of cell transformation. Upon passaging, the cells isolated from transformed colonies reverted to normal morphology, and at the same time, they lost the capacity to form colonies in soft agar, while still expressing the MN

protein. This permanent presence of MN protein in revertants ruled out alternatives A) and B) supra, that is, loss or silencing of the MN gene as a cause of reversion.

To decide among the other 3 alternatives, the revertants were superinfected with live, replication competent MLV. This virus grows in NIH3T3 cells

5   without any morphologic manifestations, and it works as a "helper" for the pGD.MN construct. Virus progeny from MLV-infected revertants represents an artificial virus complex [pGD.MN + MLV]. This consists of 2 types of virions: of standard type MLV particles and virions containing the pGD.MN genome, enveloped in structural proteins provided by the "helper" virus. This virus complex was infectious for fresh NIH3T3

10   cells; it again induced in them morphologic transformation and the capacity to form agar colonies.

Contrasting with NIH3T3 transfected with pGD.MN, all the colonies of cells infected with [pGD.MN + MLV] complex, which grew in the presence of Geneticin, were uniformly transformed and contained MN proteins. The transformants

15   once more reverted to normal phenotype although they kept producing infectious [pGD.MN + MLV] complex, which induced transformation in fresh NIH3T3 cells. This cycle of infection-transformation-reversion was repeated 3 times with the same result. This ruled out alternative C) – mutation of MN-cDNA as a cause of reversion.

Normal NIH3T3 cells formed a contact inhibited monolayer of flat cells,

20   which did not stain with Mab M75 and immunoperoxidase. Cells infected with [pGD.MN + MLV] complex were clearly transformed: they grew in a chaotic pattern and showed loss of contact inhibition. Some of the cells showed signs of apoptosis. Two passages later, the cell population totally reverted to original phenotype as a result of frequent emergence of revertants and of their selective advantages (faster growth and

25   a higher efficiency of plating). In fact, the revertants appeared to grow to a somewhat lower saturation density than the original NIH3T3 cells, showing a higher degree of contact inhibition.

The control NIH3T3 cells did not contain any MN protein (Western blot); while both transformed cells and revertants contained the same amount and the same

30   proportion of 54 and 58 kDa bands of MN protein. In a non-reducing gel, MN protein was present in the form of oligomers of 153 kDa. Consistently, by competition RIA,

approximately 40 ng MN/mg total protein was found in both of the transformed cells and revertants.

3.   Carbonic anhydrase activity and its inhibition

5   Since the carbonic anhydrase domain represents a considerable part of the MN protein (see Figure 8), tests were performed to determine whether it is indeed enzymatically active. Vero cells infected with the vaccinia.MN construct, which contained more of the MN protein than other cells used in the present experiments, served as a source of MN protein. The cells were extracted with RIPA buffer, and MN

10   protein was concentrated and partially purified by precipitation with MAb M75 and SAC. The immunoprecipitate was tested for CA activity. 78 μl of precipitate contained 1 unit of the enzyme. From the extract, the concentration of total proteins and of MN protein was determined; 1 unit of enzyme corresponded to 145 ng of MN protein or to 0.83 mg of total protein. The immunoprecipitate from Vero cells infected with control

15   virus had no enzyme activity. Activity of MN carbonic anhydrase was inhibited by acetazolamide; 1.53×10^{-8}M concentration of the drug reduced enzyme activity to 50%.

              Preliminary tests showed that confluent cultures of HeLa or of NIH3T3 cells tolerated 10^{-5} - 10^{-3}M concentration of acetazolamide for 3 days without any signs

20   of toxicity and without any effect on cell morphology. In sparse cultures, 10^{-5}M acetazolamide did not inhibit cell growth, but 10^{-4}M already caused a partial inhibition. Thus, 10^{-5}M acetazolamide was added to NIH3T3 cells freshly transformed with the [pGD.MN + MLV] complex. After 4 days of incubation, the colonies were fixed and stained. No difference was seen between cells growing in the presence or

25   absence of acetazolamide; both were indistinguishable from correctly transformed NIH3T3 cells. Thus, the enzymatic activity of carbonic anhydrase is not relevant for the transforming activity of MN protein.

4.   Cell adhesion assay

30   To determine whether or not MN protein is a cell adhesion molecule (CAM), adhesion assays were performed in plastic bacteriological Petri dishes (not treated for use with tissue culture). Cells do not adhere to the surfaces of such dishes,

unless the dishes are coated with a binding protein. NIH3T3 cells adhered, spread and grew on patches of adsorbed MN protein. Only very few cells attached outside the areas coated with MN protein.

Other variants of the experiment demonstrated that NIH3T3 cells adhered
5   and spread on patches of adsorbed collagen I and IV, fibronectin and laminin. NIH3T3 cells did not attach to dots of adsorbed gelatin, FCS or BSA.

CGL1, HeLa and Vero cells also adhered to MN protein, but 3 leukemia cell lines showed no adherence. CGL3 cells, strongly expressing MN protein adhered less efficiently to MN protein dots then did CGL1. The presence of 10^{-4}M
10   acetazolamide in the media did not affect the cell adhesion.

To confirm the specificity of adhesion, MN protein was absorbed with SAC loaded with MAb M75 (directed to MN) or MAb M67, directed to an unrelated antigen (Pastorekova et al., supra), before it was applied to the surface of the Petri dishes. Absorption with the SAC-M75 complex totally abrogated the cell binding
15   activity, whereas absorption with SAC-M67 was without any effect.

Additional Cell Adhesion Results

A shortened MN, missing TM and IC segments, is shed into the medium by 5ET1 cells (a HeLa X fibroblast hybrid, analogous to CGL3 cells that express MN
20   protein abundantly) or by Vero cells infected with VV carrying MN-cDNA with deleted TM and IC sequences. The shed MN protein was purified from the media, and tested in cell adhesion assays. The cells adhered, spread and grew only on the patches covered with adsorbed complete MN protein, but not on the dots of MN lacking TM and IC regions. Analogous results have been described also for some other adhesion
25   molecules. A variety of cells (NIH3T3, CGL1, CGL3, HeLa, XC) attached to MN protein dots suggesting that the MN receptor(s) is common on the surface of vertebrate cells.

Tests were also performed with extracellular matrix proteins or control proteins dotted on nitrocellulose. The dot-blots were treated with MN protein solution.
30   Bound MN protein was detected with MAb M75. MN protein absorbed to the dots of collagen I and IV, but not to fibronectin, laminin, gelatine or BSA.

Prospects for therapy. There are many new principles of cancer therapy

employing oncoproteins or molecules that interact with them as targets [Mendelsohn

and Lippman, "Principles of molecular cell biology of cancer: growth factors," In:

DeVita et al., eds., Cancer: principles and practice of oncology, pp. 114-133 4th ed.,

5   Philadelphia: Lippinocott (1993); DeVita et al., eds., Biologic therapy of cancer, 2nd

ed., Philadelphia: Lippinocott (1995)]. The MN protein and at least some of its ligands

(or receptors) appear to be particularly suitable for such purposes.

Example 2

10   Identification of MN's Binding Site

MN protein is a tumor-associated cell adhesion molecule (CAM). To

identify its binding site, a series of overlapping oligopeptides, spanning the N-terminal

domain of the MN protein were synthesized. The N-terminal domain is homologous to

that of proteoglycans and contains a tandem repeat of six amino acids.

15   The series of oligopeptides were tested by the cell adhesion assay

procedure essentially as described above in Example 1. The synthetic oligopeptides

were immobilized on hydrophobic plastic surfaces to see if they would mediate the

attachment, spreading and growth of cells. Also investigated were whether the

oligopeptides or antibodies inhibited attachment of cells (NIH3T3, HeLa and CGL1) to

20   purified MN protein coated onto such plastic surfaces. The MN protein was affinity

purified on agarose covalently linked to sulfonamide, as the MN protein encompasses a

CA domain.

Several of the oligopeptides were found to be biologically active: (i) when

immobilized onto the plastic, they mediate attachment of cells (NIH3T3, HeLa and to

25   CGL1); (ii) when added to the media, they compete for attachment to cells with the

immobilized MN protein; (iii) these oligopeptides, present in the media do not inhibit

attachment of cells to TC plastic, but they prevent cell-cell adhesion and formation of

intercellular contacts; (iv) treatment of immobilized MN protein and of active peptides

with MAb M75 abrogates their affinity for the cells; and (v) the binding site of MN was

30   determined to be closely related or identical to the epitope for MAb M75, at least two

copies of which are located in the 6-fold tandem repeat of 6 amino acids [aa 61-96

(SEQ ID NO: 97)] in the proteoglycan-like domain of MN protein.

It was concluded that ectopically expressed MN protein most likely participates in oncogenesis by intervention into normal cell-cell contacts. MN's binding site represents a potential target for which therapeutic agents can be designed.

5   Materials and Methods

Affinity chromatography of MN/CA IX. MN/CA IX was purified by a single cycle of adsorption - elution on sulfonamide-agarose, as described for other CAs [Falkbring et al., FEBS Letters, 24: 229 (1972)]. We used columns of p-aminoethylbenzenesulfonamide-agarose (Sigma). Columns with adsorbed MN/CA IX
10   were extensively washed with PBS (NaCl 8.0 g/l, KCl 0.2 g/l, KH_2PO_4 0.2 g/l, Na_2HPO_4 1.15 g/l, pH = 7.2) and eluted with 0.1 mM acetazolamide (Sigma). All steps of purification were carried out at 0 - 5 °C, pH 7.2, at physiological concentration of salts. Complete MN/CA IX+ was extracted with 1% Triton X-100 in PBS from Vero cells infected with vaccinia virus containing an insert of complete coding region of MN/CA
15   IX as described in Zavada et al., Int. J. Oncol., 10: 857 (1997). Before chromatography, the extract was diluted 1:6 with PBS and centrifuged for 1 h at 1500 xg. Truncated MN/CA IX ΔTM ΔIC was produced from an analogous construct except that the 3' downstream primer for PCR was: 5' CGT CTA GAA GGA ATT CAG CTA GAC TGG CTC AGC A 3' [SEQ ID NO: 117]. MN/CA IX Δ was shed into the
20   medium, from which it was affinity purified after centrifugation as above. All steps of purification were monitored by dot-blots.

Cells and media. The following cell lines were used: HeLa, CGL1 = non-tumorigenic hybrid HeLa x fibroblast, CGL3 = tumorigenic segregant from this hybrid, NIH3T3 cells = mouse fibroblasts. The origin of the cells and growth media
25   are described in Zavada et al., Int. J. Cancer, 54: 268 (1993) and Zavada et al., Int. J. Oncol., 10: 857 (1997). In addition, we used also HT29, a cell line derived from colorectal carcinoma (ATCC No. HBT-38).

Cell adhesion assay. The conditions of the assay are basically as described in Example 1. Briefly, 1μg/ml of purified MN/CA IX in 50 mM mono/bicarbonate buffer,
30   pH 9.2, was adsorbed in 30μl drops on the bottom of bacteriological 5 cm Petri dishes for 1.5 hr. Then the drops were removed by aspiration and the dishes were 3x rinsed with PBS and blocked with 50% FCS in culture medium for 30 min. There were two

variants of the test. In the first one, the whole bottom of the Petri dish was blocked with 50% FCS, and the dishes were seeded with 5 ml of cell suspension (10^5 cells/ml). After overnight incubation, the cultures were rinsed with PBS, fixed and stained. In the other variant, only the area of adsorbed MN/CA IX was blocked and on top of MN/CA IX dots

5   were added 30 μl drops of cell suspension in growth medium, containing added oligopeptides (or control without peptides). After incubation, rinsing and fixation, the cultures were stained with 0.5% Trypan blue in 50 mM Tris buffer pH 8.5 for 1 h, rinsed with water and dried. Stained areas of attached cells were extracted with 10% acetic acid, the extracts transferred to 96-well plates and absorbance was measured at

10   630 nm on microplate reader.

ELISA. Purified GST-MN [Zavada et al. (1993), supra] at concentration 10 ng /ml in carbonate buffer pH 9.2 was adsorbed for 3 h in Maxisorb strips (NUNC). After washing and blocking (1 h) with 0.05% Tween 20 in PBS, 50μl/well of the antibody + antigen mixtures were added. Final dilution of MAb 75 ascites fluid was

15   10^{-6}; concentration of the peptides varied according to their affinity for M75 so as to allow determination of 50% end-point. These mixtures were adsorbed for 1.5 h, followed by washing with Tween-PBS. Bound antibody was detected by antimouse IgG conjugate with peroxidase (SwAM-Px, SEVAC, Prague), diluted 1:1000. In the color reaction OPD (o-phenylenediamine dihydrochloride, Sigma) 1mg/ml in 0.1 M citrate

20   buffer pH 5.0 was used. To this H_2O_2 was added to final concentration 0.03%. This system is balanced so as to allow assay for antigen competing for M75 as well as for peptides binding to the epitope of immobilized GST-MN.

Peptides. The peptides used in this study were prepared by the solid phase method [Merrifield et al., IN: Gutte, B. (ed.), Peptides: Synthesis, Strucures and

25   Applications, pp. 93-169 (San Diego; Academic Press; 1995)] using the Boc/Bzl strategy. The peptide acids were prepared on PAM-resin and peptide amides on MeBHA resin. Deprotection and splitting from the resin was done by liquid hydrogen fluoride. The peptides were purified by C18 RP HPLC and characterized by amino acid analysis and FAB MS spectroscopy.

30   Western blots. MN/CA IX antigens from PAGE gels were transferred to PVDF membranes (Immobilon P, Millipore) and developed with M75, followed by

SwAM-Px (see above) and diaminobenzidine (Sigma) with H_2O_2. For dot-blots we used nitrocellulose membranes.

Phage display. Ph.D.-7 Phage Display Peptide Library kit was used for screening as recommended by manufacturer (New England Biolabs). 96-well plate was

5   coated with peptide SEQ ID NO: 106. Biopanning was carried out by incubating 2×10^{11} phage with target coated plate for 1 h. Unbound phages were washed away with TBST (50mM Tris-HCl pH 7.5, 150 mM NaCl, 0.1% Tween-20) and specifically bound phages were eluted with M75 antibody (2μg in 100 μl of TBS/well). Eluted phage was amplified and used for additional binding and amplification cycles to enrich the pool

10   in favour of binding sequence. After 5 rounds, individual clones were picked, amplified and sequenced using T7 sequencing kit (Pharmacia).

Results

Affinity chromatography of MN/CA IX protein. For purification of MN/CA

15   IX protein we decided to use affinity chromatography on sulfonamide-agarose column, described previously for other CAs [Falkbring et al., supra]. The advantages of this method are simplicity and the fact that the whole procedure is carried out under non-denaturing conditions. Vaccinia virus vector with an insert of the complete MN/CA9 cDNA, or with truncated cDNA (lacking transmembrane and intracellular

20   domains) was employed as a source of MN/CA IX protein.

A single cycle of adsorption - elution yielded relatively pure proteins: MN/CA IX+ gave 2 bands of 54 and 58 kDa, MN/CA IXΔ of 54.5 and 56 kDa. These proteins strongly reacted with MAb M75 on Western blots. In extracts from HeLa, CGL3 and HT29 the blot revealed 2 bands of the same size as MN/CA IX+ purified

25   from vaccinia virus construct.

Adhesion of cells to MN/CA IX protein. MN/CA IX immobilized on hydrophobic plastic enabled attachment, spreading and growth of cells. Extremely low concentrations of MN/CA IX corresponding to 1 μg/ml of purified protein in adsorption buffer were sufficient to cause this effect; other cell adhesion molecules are used in 10 -

30   50x higher concentrations. Only complete MN/CA IX protein was active in cell adhesion test, truncated MN/CA IX did not support cell adhesion at all or it showed only a low adhesion activity and in some instances it even acted as a cell "repellent".

Treatment of the dots of immobilized MN/CA IX with MAb M75 abrogated its capacity to attach the cells, but the control MAb M16, irrelevant for MN/CA IX had no effect. Blocking of cell attachment by M75 shows that the epitope is identical to or overlapping with the binding site of MN/CA IX for cell receptors.

5   Identification of the epitope recognized by Mab M75. Preliminary mapping of M75 epitope employing partial sequences of extracellular parts of MN/CA9 cDNA expressed from bacterial vectors and tested on Western blots located it in PG region. For exact mapping, our strategy was to synthesize partially overlapping oligopeptides of 15-25 aa covering the PG domain and test them in competition ELISA

10   with M75. According to the results, this was followed by a series of 6-12 aa oligopeptides. A major part of the PG domain consists of a 6-fold tandem repeat of 6 aa (aa 61 - 96) [SEQ ID NO: 97]; 4 repeats are identical (GEEDLP) [SEQ ID NO: 98] and 2 contain 2 aa exchanged (SEEDSP [SEQ ID NO: 141] and REEDPP [SEQ ID NO: 142]).

Following are the results of competition ELISA with recombinant MN/CA

15   IX and oligopetides synthesized according to partial sequences of the PG region. MN/CA IX+ and Δ produced in mammalian cells possessed a higher serological activity than any other protein or peptide included in this experiment; fusion protein GST-MN synthesized in bacteria was less active. The following peptides span the PG region: GGSSGEDDPLGEEDLPSEEDSPC (aa 51-72) [SEQ ID NO: 104];

20   GEEDLPSEEDSPREEDPPGEEDLPGEC (aa 61-85) [SEQ ID NO: 105]; EDPPGEEDLPGEEDLPGEEDLPEVC (aa 75-98) [SEQ ID NO: 106]; and EVKPKSEEEGSLKLE (aa 97 - 111) [SEQ ID NO: 118]. SEQ ID NOS: 104 and 106 caused 50% inhibition at 1ng/ml. Those 2 oligopeptides are mutually non-overlapping, thus the epitope is repeated in both of them. SEQ ID NO: 105 was 1000x less active,

25   probably due to a different conformation. SEQ ID NO: 118 was inactive; thus it does not contain the M75 epitope.

The next step for identifying the epitope was to synthesize oligopeptides containing all circular permutations of the motif GEEDLP [SEQ ID NO: 98] repeated twice. All 6 of the following dodecapeptides [SEQ ID NOS: 119-124] were

30   serologically active (2 more and 4 less so): GEEDLPGEEDLP [SEQ ID NO: 119]; EEDLPGEEDLPG [SEQ ID NO: 120]; EDLPGEEDLP [SEQ ID NO: 121]; DLPGEEDLPGEE [SEQ ID NO: 122]; LPGEEDLPGEED [SEQ ID NO: 123]; and PGEEDLPGEEDL [SEQ ID

66

NO: 124]. The following series of 7 aa sequences, flanked by alanine on both ends
were tested: APGEEDLPA [SEQ ID NO: 125]; AGEEDLPGA [SEQ ID NO: 126];
AEEDLPGEA [SEQ ID NO: 127]; AEDLPGEEA [SEQ ID NO: 128]; ADLPGEEDA [SEQ ID
NO. 129]; and ALPGEEDLA [SEQ ID NO: 130]. The results showed that the minimum

5   serologically active sequence is the oligopeptide APGEEDLPA [SEQ ID NO: 125]. SEQ
ID NOS: 127-130 proved negative in competition at 100 μg/ml. Further, none of the
following still shorter oligopeptides (6 + 2aa) competed in ELISA for M75: AGEEDLPA
[SEQ ID NO: 131]; AEEDLPGA [SEQ ID NO: 132]; AEDLPGEA [SEQ ID NO: 133];
ADLPGEEA [SEQ ID NO: 134]; ALPGEEDA [SEQ ID NO: 135]; and APGEEDLA [SEQ

10   ID NO: 136].

In the oligopeptides of SEQ ID NOS: 104, 105, 106 and 118, the C-
terminal amino acid was present as an acid, whereas in all the other oligopeptides, the
C-terminal amino acid was present as an amide. It is clear that the affinity between
these oligopeptides and MAb M75 very strongly increases with the size of peptide

15   molecule.

Attempts to demonstrate adhesion of cells to immobilized oligopeptides.
Our initial plan was to follow the pioneering work of Piersbacher and Ruoslahti, PNAS,
81: 5985 (1984). They linked tested oligopeptides to adsorbed bovine serum albumin
by cross-linking agent SPDP (N-succinimidyl 3[pyridylhydro] propionate). This is why

20   we added onto the C-end of oligopeptides SEQ ID NOS: 104-106 cysteine, which
would enable oriented linking to adsorbed albumin. We demonstrated linking of the
peptides directly in Petri dishes by immunoperoxidase staining with M75.
Unfortunately, CGL1 or CGL3 cells adhered to control albumin treated with SPDP and
blocked with ethanolamine (in place of oligopeptides) as strongly as to BSA dots with

25   linked oligopeptides. We were unable to abrogate this non-specific adhesion.
Oligopeptides SEQ ID NOS: 104-106 adsorb only very poorly to bacteriological Petri
dishes, thereby not allowing the performance of the cell adhesion assay.

Alternatively, we tested inhibition of cell adhesion to MN/CA IX dots by
oligopeptides added to the media together with the cell suspension, as described by

30   Piersbacher and Ruoslahti, supra.. All peptides SEQ ID NOS: 104-106 and 118-136,
were tested at concentrations of 100 and 10 μg/ml. None of them inhibited
reproducibly the adhesion of CGL1 cells.

Oligopeptides with affinity to M75 epitope which inhibit cell adhesion to
MN/CA IX. As an alternative to monoclonal antibodies, we set out to select
oligopeptides exerting affinity to M75 epitope as well as to MN/CA IX receptor binding
site from a phage display library of random heptapeptides - Ph.D.-7. Our aim was to

5   select phages containing the desired heptapeptides by panning on immobilized peptide
SEQ ID NO: 106 and subsequent elution with M75. Eluted phage was multiplied in
appropriate bacteria and subjected to 4 more cycles of panning and elution. From the
selected phage population, 10 plaques were picked, amplified and the
heptapeptide-coding region was sequenced. Only 3 heptapeptides were represented.

10   Those three heptapeptides, after adding alanine on both sides, are the following
nonapeptides: AKKMKRRKA [SEQ ID NO: 137]; AITFNAQYA [SEQ ID NO: 138]; and
ASASAPVSA [SEQ ID NO: 139]. The last heptapeptide, synthesized again with added
terminal alanines as nonapeptide AGQTRSPLA [SEQ ID NO: 140], was identified by
panning on GST-MN and eluted with acetazolamide. This last peptide has affinity to the

15   active site of MN/CA IX carbonic anhydrase. We synthesized these peptides of 7 + 2
aa and tested them in competition ELISA and in cell adhesion inhibition. Both tests
yielded essentially consistent results: peptide SEQ ID NO: 138 showed the highest
activity, peptide SEQ ID NO: 137 was less active, peptide SEQ ID NO: 139 was
marginally positive only in ELISA, and peptide SEQ ID NO: 140 was inactive. In all of

20   those 4 nonapeptides, the C-terminal amide was present as amide.

Discussion

            Purification of transmembrane proteins like MN/CA IX often poses
technical problems because they tend to form aggregates with other membrane proteins

25   due to their hydrophobic TM segments. To avoid this, we engineered truncated MN/CA
IX ΔICΔTM, which is secreted into the medium. Indeed, truncated MN/CA IX was
obtained in higher purity than MN/CA IX+. Unfortunately, this protein was of little use
for our purposes, since it was inactive in the cell adhesion assay. Such a situation has
also been described for other cell adhesion molecules: their shed, shortened form

30   either assumes an inactive conformation, or it adsorbs to hydrophobic plastic "upside
down," while complete proteins adsorb by hydrophobic TM segments in the "correct"
position.

MN/CA IX protein forms oligomers of 150 kDa, linked by disulfidic

bonds. It was not known whether these are homo- or hetero-oligomers, but PAGE and

Western blot analysis suggest that these are probably homo-oligomers, most likely

trimers, since on the gel stained with Coomassie Blue no additional bands of intensity

5   comparable to 2 bands specific for MN/CA IX appeared. It is also unlikely that there

could exist an additional protein co-migrating with one of the 2 major MN/CA IX bands,

since the intensity of their staining on the gel and on Western blots is well comparable.

There can be no doubt on the specificity of cell attachment to purified

MN/CA IX+. It is abrogated by specific MAb M75, at a dilution 1:1000 of ascites fluid.

10   This is a correction to our previous report in Zavada et al., Int. J. Oncol., 10: 857 (1997)

in which we observed that MN/CA IX produced by vaccinia virus vector and fusion

protein GST-MN support cell adhesion, but we did not realize that GST anchor itself

contains another binding site, which is not blocked by M75.

MAb M75 reacts excellently with MN/CA IX under any circumstances -

15   with native antigen on the surface of living cells, with denatured protein on Western

blots and with antigen in paraffin sections of biopsies fixed with formaldehyde,

suggesting that the epitope is small and contiguous. In competition ELISA the smallest

sequence reactive with M75 was 7 + 2 aa, but the affinity between M75 and tested

peptides strongly depended on their molecular weight. Complete MN/CA IX was

20   100,000x more active than the smallest serologically active peptide in terms of

weight/volume concentration. In terms of molar concentration this difference would be

150,000,000x. Oligopeptides of intermediate size also showed intermediate activities.

It remains to be elucidated whether such differences in activity are due to the

conformation depending on the size of the molecule, or to the fact that complete

25   MN/CA IX contains several copies of the epitope, but the smallest molecule only one.

Considering the possibility that the epitope is identical with the cell

adhesion structure in MN/CA IX, we can understand why we failed to detect inhibition

of cell adhesion by the oligopeptides. The binding site is just not as simple as the

prototype peptide, RGD [Winter, J., IN Cleland and Craik (eds.), Protein Engineering.

30   Principles and Practice, pp. 349-369 (N.Y.; Wiley-Liss; 1996)].

Naturally, one can argue that the size of MN/CA IX is about the same as of

immunoglobulin molecule, and that binding of M75 to its epitope may sterically hinder

a different sequence of cell attachment site. This objection has been made unlikely by
blocking of both M75 epitope and of cell binding site by nonapeptides 7 + 2 aa. That
result strongly suggests that the epitope and the binding site are indeed identical.

MN/CA IX and its PG region in particular appears to be a potential target
5   molecule for therapy for the following reasons: (i) it is exposed on the cell surface; (ii) it
is present in high percentage of certain human carcinomas; (iii) it is normally expressed
MN/CA IX in the mucosa of alimentary tract which is not accessible to circulating
antibodies, in contrast with the tumors; (iv) it is not shed (or only minimally) into the
body fluids; (v) the motif GEEDLP [SEQ ID NO: 98] is repeated 18 x on the surface of
10   every MN/CA IX molecule. Oligopeptide display libraries are being employed in the
first steps to develop new drugs [Winter, J., supra]. Selected oligopeptides can serve as
lead compounds for the computerized design of new molecules, with additional
properties required from a drug [DeCamp et al., IN Cleland and Craik (eds.), supra at
pp. 467-505].

15

Example 3

Identification of Peptides Binding to
MN Protein Using Phage Display

20   (a) To identify peptides that are recognized by MN protein, a heptapeptide
phage display library [Ph.D.®-7 Peptide 7-mer Library Kit (phage display peptide library
kit); New England Biolabs; Beverly, MA (USA)] was screened. In screening the library,
a selection process, i.e., biopanning [Parmley and Smith, Gene, 73: 308 (1988); Noren,
C.J., NEB Transcript, 8(1): 1 (1996)] was carried out by incubating the phages encoding
25   the peptides with a plate coated with MN protein, washing away the unbound phage,
eluting and amplifying the specifically bound phage.

The target MN protein in this process was a glutathione-S-transferase
(GST) MN fusion protein (GST-MN). GST-MN is a recombinantly produced fusion
protein expressed from pGEX-3X-MN containing the cDNA for the MN protein without
30   the signal peptide. GST-MN was produced in bacteria under modified cultivation
conditions (decreased optical density, decreased temperature). Such cultivation
prevented premature termination of translation and resulted in synthesis of the protein
molecules which were in vast majority of the full length. The GST-MN protein was

used for coating of the wells and binding the relevant phages. The bound phages were
then eluted by acetazolamide, amplified and used for two additional rounds of
screening.

After sequencing of several independent phage clones obtained after the
third round of screening, the following heptapeptides were obtained:

      (1) GETRAPL (SEQ ID NO: 107)

      (2) GETREPL (SEQ ID NO: 108)

      (3) GQTRSPL (SEQ ID NO: 109)

      (4) GQTRSPL ("   )

      (5) GQTRSPL ("   )

      (6) GQTRSPL ("   )

      (7) GQTRSPL ("   )

The heptapeptides show very similar or identical sequences indicating that the binding
is specific. The fact that phages bearing these heptapeptides were eluted by
acetazolamide, an inhibitor of carbonic anhydrase activity, indicates that the peptides
bind to the CA domain of MN protein.

(b) Analogous screening of the heptapeptide phage display library is done
using collagen I, shown to bind MN protein, for elution of phages. Different peptide(s)
binding to different part(s) of the MN protein molecule are expected to be identified.
After identifying such MN-binding peptides, the corresponding synthetic peptides shall
then be analysed for their biological effects.

Example 4

Accessibility In Vivo of MN Protein Expressed in
Tumor Cells and in Stomach

Lewis rats (384g) carrying a BP6 subcutaneous tumor (about 1 cm in
diameter) expressing rat MN protein were injected intraperitoneally (i.p.) with ^{125}I-M75
Mab (2.5 x 10^6 cpm). Five days later, 0.5-1g pieces of the tumor and organs were
weighed and their radioactivity was measured by a gamma counter.

Table 2? summarizes the results. The highest radioactivity was present in
the tumor. Relatively high radioactivity was found in the liver and kidney, apparently
reflecting the clearance of mouse IgG from the blood. The stomach continued a

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relatively low level of radioactivity, indicating that the M75 Mab had only limited access to MN protein exposed in the gastric mucosa.

TABLE 2

Distribution of radioactivity of ^{125}I-M75 in rat organs and in the tumor

Organ	cpm/g				
Kidney	2153	2184			
Spleen	653	555			
Liver	1993	1880			
Lung	1183	1025			
Blood	1449				
Heart	568	477			
Stomach	1184	1170			
Testis	812	779			
Tail	647				
Tumor	3646	4058	3333	8653	3839

Example 5

FACS Analysis of MN Protein Expression
in CGL3 Cells – Apoptosis

A FACS investigation was designed to determine the conditions that influence the synthesis of MN protein and to analyse the cell cycle distribution of MN-positive versus MN-negative cells in a CGL3 population stimulated to apoptosis. Previous Western blotting analyses have shown CGL3 cells to express a relatively high amount of MN protein under different cultivation conditions. CGL3 cells are considered a constitutive producer of MN proteins. However, Western blotting does not recognize small differences in the level of protein. In contrast FACS allows the detection of individual MN-positive cells, a calculation of their percentage in the analysed population, an estimation of the level of MN protein in the cells, and a determination of the cell cycle distribution.

To study the effect of cultivation conditions on MN expression in CGL3 cells, the CGL3 cells were plated in different relative densities and serum concentrations. Three days after plating, the cells were collected, surface labeled by M75 Mab followed by FITC-conjugated anti-mouse IgG and immediately analysed by

5   FACS.

The analysis showed that in adherent cells, MN expression is dependent on cell density as is HeLa cells. However, low density cultures still produced detectable amounts of MN protein. In low density cultures, serum concentration does not seem to play a role. In relatively high density cultures, a decreasing serum

10   concentration resulted in slightly diminished MN expression, probably due to a lower density that the cells were able to reach during the three days of cultivation.

The effect of the actual cell density is remarkable, and MN expression (detectable in 15-90% of the cells) represents a very sensitive monitoring factor. In all experiments, there was about a 5% higher percentage of cycling cells in the MN-

15   positive part of the population, compared to the MN-negative part. That fact prompted the analysis of the cell cycle distribution of MN-positive CGL3 cells under unfavorable growth conditions, that is, after induction of apoptosis.

Apoptosis

20   CGL3 cells were stimulated to apoptotic death by several drugs, including cycloheximide, actimonycin D and dexamethasone. The FACS study showed that the onset of apoptosis is delayed in MN-positive cells suggesting a protective role of MN in this process. It was also observed that the induction of apoptosis resulted in the down-regulation of MN expression in a time-dependent manner. That same phenomenon

25   was described for Bcl-2 anti-apoptotic protein, and there is existing opinion that the down-regulation of certain regulatory genes during apoptosis sensitizes the cells to undergo apoptotic death. To prove the role of MN in apoptosis, a similar study with cells transfected by MN cDNA is to be performed.

The preliminary results indicate the possible involvement of MN in the

30   suppression of apoptosis. The recent view that tumors arise both as a consequence of increased proliferation and decreased cell death appears to be consistent with the association of the MN protein with tumors in vivo.

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ATCC Deposits

The materials listed below were deposited with the American Type Culture Collection (ATCC) now at 10810 University Blvd., Manassus, Virginia 20110-2209 (USA). The deposits were made under the provisions of the Budapest Treaty on

5   the International Recognition of Deposited Microorganisms for the Purposes of Patent Procedure and Regulations thereunder (Budapest Treaty). Maintenance of a viable culture is assured for thirty years from the date of deposit. The hybridomas and plasmids will be made available by the ATCC under the terms of the Budapest Treaty, and subject to an agreement between the Applicants and the ATCC which assures

10   unrestricted availability of the deposited hybridomas and plasmids to the public upon the granting of patent from the instant application. Availability of the deposited strain is not to be construed as a license to practice the invention in contravention of the rights granted under the authority of any Government in accordance with its patent laws.

Hybridoma	Deposit Date	ATCC #
VU-M75	September 17, 1992	HB 11128
MN 12.2.2	June 9, 1994	HB 11647

15

Plasmid	Deposit Date	ATCC #
A4a	June 6, 1995	97199
XE1	June 6, 1995	97200
XE3	June 6, 1995	97198

20

The description of the foregoing embodiments of the invention have been

25   presented for purposes of illustration and description. They are not intended to be exhaustive or to limit the invention to the precise form disclosed, and obviously many modifications and variations are possible in light of the above teachings. The embodiments were chosen and described in order to explain the principles of the invention and its practical application to enable thereby others skilled in the art to

30   utilize the invention in various embodiments and with various modifications as are suited to the particular use contemplated.

All references cited herein are hereby incorporated by reference.

CLAIMS

1. An organic or inorganic molecule that binds specifically to a site on
MN protein to which vertebrate cells adhere in a cell adhesion assay, wherein said
5   molecule when tested in vitro inhibits the adhesion of cells to MN protein.

2. The organic or inorganic molecule of Claim 1 wherein said molecule,
when in contact with a vertebrate preneoplastic or neoplastic cell that abnormally
expresses MN protein, inhibits the growth of said cell.
10

3. The molecule of Claims 1 and 2 which is organic.

4. The molecule of Claim 3 which is a protein or a polypeptide.

15   5. The molecule of Claim 4 wherein said protein or polypeptide
comprises an amino acid sequence selected from the group consisting of SEQ ID NOS:
107, 108, 109, 137 and 138.

6. The molecule of Claim 4 wherein said polypeptide is selected from the
20   group consisting of SEQ ID NOS: 107, 108, 109, 137 and 138.

7. The molecule of Claim 1 wherein the site on the MN protein to which
said vertebrate cells adhere in said cell adhesion assay is within the proteoglycan-like
domain or within the carbonic anhydrase domain of the MN protein.
25

8. The molecule of Claim 1 wherein the site on the MN protein
comprises an amino acid sequence selected from the group consisting of SEQ ID NOS:
10 and 97-106.

30   9. The molecule of Claim 1 wherein the site on the MN protein has an
amino acid sequence selected from the group consisting of SEQ ID NOS: 10 and 97-
106.

10. The molecule of Claim 1 wherein said vertebrate cells are mammalian.

11. The molecule of Claim 1 wherein said vertebrate cells are human.

5

12. A MN protein or MN polypeptide which mediates attachment of vertebrate cells in a cell adhesion assay, wherein said MN protein or MN polypeptide when introduced into the extracellular fluid environment of vertebrate cells prevents the formation of intercellular contacts and the adhesion of said vertebrate cells to each

10 other.

13. The MN protein or MN polypeptide of Claim 12 which when introduced into the extracellular fluid environment of a vertebrate preneoplastic or neoplastic cell that abnormally expresses MN protein, inhibits the growth of said cell.

15

14. The MN protein or MN polypeptide of Claim 12 whose amino acid sequence is from SEQ ID NO: 97, from SEQ ID NO: 50, or from SEQ ID NO: 51.

15. The MN protein or MN polypeptide of Claim 12 whose amino acid

20 sequence is from SEQ ID NO: 50.

16. The MN protein or MN polypeptide of Claim 12 which comprises an amino acid sequence selected from the group consisting of SEQ ID NOS: 10 and 97-106.

25

17. The MN polypeptide of Claim 12 that is selected from the group consisting of SEQ ID NOS: 10 and 97-106.

18. The MN protein or MN polypeptide of Claim 12 that is specifically

30 bound by either the M75 monoclonal antibody that is secreted from the hybridoma VU-M75, which was deposited at the American Type Culture Collection under ATCC No. HB 11128, or by the MN12 monoclonal antibody that is secreted from the hybridoma

MN 12.2.2, which was deposited at the American Type Culture Collection under ATCC
No. HB 11647, or by both said monoclonal antibodies.

19. A method of identifying a site on an MN protein to which vertebrate
cells adhere by testing a series of overlapping polypeptides from said MN protein in a
cell adhesion assay with vertebrate cells, and determining that if cells adhere to a
polypeptide from said series, that said polypeptide comprises a site on said MN protein
to which vertebrate cells adhere.

20. A vector comprising an expression control sequence operatively
linked to a nucleic acid encoding the variable domains of a MN-specific antibody,
wherein said domains are separated by a flexible linker polypeptide, and wherein said
vector, when transfected into a vertebrate preneoplastic or neoplastic cell that
abnormally expresses MN protein, inhibits the growth of said cell.

21. The vector of Claim 20 wherein said expression control sequence
comprises the MN gene promoter operatively linked to said nucleic acid.

22. The vector of Claim 20 wherein said flexible linker polypeptide has
the amino acid sequence of SEQ ID NO: 116.

23. The vector of Claim 20 wherein said expression control sequence
comprises a nucleotide sequence selected from the group consisting of SEQ ID NO: 21,
SEQ ID NO: 27 and SEQ ID NO: 91.

24. A vector comprising a nucleic acid that encodes a cytotoxic protein
or cytotoxic polypeptide operatively linked to the MN gene promoter, wherein said
vector, when transfected into a vertebrate preneoplastic or neoplastic cell that
abnormally expresses MN protein, inhibits the growth of said cell.

25. The vector of Claim 24 wherein said cytotoxic protein is HSV
thymidine kinase.

26. The vector according to Claim 24 wherein said vector further comprises a nucleic acid encoding a cytokine operatively linked to said MN gene promoter.

5   27. The vector of Claim 26 wherein said cytokine is interferon or interleukin-2.

28. A repressor complex that binds to the MN gene promoter.

10   29. The repressor complex of Claim 28 that binds to SEQ ID NO: 115 of the MN gene promoter.

30. The repressor complex of Claim 29 comprising two proteins having molecular weights of 35 and 42 kilodaltons, respectively.

15

FIG._1A

```
  1                          M   A   P   L   C   P   S   P   W   L   P   L            12
  1   ACA GTC AGC CGC ATG GCT CCC CTG TGC CCC AGC CCC TGG CTC CCT CTG                 48

 13   L   L   I   P   A   P   A   G   L   T   V   Q   L   L   L   S                    28
 49   TTG CTG ATC CCG GCC CCT GCT GGC CTC ACT GTG CAA CTG CTG CTG TCA                 96

 29   D   E   S   P   L   G   S   G   G   S   G   E   R   P   L   Q                    44
 97   GAT GAG TCC CCC TTG GGA TCT GGA GGC AGT GGA GAA CGG CCC CTG CAG                144

 45   E   D   E   D   E   S   G   D   E   P   E   E   R   P   D   L                    60
145   GAG GAT GAG GAC GAA TCA GGG GAC GAG CCT GAG GAG AGA CCC GAC CTG                192

 61   G   E   E   E   E   E   G   S   S   D   D   E   R   P   P   D                    76
193   GGC GAG GAG GAG GAA GAG GGG TCA TCA GAT GAT GAG AGA CCC CCC GAT                240

 77   P   P   G   E   E   G   E   G   D   P   L   D   E   E   G   E                    92
241   CCA CCC GGA GAG GAG GGA GAG GGA GAT CCT CTA GAT GAG GAG GGA GAG                288

 93   E   L   P   E   K   S   A   E   E   E   E   E   G   S   E   E                   108
289   GAG CTA CCT GAA AAA TCA GCT GAG GAA GAA GAA GAG GGA TCC GAA GAG                336

109   K   E   S   K   P   V   T   E   D   R   P   D   K   E   G   L                   124
337   AAG GAG TCA AAA CCT GTT ACT GAG GAC AGG CCT GAC AAA GAA GGA CTG                384

125   Q   N   A   H   R   D   D   K   E   R   E   D   D   D   D   E                   140
385   CAG AAT GCC CAC AGG GAC GAC AAA GAA AGG GAA GAT GAC GAT GAC GAA                432

141   W   R   Y   G   D   P   P   W   R   S   V   P   D   Q   S   H                   156
433   TGG CGC TAT GGA GAC CCC CCG TGG CGG TCC GTG CCC GAC CAG AGT CAT                480

157   A   R   G   F   Q   S   P   V   V   D   I   R   P   Q   L   A                   172
481   GCG CGC GGC TTC CAG TCC CCG GTG GTG GAT ATC CGC CCC CAG CTC GCC                528
```

FIG._1B

Position labels at right of each row (amino acid / nucleotide):
188/576, 204/624, 220/672, 236/720, 252/768, 268/816, 284/864, 300/912, 316/960, 332/1008, 348/1056

```
173                                                                                              188
529                                                                                              576
 F   C   P   A   L   R   P   L   E   L   L   G   F   Q   L   P
TTC TGC CCG GCC CTG CGC CCC CTG GAA CTC CTG GGC TTC CAG CTC CCG

189                                                                                              204
577                                                                                              624
 P   L   P   E   L   R   R   N   N   G   H   S   V   Q   L   L
CCG CTC CCA GAA CTG CGC CGC AAC AAT GGC CAC AGT GTG CAA CTG CTG

205                                                                                              220
625                                                                                              672
 T   L   P   P   G   L   E   M   A   L   G   P   E   R   E   Y
ACC CTG CCT CCT GGG CTA GAG ATG GCT CTG GGT CCC GAG CGG GAG TAC

221                                                                                              236
673                                                                                              720
 R   A   Q   L   L   H   H   H   W   L   G   A   G   R   P   G
CGG GCT CAG CTG CTG CAT CAC CAC TGG CTG GGG GCA GGT CGT CCG GGC

237                                                                                              252
721                                                                                              768
 S   E   H   T   V   E   G   H   R   T   A   P   E   I   H   V
TCG GAG CAC ACT GTG GAA GGC CAC CGT ACT GCC CCT GAG ATC CAC GTG

253                                                                                              268
769                                                                                              816
 V   H   L   S   A   T   F   A   A   S   A   E   A   L   G   R
GTT CAC CTC AGC GCC ACC TTT GCC GCC AGC GCC GAG GCC TTG GGG CGC

269                                                                                              284
817                                                                                              864
 P   G   G   L   A   V   L   A   A   G   E   E   E   G   P   E
CCG GGA GGC CTG GCC GTG TTG GCC GCC GGC GAG GAG GAG GGC CCG GAA

285                                                                                              300
865                                                                                              912
 E   N   S   A   Y   E   Q   L   L   S   R   L   E   E   I   A
GAA AAC AGT GCC TAT GAG CAG TTG CTG TCT CGC TTG GAA GAA ATC GCT

301                                                                                              316
913                                                                                              960
 E   E   G   S   E   T   Q   P   V   P   G   D   I   S   A   L
GAG GAA GGC TCA GAG ACT CAG CCA GTC CCA GGA GAC ATA TCT GCA CTC

317                                                                                              332
961                                                                                              1008
 L   P   S   D   F   S   R   Y   Q   Y   E   G   G   S   L   T
CTG CCC TCT GAC TTC AGC CGC TAC CAA TAT GAG GGG GGG TCT CTG ACT

333                                                                                              348
1009                                                                                             1056
 T   P   P   A   Q   Q   G   V   I   W   T   V   F   N   Q   T
ACA CCC CCC GCC CAG CAG GGT GTC ATC TGG ACT GTG TTT AAC CAG ACA
```

```
349              V   M   L   S   A   K   Q   L   H   T   L   S   D   T   L   W   364
1057            GTG ATG CTG AGT GCT AAG CAG CTC CAC ACC CTC TCT GAC ACC CTG TGG  1104

365              G   P   G   D   S   R   L   Q   N   F   R   A   T   Q   P        380
1105            GGA CCT GGT GAC TCT CGG CTA CAG AAC TTC CGA GCG ACG CAG CCT       1152

381              L   N   G   R   V   I   E   A   S   F   P   D   V   G   D   S   396
1153            TTG AAT GGG CGA GTG ATT GAG GCC TCC TTC CCT GGA GTG GAC AGC       1200

397              S   P   R   A   A   A   E   Q   V   L   C   L   A   A           412
1201            AGT CCT CGG GCT GCT GCT GAG CAG GTC CTG TGC CTG GCT GCT          1248

413              G   D   I   L   A   V   Q   L   L   F   G   L   F   A   T   S   428
1249            GGT GAC ATC CTA GCC GTC CAG CTG CTC TTT GGC CTC TTT GCT ACC AGC   1296

429              V   A   F   L   V   Q   M   Q   R   R   H   R   Q   A   V       444
1297            GTC GCG TTC CTT GTG CAG ATG AGA CAG AGG CAC AGA AGG GCT GTC       1344

445              G   V   S   Y   R   P   A   E   V   A   E   T   G   A   *       460
1345            GGG GTG AGC TAC CGC CCA GCA GAG GTA GCC GAG ACT GGA GCC TAG       1392

                AGG CTG GAT CTT GGA GAA TGT CCT GAG AAG CCA GCC AGA GGC ATC TGA GGG  1440
1393

                GGA GCC GGT AAC TGT CCT GTC CTG CTC ATT ATG CCA CTT CCT TTT AAC  1488
1441

1489            TGC CAA GAA ATT TTT TAA AAT AAA TAT TTA TAA T                    1522
```

```
   1  ggatcctgtt gactcgtgac cttaccccca accctgtgct ctctgaaaca tgagctgtgt
  61  ccactcaggg ttaaatggat taaggggcgt gcaagatgtg ctttgttaaa cagatgcttg
 121  aaggcagcat gctcgttaag agtcatcacc aatccctaat ctcaagtaat caggacaca
 181  aacactgcgg aaggccgcag ggtcctctgc ctaggaaaac cagagacctt tgttcacttg
 241  tttatctgac cttccctcca ctattgtcca tgaccctgcc aaatcccct  ctgtgagaaa
 301  cacccaagaa ttatcaataa aaaaataaat ttaaaaaaaa aatacaaaaa aaaaaaaaaa
 361  aaaaaaaaaa gacttacgaa tagttattga taaatgaata gctattggta aagccaagta
 421  aatgatcata ttcaaaacca gacggccatc atcacagctc aagtctacct gattgatct
 481  ctttatcatt gtcattcttt ggattcacta gattagtcat catcctcaaa attctcccc
 541  aagttctaat tacgttccaa acattaggg  gttacatgaa gcttgaacct actaccttct
 601  ttgcttttga gccatgagtt gtaggaatga tgagtttaca ccttacatgc tggggattaa
 661  tttaaacttt acctctaagt cagttgggta gcctttggct tattttgta  gctaattttg
 721  tagttaatgg atgcactgtg aatcttgcta tgatagtttt cctccacact ttgccactag
 781  gggtaggtag gtactcagtt ttcagtaatt gctacctaa  gaccctaagc cctatttctc
 841  ttgtactggc ctttatctgt aatatgggca tatttaatac aatataattt ttggagtttt
 901  tttgtttgtt tgtttttttg tttttttgag acggagtctt gcatctgtca tgcccaggct
 961  ggagtagcag tggtgccatc tcggctcact gcaagctcca ctcccgagt  tcacgccatt
1021  ttcctgcctc agcctcccga gtagctggga ctacaggcgc ccgccaccat gcccggctaa
1081  ttttttgtat tttttggtag a         tttggtaga  gacggggttt caccgtgtta tctcgatctc
1141  ctgacttcgt gatccaccg  cctcggcctc ccaaagtcct gggattacag gtgtgagcca
1201  ccgcacctgg ccaattttt  gagtctttta aagtaaaaat atgtcttgta agctggtaac
1261  tatggtacat ttcctttat  taatgtggtg ctgacggtca tataggttct tttgagtttg
1321  gcatgcatat gctactttt  gcagtccttt cattacattt tctctcttc  attgaagag
1381  catgttatat cttttagctt cacttggctt aaaaggttct ctcattagcc taacacagtg
1441  tcattgttgg taccacttgg atcataagtg gaaaaacagt caagaaattg cacagtaata
1501  cttgtttgta agaggggatga ttcaggtgaa tctgacacta agaaactccc ctacctgagg
1561  tctgagattc ctctgacatt gctgtatata ggcttttcct ttgacagcct gtgactgcgg
1621  actattttc  ttaagcaaga tatgctaaag tttgtgagc  cttttccag  agagaggtct
1681  catatctgca tcaagtgaga acatataatg tctgcatgtt tccatatttc aggaatgttt
1741  gcttgtgttt tatgctttta tatagacagg gaaacttgtt cctcagtgac ccaaaagagg
1801  tgggaattgt tattggatat catcattggc ccacgctttc tgacctttga aacaattaag
1861  ggttcataat ctcaattctg tcagaattgg tacaagaaat agctgctatg tttcttgaca
1921  ttccacttgg taggaaataa gaatgtgaaa ctcttcagtt ggtgtgtgtc cct?gtttttt
```

FIG._2A

```
1981  ttgcaatttc cttcttactg tgttaaaaaa aagtatgatc ttgtctgag  aggtgaggca
2041  ttcttaatca tgatctttaa agatcaataa tataatcctt tcaaggatta tgtctttatt
2101  ataataaaga taatttgtct ttaacagaat caataatata atcccttaaa ggattatatc
2161  tttgctgggc gcagtggctc acacctgtaa tcccagcact ttgggtggcc aaggtggaag
2221  gatcaaattt gcctacttct atattatctt ctaaagcaga attcatctct cttcccctcaa
2281  tatgatgata ttgacagggt ttgccctcac tcactagatt gtgagctcct gctcagggca
2341  ggtagcgttt tttgtttttg tacagtctca ctcctgagta gctgggacta ccattacacc
2401  cccaggccag agtgcaatgg tacagtctca gctcactgca gcctcaaccg cctcggctca
2461  aaccatcatc ccatttcagc ctcctgagta gctgggacta caggcacatg ccattacacc
2521  tggctaattt ttttgtattt ctagtagaga caggttttgg ccatgttgcc cgggctggtc
2581  tcgaactcct ggactcaagc aatccaccca cctcagcctc ccaaaatgag ggaccgtgtc
2641  ttattcattt ccatgtccct agtccatagc ccagtgctgg acctatggta gtactaaata
2701  aatatttgtt gaatgcaata gtaaatagca tttcagggag caagaactag attaacaaag
2761  gtggtaaaag gtttggagaa aaaaataata gtttaatttg gctagagtat gagggagagt
2821  agtaggagac aagatggaaa ggtctcttgg gcaaggtttt gaaggaagtt ggaagtcaga
2881  agtacacaat gtgcatatcg tggcaggcag tggggagcca atgaaggctt ttgagcagga
2941  gagtaatgtg ttgaaaaata aatataggtt aaacctatca gagcccctct gacacataca
3001  cttgctttc attcaagctc aagtttgtct cccacatacc cattacttaa ctcaccctcg
3061  ggctccccta gcagcctgcc ctacctcttt acctgcttcc tggtggagtc agggatgtat
3121  acatgagctg cttcccctct cagccagagg acatggggggg cccagctcc cctgcctttc
3181  cccttctgtg cctggagctg ggaagcaggc cagggttagc tgaggctggc tggcaagcag
3241  ctgggtggtg ccagggagag cctgcatagt gccaggtggt gccttgggtt ccaagctagt
3301  ccatggcccc gataaccttc tgcctgtgca cacacctgcc cctcactcca cccccatcct
3361  agctttggta tgggggagag ggcacagggc cagacaaacc tgtgagactt tggctccatc
3421  tctgcaaaag ggcgctctgt gagtcagcct gctcccctcc agcttgctc ctcccccacc
3481  cagctctcgt ttccaatgca cgtacagccc gtacacaccg tgtgctggga caccCACAG
3541  TCAGCCGCAT GGCTCCCCTG TGCCCCAGCC CCTGGCTCCC CCGGCTGGC CCGGCCCCTG
3601  CTCCAGGCCT CACTGTGCAA CTGCTGCTGT CTGCTGCTGT CACTGCTGCT GTCCATCCCC
3661  AGAGGTTGCC CCGGATGCAG GAGGATTCCC CCTTGGGAGG AGGCTCTTCT GGGGAAGATG
3721  ACCCACTGGG CGAGGAGGA CTGCCCAGTG AAGAGGATTC ACCCAGAGAG GAGGATCCAC
3781  CCGGAGAGGA GGATCTACCT GGAGAGGAGG ATCTACCTGG AGAGGAGGAT CTACCTGAAG
3841  TTAAGCCTAA ATCAGAAGAA GAGGGCTCCC TGAAGTTAGA GGATCTACCT ACTGTTGAGG
3901  CTCCTGAGA TCCTCAAGAA CCCCAGAATA ATGCCCACAG GGACAAAGAA Ggtaagtggt
```

FIG._2B

```
3961  catcaatctc caaatccagg ttccaggagg ttcatgactc cctcccata cccagccta
4021  ggctctgttc actcagggaa ggaggggaga ctgtactccc cacagaagcc cttccagagg
4081  tcccatacca atatccccat ccccactctc ggaggtagaa agggacagat gtggagagaa
4141  aataaaaagg gtgcaaaagg agagaggtga gctgaggtga atgggagaga agggggaggc
4201  tggagagag aaagggatga gaactgcaga tgagagaaaa aatgtgcaga cagaggaaaa
4261  aaataggtgg agaaggagag tcagagagtt tgaggggaag agaaaaggaa agcttgggag
4321  gtgaagtggg taccagagac aagcaagaag agctggtaga agtcatctca tcttaggcta
4381  caatgaggaa ttgagaccta ggaagaaggg acacagcagg tagagaaacg tggcttcttg
4441  actcccaagc caggaatttg gggaaagggg ttggagacca tacaaggcag agggatgagt
4501  ggggagaaga aagagaaaga aaaggaaaga tggtgtactc actcatttgg gactcaggac
4561  tgaagtgccc actcactttt tttttttttt tttttgagac aaactttcac tttttgttgcc
4621  caggctggag tgcaatggcg cgatctcggc tcactgcaac ctccacctcc cgggttcaag
4681  tgattctcct gcctcagcct ctagccaagt agctgcgatt acaggcatgc gccaccacgc
4741  ccggctaatt tttgtatttt tagtagagac gggtttcgc catgttggtc agctggtct
4801  cgaactcctg atctcaggtg atccaaccac cctggcctcc caaagtgctg ggattatagg
4861  cgtgagccac agcgcctggc ctgaagcagc cactcacttt tacagaccct aagacaatga
4921  ttgcaagctg gtaggattgc tgtttggccc acccagctgc ggtgttgagt ttgggtgcgg
4981  tctcctgtgc tttgcacctg gcccgcttaa ggcatttgtt acccgtaatg ctcctgtaag
5041  gcatctgcgt ttgtgacatc gtttggtcg ccaggaaggg attggggctc taagcttgag
5101  cggttcatcc ttttcattta tacagGGGAT tacagGACCA GAGTC ATTGGCGCTA TGGAGgtgag
5161  acacccaccc gctgcacaga cccaatctgg gaacccagct ctgtggatct ccctacagc
5221  cgtccctgaa cactggtccc gggcgtccca cccgccgccc accgtcccac ccctccacct
5281  tttctacccg ggttccctaa gttcctgacc taggcgtcag acttcctcac tatactctcc
5341  caccccagGC GACCCGCCCT GGCCCCGGGT GTCCCCAGCC TGCGCGGGCC GCTTCCAGTC
5401  CCCGGTGGAT ATCCGCCCCC AGCTCGCCGC CTTCTGCCCG GCCCTGCGCC CCCTGGAACT
5461  CCTGGGCTTC CAGCTCCCGC CGCTCCCAGA ACTGCGCCTG CGCAACAATG GCCACAGTGg
5521  tgaggggtc tccccgccga gacttgggga tggggcgggg cgcagggaag ggaaccgtcg
5581  cgcagtgcct gcccggggt tgggctggcc ctaccgggcg gggccggctc acttgcctct
5641  ccctacgcag TGCAACTGAC CCTGCCTCCT GGGCTAGAGA TGGCTCTGGG TCCCGGGCGG
5701  GAGTACCGGG CTCTGCAGCT GCATCTGCAC TGGGGGGCTG CAGGTCGTCC GGGCTCGGAG
5761  CACACTGTGG AAGGCCACCG TTTCCCTGCC GAGgtgagcg cggactggcc gagaaggggc
5821  aaggagcgg ggcggacggg ggccagagac gtggccctct cctacccctcg tgtcctttc
5881  agATCCACGT GGTTCACCTC AGCACCGCCT TGCCCAGAGT TGACGAGGCC TTGGGGGGCC
```

```
5941 CGGGAGGCCT GGCCGTGTTG GCCGCCTTTC TGGAGgtacc agatcctgga cacccctac
6001 tccccgcttt cccatcccat gctcctcccg gactctatcg tggagccaga gacccatcc
6061 cagcaagctc actcaggccc ctggctgaca aactcattca cgcactgttt gttcatttaa
6121 cacccactgt gaaccaggca ccagccccca acaaggattc tgaagctgta ggtcctgcc
6181 tctaaggagc ccacagccag tggggaggc tgacatgaca gacacatagg aaggacatag
6241 taaagatggt ggtcacagag gaggtgacac ttaaagcctt cactggtaga aaagaaaagg
6301 aggtgttcat tgcagaggaa acagaatgtg caaagactca gaatatggcc tatttaggga
6361 atggctacat acaccatgat tagaggaggc ccagtaaagg gaagggatgg tgagatgcct
6421 gctaggttca ctcactcact tttatttatt tattatttt tttgacagtc tctctgtcgc
6481 ccaggctgga gtgcagtggt gtgatcttgg gtcactgcaa cttccgcctc ccggttcaa
6541 gggattctcc tgcctcagct tcctgagtag ctggggttac caccatgccc
6601 agctaatttt tttttgtatt tttagtagac agggtttcac aggctggtct
6661 caaactcctg gcctcaagtg atccgcctga ctcagcctac attacaagtg
6721 tgagccaccg tgcccagcca cactcactga ttctttaatg agcacaaagt
6781 tcagagaaat gcctccatca tagcatgtca atatgttcat tcatgatgtt
6841 cttaacatta ggttcataag caaaataaga aaaaagaata gaagtggcat
6901 gtcaggacct cacctgaaaa gccaaacaca gaatcatgaa gaggtgacac
6961 caacacaaag gtgtatatat ggtttcctgt ggggagtatg gcagtgagtg
7021 agactgcaaa cgtcagagagg gcacgggtca ctgagagcct taaagtgggc
7081 tctctcccctc tctctccagc ttgtcattga aaaccagtcc ttggttcgca
7141 cagcaagagt acatagagtt tgaaataata cataggattt acactgtctc
7201 taaaaaaaaa aacaacagca acaacaaaaa gcaacaacca ttacaatttt atgttccctc
7261 agcattctca gagctgagga atgggagagg actatgggaa cccccttcat gttccggcct
7321 tcagccatgg ccctggatac atgcactcat ctgtcttaca atgtcattcc cccagGAGGG
7381 CCCGGAAGAA AACAGTGCCT ATGAGCAGTT GCTGTCTCGC TTGGAAGAAA TCGCTGAGGA
7441 AGgtcagttt gttggtctgg ccactaatct ctgtggccta gttcataaag aatcacccctt
7501 tggagcttca ggtctgaggc tggagatggg ctcccctccag tgcaggaggg attgaagcat
7561 gagccagcgc tcatcttgat aataaccatg aagctgacag acacagttac ccgcaaacgg
7621 ctgcctacag attgaaaaacc aagcaaaaac cgccgggcac ggtggctcac gcctgtaatc
7681 ccagcacttt gggaggccaa ggcaggtgga tcacgaggtc aagagatcaa gaccatcctg
7741 gccaacatgg tgaaaccccca tctctactaa aaatacgaaa aaatagccag gcgtggtggc
7801 gggtgcctgt aatcccagct actcgggagg ctgaggcagg agaatggcat gaacccggga
7861 ggcagaagtt gcagtgagcc gagatcgtgc cactgcactc cagcctgggc aacagagcga
```

FIG.__2D

```
7921  gactcctgtc  tcaaaaaaaa  aaaaaaaaaa  gaaaaccaa   caaaaaccaa   aatgagacaa
7981  aaaaaacaag  accaaaaaat  ggtgtttgga  aattgtcaag  gtcaagtctg   gagagctaaa
8041  cttttctga   gaactgttta  tcttaataa   gcatcaaata  tttaacttt    gtaaatactt
8101  ttgttggaaa  tcgttctctt  cttagtcact  cttgggtcat  tttaaatctc   acttactcta
8161  ctagaccttt  taggtttctg  ctagactagg  tagaactctg  cctttgcatt   tcttgtgtct
8221  gttttgtata  gttatcaata  ttcatattta  tttacaagtt  attcagatca   tttttttctt
8281  tcttttttt   tttttttttt  tttttttacat  cttagtaga   gacagggttt   caccatattg
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FIG._2F

FIG._2A
FIG._2B
FIG._2C
FIG._2D
FIG._2E
FIG._2F

FIG._2

FIG._3

FIG._4

FIG._8

FIG._5

MN/CAIX	SSG	EDDPLG	EEDLPS	EEDSPR	EEDPPG	EEDLPG	EEDLPE	VKPKSE	EEGSLK	LE
	: .	: .	. . : .	. . .	. . . .	. . .	. . . .	. .	. . . .	. .
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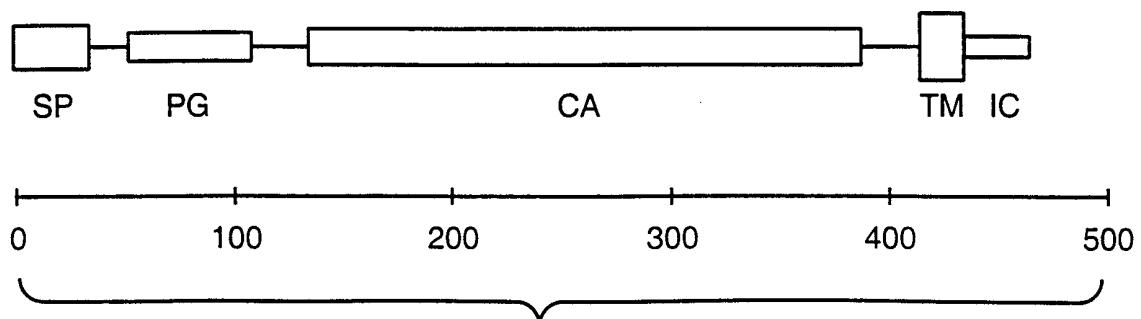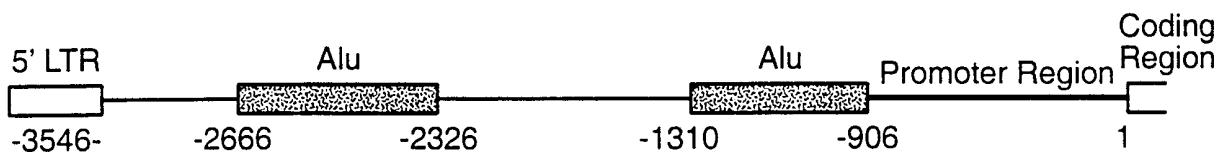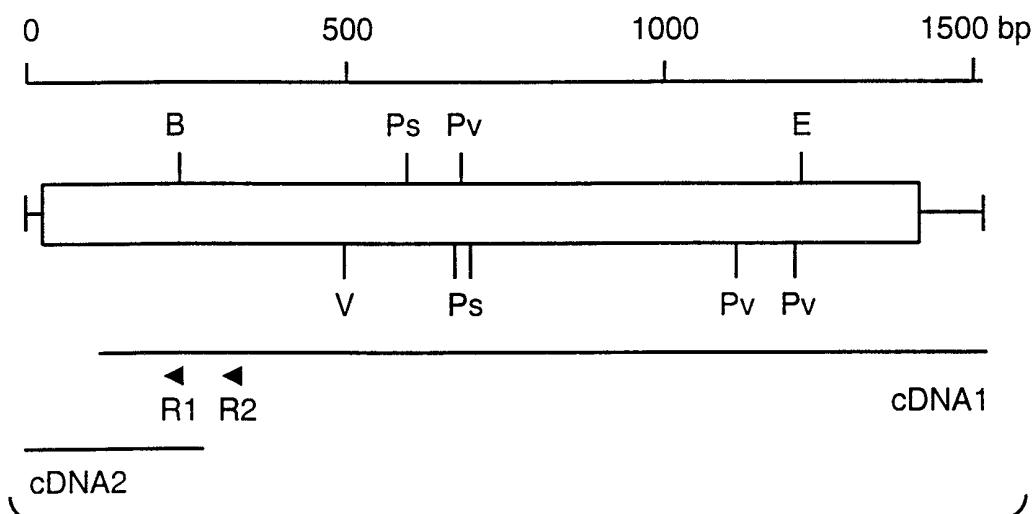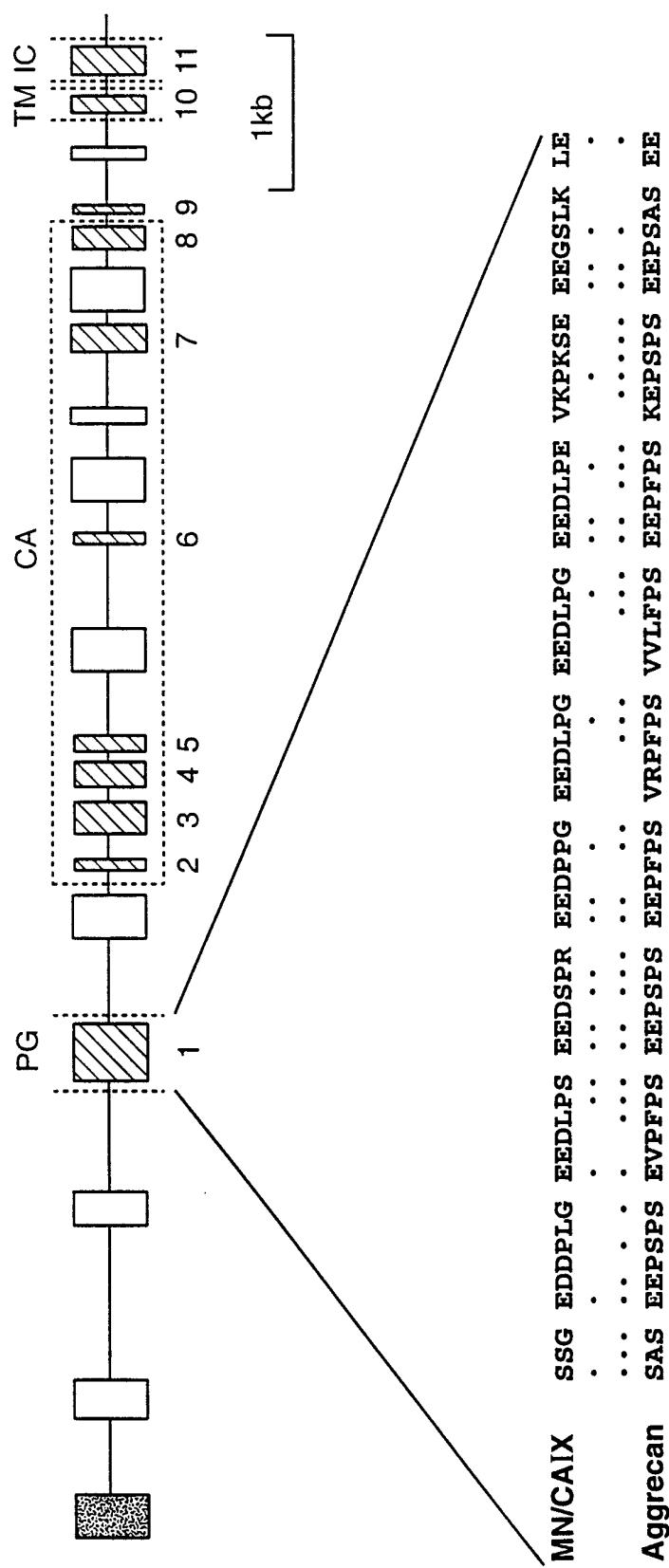
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                         AP2

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                               VII                          p53

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                                                      AP1         III  Inr

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FIG._6

FIG._7
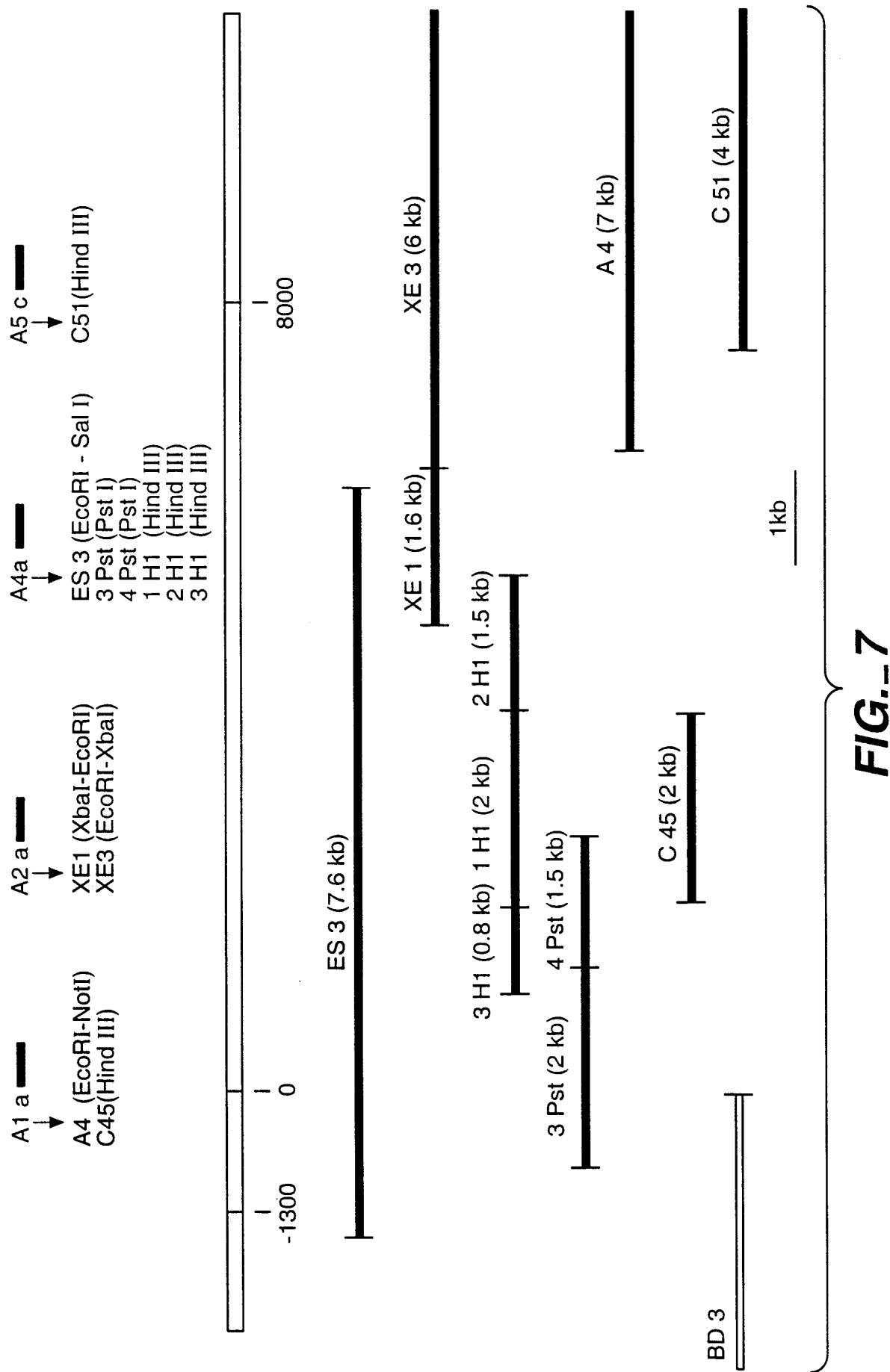
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            -20   -15   -10

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Glu Glu Asp Leu Pro Ser Glu Glu Asp Ser Pro Arg Glu Glu Asp Pro
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ccc gga gag gag gat cta cct gga gag gag gat cta cct gga gag gag    291
Pro Gly Glu Glu Asp Leu Pro Gly Glu Glu Asp Leu Pro Gly Glu Glu
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Asp Leu Pro Glu Val Lys Pro Lys Ser Glu Glu Glu Gly Ser Leu Lys
                60                  65                  70


tta gag gat cta cct act gtt gag gct cct gga gat cct caa gaa ccc    387
Leu Glu Asp Leu Pro Thr Val Glu Ala Pro Gly Asp Pro Gln Glu Pro
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Gln Asn Asn Ala His Arg Asp Lys Glu Gly Asp Asp Gln Ser His Trp
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Arg Tyr Gly Gly Asp Pro Pro Trp Pro Arg Val Ser Pro Ala Cys Ala
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Gly Arg Phe Gln Ser Pro Val Asp Ile Arg Pro Gln Leu Ala Ala Phe
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tgc ccg gcc ctg cgc ccc ctg gaa ctc ctg ggc ttc cag ctc ccg ccg    579
Cys Pro Ala Leu Arg Pro Leu Glu Leu Leu Gly Phe Gln Leu Pro Pro
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ctc cca gaa ctg cgc ctg cgc aac aat ggc cac agt gtg caa ctg acc    627
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ctg cct cct ggg cta gag atg gct ctg ggt ccc ggg cgg gag tac cgg    675
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gct ctg cag ctg cat ctg cac tgg ggg gct gca ggt cgt ccg ggc tcg    723
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His Leu Ser Thr Ala Phe Ala Arg Val Asp Glu Ala Leu Gly Arg Pro
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Gly Gly Leu Ala Val Leu Ala Ala Phe Leu Glu Glu Gly Pro Glu Glu
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gaa ggc tca gag act cag gtc cca gga ctg gac ata tct gca ctc ctg    963
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ccg ccc tgt gcc cag ggt gtc atc tgg act gtg ttt aac cag aca gtg   1059
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atg ctg agt gct aag cag ctc cac acc ctc tct gac acc ctg tgg gga   1107
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cct ggt gac tct cgg cta cag ctg aac ttc cga gcg acg cag cct ttg   1155
Pro Gly Asp Ser Arg Leu Gln Leu Asn Phe Arg Ala Thr Gln Pro Leu
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aat ggg cga gtg att gag gcc tcc ttc cct gct gga gtg gac agc agt   1203
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Pro Arg Ala Ala Glu Pro Val Gln Leu Asn Ser Cys Leu Ala Ala Gly
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Leu His Leu His Trp Gly Ala Ala Gly Arg Pro Gly Ser Glu His Thr
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Val Glu Gly His Arg Phe Pro Ala Glu Ile His Val Val His Leu Ser
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Thr Ala Phe Ala Arg Val Asp Glu Ala Leu Gly Arg Pro Gly Gly Leu
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Ala Val Leu Ala Ala Phe Leu Glu Glu Gly Pro Glu Glu Asn Ser Ala
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Glu Thr Gln Val Pro Gly Leu Asp Ile Ser Ala Leu Leu Pro Ser Asp
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Phe Ser Arg Tyr Phe Gln Tyr Glu Gly Ser Leu Thr Thr Pro Pro Cys
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Ser Arg Leu Gln Leu Asn Phe Arg Ala Thr Gln Pro Leu Asn Gly Arg
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Val Ile Glu Ala Ser Phe Pro Ala Gly Val Asp Ser Ser Pro Arg Ala
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5

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tggagagaaa ataaaaaggg tgcaaaagga gagaggtgag ctggatgaga tgggagagaa 240

ggggggaggct ggagaagaga aagggatgag aactgcagat gagagaaaaa atgtgcagac 300

agaggaaaaa aataggtgga gaaggagagt cagagagttt gaggggaaga gaaaaggaaa 360

gcttgggagg tgaagtgggt accagagaca agcaagaaga gctggtagaa gtcatctcat 420

cttaggctac aatgaggaat tgagacctag gaagaaggga cacagcaggt agagaaacgt 480

ggcttcttga ctcccaagcc aggaatttgg ggaaaggggt tggagaccat acaaggcaga 540

gggatgagtg gggagaagaa agaagggaga aaggaaagat ggtgtactca ctcatttggg 600

actcaggact gaagtgccca ctcacttttt tttttttttt ttttgagaca aactttcact 660

tttgttgccc aggctggagt gcaatggcgc gatctcggct cactgcaacc tccacctccc 720
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gggttcaagt gattctcctg cctcagcctc tagccaagta gctgcgatta caggcatgcg 780

ccaccacgcc cggctaattt ttgtattttt agtagagacg gggtttcgcc atgttggtca 840

ggctggtctc gaactcctga tctcaggtga tccaaccacc ctggcctccc aaagtgctgg 900

gattataggc gtgagccaca gcgcctggcc tgaagcagcc actcactttt acagaccctа 960

agacaatgat tgcaagctgg taggattgct gtttggccca cccagctgcg gtgttgagtt 1020

tgggtgcggt ctcctgtgct ttgcacctgg cccgcttaag gcatttgtta cccgtaatgc 1080

tcctgtaagg catctgcgtt tgtgacatcg ttttggtcgc caggaaggga ttggggctct 1140

aagcttgagc ggttcatcct tttcatttat acag                              1174
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<210> 40
<211> 193
<212> DNA
<213> HUMAN

<220>
<221> intron
<222> (1)..(193)
<223> 2nd MN intron

<400> 40
gtgagacacc cacccgctgc acagacccaa tctgggaacc cagctctgtg gatctcccct 60

acagccgtcc ctgaacactg gtcccgggcg tcccacccgc cgcccaccgt cccaccccct 120

caccttttct acccgggttc cctaagttcc tgacctaggc gtcagacttc ctcactatac 180

tctcccaccc cag                                                     193
```


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<210> 41
<211> 131
<212> DNA
<213> HUMAN

<220>
<221> intron
<222> (1)..(131)
<223> 3rd MN intron

<400> 41
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gtgagggggt ctccccgccg agacttgggg atggggcggg gcgcagggaa gggaaccgtc 60

gcgcagtgcc tgcccggggg ttgggctggc cctaccgggc ggggccggct cacttgcctc 120

tccctacgca g   131

<210> 42
<211> 89
<212> DNA
<213> HUMAN

<220>
<221> intron
<222> (1)..(89)
<223> 4th MN intron

<400> 42
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gccctctcct accctcgtgt cctttcag   89

<210> 43
<211> 1400
<212> DNA
<213> HUMAN

<220>
<221> intron
<222> (1)..(1400)
<223> 5th MN intron

<400> 43
gtaccagatc ctggacaccc cctactcccc gctttcccat cccatgctcc tcccggactc 60

tatcgtggag ccagagaccc catcccagca agctcactca ggccctggc tgacaaactc 120

attcacgcac tgtttgttca tttaacaccc actgtgaacc aggcaccagc ccccaacaag 180

gattctgaag ctgtaggtcc ttgcctctaa ggagcccaca gccagtgggg gaggctgaca 240

tgacagacac ataggaagga catagtaaag atggtggtca cagaggaggt gacacttaaa 300

gccttcactg gtagaaaaga aaaggaggtg ttcattgcag aggaaacaga atgtgcaaag 360

actcagaata tggcctattt agggaatggc tacatacacc atgattagag gaggcccagt 420

26

aaagggaagg gatggtgaga tgcctgctag gttcactcac tcacttttat ttatttattt 480

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tgcaacttcc gcctcccggg ttcaagggat tctcctgcct cagcttcctg agtagctggg 600

gttacaggtg tgtgccacca tgcccagcta attttttttt gtatttttag tagacaggt 660

ttcaccatgt tggtcaggct ggtctcaaac tcctggcctc aagtgatccg cctgactcag 720

cctaccaaag tgctgattac aagtgtgagc caccgtgccc agccacactc actgattctt 780

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ttcatactct taggttcatg atgttcttaa cattaggttc ataagcaaaa taagaaaaaa 900

gaataataaa taaagaagt ggcatgtcag gacctcacct gaaaagccaa acacagaatc 960

atgaaggtga atgcagaggt gacaccaaca caaaggtgta tatatggttt cctgtgggga 1020

gtatgtacgg aggcagcagt gagtgagact gcaaacgtca gaagggcacg ggtcactgag 1080

agcctagtat cctagtaaag tgggctctct ccctctctct ccagcttgtc attgaaaacc 1140

agtccaccaa gcttgttggt tcgcacagca agagtacata gagtttgaaa taatacatag 1200

gattttaaga gggagacact gtctctaaaa aaaaaaacaa cagcaacaac aaaaagcaac 1260

aaccattaca attttatgtt ccctcagcat tctcagagct gaggaatggg agaggactat 1320

gggaacccc ttcatgttcc ggccttcagc catggccctg gatacatgca ctcatctgtc 1380

ttacaatgtc attcccccag   1400

<210> 44
<211> 1334
<212> DNA
<213> HUMAN

<220>
<221> intron
<222> (1)..(1334)
<223> 6th MN intron

<400> 44
gtcagtttgt tggtctggcc actaatctct gtggcctagt tcataaagaa tcaccctttg 60

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gccagcgctc atcttgataa taaccatgaa gctgacagac acagttaccc gcaaacggct 180

gcctacagat tgaaaaccaa gcaaaaaccg ccgggcacgg tggctcacgc ctgtaatccc 240

agcactttgg gaggccaagg caggtggatc acgaggtcaa gagatcaaga ccatcctggc 300

caacatggtg aaaccccatc tctactaaaa atacgaaaaa atagccaggc gtggtggcgg 360

gtgcctgtaa tcccagctac tcgggaggct gaggcaggag aatggcatga acccgggagg 420

cagaagttgc agtgagccga gatcgtgcca ctgcactcca gcctgggcaa cagagcgaga 480

ctcttgtctc aaaaaaaaaa aaaaaaaga aaaccaagca aaaccaaaa tgagacaaaa 540

aaaacaagac caaaaaatgg tgtttggaaa ttgtcaaggt caagtctgga gagctaaact 600

ttttctgaga actgtttatc tttaataagc atcaaatatt ttaactttgt aaatactttt 660

gttggaaatc gttctcttct tagtcactct tgggtcattt taaatctcac ttactctact 720

agacctttta ggtttctgct agactaggta gaactctgcc tttgcatttc ttgtgtctgt 780

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tcccttccct cccaccttcc cttctctcct tcctttcttt cttcctctct tgcttcctca 1140

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ggcctgcact tagtgaagaa gtggtctcag agttgagtta ccttggcttc tgggaggtga 1260

aactgtatcc ctataccctg aagctttaag ggggtgcaat gtagatgaga ccccaacata 1320

gatcctcttc acag                                                  1334
```

<210> 45
<211> 512
<212> DNA

<213> HUMAN

<220>
<221> intron
<222> (1)..(512)
<223> 7th MN intron

<400> 45
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gagaaacagg agaagaaaga aatcaaggct gggctctgtg gcttacgcct ataatcccac 120

cacgttggga ggctgaggtg ggagaatggt ttgagccuag gagttcaaga caaggcgggg 180

caacatagtg tgaccccatc tctaccaaaa aaaccccaac aaaaccaaaa atagccgggc 240

atggtggtat gcggcctagt cccagctact caaggaggct gaggtgggaa gatcgcttga 300

ttccaggagt ttgagactgc agtgagctat gatcccacca ctgcctacca tctttaggat 360

acatttattt atttataaaa gaaatcaaga ggctggatgg ggaatacagg agctggaggg 420

tggagccctg aggtgctggt tgtgagctgg cctgggaccc ttgtttcctg tcatgccatg 480

aacccacccca cactgtccac tgacctccct ag   512

<210> 46
<211> 114
<212> DNA
<213> HUMAN

<220>
<221> intron
<222> (1)..(114)
<223> 8th MN intron

<400> 46
gtacagcttt gtctggtttc ccccagcca gtagtccctt atcctcccat gtgtgtgcca 60

gtgtctgtca ttggtggtca cagcccgcct ctcacatctc cttttctct ccag   114

<210> 47
<211> 617
<212> DNA
<213> HUMAN

```
<220>
<221> intron
<222> (1)..(617)
<223> 9th MN intron

<400> 47
gtgagtctgc ccctcctctt ggtcctgatg ccaggagact cctcagcacc attcagcccc 60

agggctgctc aggaccgcct ctgctccctc tccttttctg cagaacagac cccaacccca 120

atattagaga ggcagatcat ggtggggatt cccccattgt ccccagaggc taattgatta 180

gaatgaagct tgagaaatct cccagcatcc ctctcgcaaa agaatccccc cccctttttt 240

taaagatagg gtctcactct gtttgcccca ggctggggtg ttgtggcacg atcatagctc 300

actgcagcct cgaactccta ggctcaggca atcctttcac cttagcttct caaagcactg 360

ggactgtagg catgagccac tgtgcctggc cccaaacggc cctttactt ggctttagg 420

aagcaaaaac ggtgcttatc ttacccttc tcgtgtatcc accctcatcc cttggctggc 480

ctcttctgga gactgaggca ctatggggct gcctgagaac tcggggcagg ggtggtggag 540

tgcactgagg caggtgttga ggaactctgc agaccctct tccttcccaa agcagccctc 600

tctgctctcc atcgcag                                                617


<210> 48
<211> 130
<212> DNA
<213> HUMAN

<220>
<221> intron
<222> (1)..(130)
<223> 10th MN intron

<400> 48
gtattacact gacccttct tcaggcacaa gcttccccca cccttgtgga gtcacttcat 60

gcaaagcgca tgcaaatgag ctgctcctgg ccagttttc tgattagcct ttcctgttgt 120

gtacacacag                                                        130


<210> 49
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<211> 1401
<212> DNA
<213> HUMAN

<400> 49
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cctccacctc ccgggttcaa gtgattctcc tgcctcagcc tctagccaag tagctgcgat 120

tacaggcatg cgccaccacg cccggctaat ttttgtattt ttagtagaga cggggtttcg 180

ccatgttggt caggctggtc tcgaactcct gatctcaggt gatccaacca ccctggcctc 240

ccaaagtgct gggattatag gcgtgagcca cagcgcctgg cctgaagcag ccactcactt 300

ttacagaccc taagacaatg attgcaagct ggtaggattg ctgtttggcc cacccagctg 360

cggtgttgag tttgggtgcg gtctcctgtg ctttgcacct ggcccgctta aggcatttgt 420

tacccgtaat gctcctgtaa ggcatctgcg tttgtgacat cgttttggtc gccaggaagg 480

gattggggct ctaagcttga gcggttcatc cttttcattt atacagggga tgaccagagt 540

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tctgtggatc tcccctacag ccgtccctga acactggtcc cgggcgtccc accgccgcc 660

caccgtccca ccccctcacc ttttctaccc gggttcccta agttcctgac ctaggcgtca 720

gacttcctca ctatactctc ccaccccagg cgacccgccc tggccccggg tgtcccagc 780

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ggccctgcgc ccctggaac tcctgggctt ccagctcccg ccgctcccag aactgcgcct 900

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ttgacgaggc cttggggcgc ccgggaggcc tggccgtgtt ggccgccttt ctggaggtac 1380

cagatcctgg acacccccta c   1401

<210> 50
<211> 59
<212> PRT
<213> HUMAN

<400> 50
Ser Ser Gly Glu Asp Asp Pro Leu Gly Glu Glu Asp Leu Pro Ser Glu
  1   5   10   15

Glu Asp Ser Pro Arg Glu Glu Asp Pro Pro Gly Glu Glu Asp Leu Pro
              20   25   30

Gly Glu Glu Asp Leu Pro Gly Glu Glu Asp Leu Pro Glu Val Lys Pro
          35   40   45

Lys Ser Glu Glu Glu Gly Ser Leu Lys Leu Glu
      50   55

<210> 51
<211> 257
<212> PRT
<213> HUMAN

<400> 51
Gly Asp Asp Gln Ser His Trp Arg Tyr Gly Gly Asp Pro Pro Trp Pro
  1   5   10   15

Arg Val Ser Pro Ala Cys Ala Gly Arg Phe Gln Ser Pro Val Asp Ile
              20   25   30

Arg Pro Gln Leu Ala Ala Phe Cys Pro Ala Leu Arg Pro Leu Glu Leu
          35   40   45

Leu Gly Phe Gln Leu Pro Pro Leu Pro Glu Leu Arg Leu Arg Asn Asn
      50   55   60

Gly His Ser Val Gln Leu Thr Leu Pro Pro Gly Leu Glu Met Ala Leu
  65   70   75   80

Gly Pro Gly Arg Glu Tyr Arg Ala Leu Gln Leu His Leu His Trp Gly
                  85   90   95

32

```
Ala Ala Gly Arg Pro Gly Ser Glu His Thr Val Glu Gly His Arg Phe
            100             105             110

Pro Ala Glu Ile His Val Val His Leu Ser Thr Ala Phe Ala Arg Val
        115             120             125

Asp Glu Ala Leu Gly Arg Pro Gly Gly Leu Ala Val Leu Ala Ala Phe
    130             135             140

Leu Glu Glu Gly Pro Glu Glu Asn Ser Ala Tyr Glu Gln Leu Leu Ser
145             150             155             160

Arg Leu Glu Glu Ile Ala Glu Glu Gly Ser Glu Thr Gln Val Pro Gly
            165             170             175

Leu Asp Ile Ser Ala Leu Leu Pro Ser Asp Phe Ser Arg Tyr Phe Gln
            180             185             190

Tyr Glu Gly Ser Leu Thr Thr Pro Pro Cys Ala Gln Gly Val Ile Trp
        195             200             205

Thr Val Phe Asn Gln Thr Val Met Leu Ser Ala Lys Gln Leu His Thr
    210             215             220

Leu Ser Asp Thr Leu Trp Gly Pro Gly Asp Ser Arg Leu Gln Leu Asn
225             230             235             240

Phe Arg Ala Thr Gln Pro Leu Asn Gly Arg Val Ile Glu Ala Ser Phe
            245             250             255

Pro



<210> 52
<211> 20
<212> PRT
<213> HUMAN

<400> 52
Ile Leu Ala Leu Val Phe Gly Leu Leu Phe Ala Val Thr Ser Val Ala
  1           5               10              15

Phe Leu Val Gln
            20
```

```
<210> 53
<211> 25
<212> PRT
<213> HUMAN

<400> 53
Met Arg Arg Gln His Arg Arg Gly Thr Lys Gly Gly Val Ser Tyr Arg
 1               5                  10                  15

Pro Ala Glu Val Ala Glu Thr Gly Ala
             20                  25


<210> 54
<211> 59
<212> PRT
<213> HUMAN

<400> 54
Ser Ala Ser Glu Glu Pro Ser Pro Ser Glu Val Pro Phe Pro Ser Glu
 1               5                  10                  15

Glu Pro Ser Pro Ser Glu Glu Pro Phe Pro Ser Val Arg Pro Phe Pro
             20                  25                  30

Ser Val Val Leu Phe Pro Ser Glu Glu Pro Phe Pro Ser Lys Glu Pro
         35                  40                  45

Ser Pro Ser Glu Glu Pro Ser Ala Ser Glu Glu
     50                  55


<210> 55
<211> 470
<212> RNA
<213> HUMAN

<400> 55
cauggccccg auaaccuucu gccugugcac acaccugccc cucacuccac ccccauccua 60

gcuuugguau gggggagagg gcacagggcc agacaaaccu gugagacuuu ggcuccaucu 120

cugcaaaagg gcgcucugug agucagccug cuccccucca ggcuugcucc uccccacccc 180

agcucucguu uccaaugcac guacagcccg uacacaccgu gugcugggac accccacagu 240

cagccgcaug gcuccccugu gccccagccc cuggcucccu cguugaucc cggccccugc 300
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uccaggccuc acugugcaac ugcugcuguc acugcugcuu cuggugccug uccauccccca 360

gagguugccc cggaugcagg aggauuccccc cuugggagga ggcucuucug gggaagauga 420

cccacugggc gaggaggauc ugcccaguga agaggauuca cccagagagg   470

<210> 56
<211> 292
<212> DNA
<213> HUMAN

<400> 56
gttttttga dacggagtct tgcatctgtc atgcccaggc tggagtagca gtggtgccat 60

ctcggctcac tgcaagctcc acctcccgag ttcacgccat tttcctgcct cagcctcccg 120

agtagctggg actacaggcg cccgccacca tgcccggcta attttttgta tttttggtag 180

agacggggtt tcaccgtgtt agccagaatg gtctcgatct cctgacttcg tgatccaccc 240

gcctcggcct cccaaagttc tgggattaca ggtgtgagcc accgcacctg gc   292

<210> 57
<211> 262
<212> DNA
<213> HUMAN

<400> 57
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cagctcactg cagcctcaac cgcctcggct caaaccatca tcccatttca gcctcctgag 120

tagctgggac tacaggcaca tgccattaca cctggctaat ttttttgtat ttctagtaga 180

gacagggttt ggccatgttg cccgggctgg tctcgaactc ctggactcaa gcaatccacc 240

cacctcagcc tcccaaaatg ag   262

<210> 58
<211> 2501
<212> DNA
<213> HUMAN

<220>
<221> misc_feature

<222> (1)..(2501)

<400> 58

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agggttaaat ggattaaggg cggtgcaaga tgtgctttgt taaacagatg cttgaaggca   120

gcatgctcgt taagagtcat caccaatccc taatctcaag taatcaggga cacaaacact   180

gcggaaggcc gcagggtcct ctgcctagga aaaccagaga cctttgttca cttgtttatc   240

tgaccttccc tccactattg tccatgaccc tgccaaatcc ccctctgtga gaaacaccca   300

agaattatca ataaaaaat aaatttaaaa aaaaaataca aaaaaaaaa aaaaaaaaa   360

aaaagactta cgaatagtta ttgataaatg aatagctatt ggtaaagcca agtaaatgat   420

catattcaaa accagacggc catcatcaca gctcaagtct acctgatttg atctctttat   480

cattgtcatt ctttggattc actagattag tcatcatcct caaaattctc ccccaagttc   540

taattacgtt ccaaacattt aggggttaca tgaagcttga acctactacc ttctttgctt   600

ttgagccatg agttgtagga atgatgagtt tacaccttac atgctgggga ttaatttaaa   660

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tggcctttat ctgtaatatg ggcatattta atacaatata attttttggag ttttttttgtt   900

tgtttgtttg tttgtttttt tgagacggag tcttgcatct gtcatgccca ggctggagta   960

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ctggccaatt ttttgagtct tttaaagtaa aaatatgtct tgtaagctgg taactatggt   1260

acatttcctt ttattaatgt ggtgctgacg gtcatatagg ttcttttgag tttggcatgc   1320

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tttcttaagc aagatatgct aaagttttgt gagccttttt ccagagagag gtctcatatc 1680

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ttggtaggaa ataagaatgt gaaactcttc agttggtgtg tgtccctngt tttttgcaa 1980

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atcatgatct ttaaagatca ataatataat cctttcaagg attatgtctt tattataata 2100

aagataattt gtctttaaca gaatcaataa tataatccct taaaggatta tatctttgct 2160

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atttgcctac ttctatatta tcttctaaag cagaattcat ctctcttccc tcaatatgat 2280

gatattgaca gggtttgccc tcactcacta gattgtgagc tcctgctcag ggcaggtagc 2340

gttttttgtt tttgtttttg tttttctttt ttgagacagg gtcttgctct gtcacccagg 2400

ccagagtgca atggtacagt ctcagctcac tgcagcctca accgcctcgg ctcaaaccat 2460

catcccattt cagcctcctg agtagctggg actacaggca c                       2501
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<210>  59
<211>  292
<212>  DNA
<213>  HUMAN

<220>
<221>  misc_feature
<222>  (1)
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<400> 59

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tcggctcact gcaagctcca cctcccgagt tcacgccatt ttcctgcctc agcctcccga 120

gtagctggga ctacaggcgc ccgccaccat gcccggctaa ttttttgtat ttttggtaga 180

gacggggttt caccgtgtta gccagaatgg tctcgatctc ctgacttcgt gatccacccg 240

cctcggcctc ccaaagttct gggattacag gtgtgagcca ccgcacctgg cc   292

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agctgggact acaggcacat gccattacac ctggctactt ttttgtatt tctagtagag 180

acagggtttg gccatgttgc ccgggctggt ctcgaactcc tggactcaag caatccaccc 240

acctcagcct cccaaaatga gg   262

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<212> DNA
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<400> 61

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aagtagctgc gattacaggc atgcgccacc acgcccggct aattttttgta tttttagtag 180

agacggggtt tcgccatgtt ggtcaggctg gtctcgaact cctgatctca ggtgatccaa 240

ccaccctggc ctcccaaagt gctgggatta taggcgtgag ccacagcgcc tggc   294

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gtgtgtgcca ccatgcccag ctaattttt tttgtatttt tagtagacag ggtttcacca 180

tgttggtcag gctggtctca aactcctggc ctcaagtgat ccgcctgact cagcctacca 240

aagtgctgat tacaagtgtg agccaccgtg cccagc   276

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aaatacgaaa aaatagccag gcgtggtggc gggtgcctgt aatcccagct actcgggagg 180

ctgaggcagg agaatggcat gaacccggga ggcagaagtt gcagtgagcc gagatcgtgc 240

cactgcactc cagcctgggc aacagagcga gactcttgtc tcaaaaaaa   289

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caaaaaaacc ccaacaaaac caaaaatagc cgggcatggt ggtatgcggc ctagtcccag 180

ctactcaagg aggctgaggt gggaagatcg cttgattcca ggagtttgag actgcagtga 240

gctatgatcc caccactgcc taccatcttt aggatacatt tatttattta taaaagaa   298

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ctgaccttgt gatccaccag cctcggcctc ccaaagtgct gggat   105

<210> 66
<211> 83
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<400> 66
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aggcatgagc cactgtgcct ggc   83

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<400> 67
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<400> 69

cagtcgtgag g   11

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<400> 72
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<400> 73
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atacagggga t   11

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ccccaggagg g   11

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<400> 84
ctccagtcca g   11

<210> 85
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<400> 85

tcgcaggtga ca   12

<210> 86
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<400> 86
acacagaagg g   11

<210> 87
<211> 377
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            20   25   30

Asp Ser Pro Arg Glu Glu Asp Pro Pro Gly Glu Glu Asp Leu Pro Gly
         35   40   45

Glu Glu Asp Leu Pro Gly Glu Glu Asp Leu Pro Glu Val Lys Pro Lys
     50   55   60

Ser Glu Glu Glu Gly Ser Leu Lys Leu Glu Asp Leu Pro Thr Val Glu
 65   70   75   80

Ala Pro Gly Asp Pro Gln Glu Pro Gln Asn Asn Ala His Arg Asp Lys
                 85   90   95

Glu Gly Asp Asp Gln Ser His Trp Arg Tyr Gly Gly Asp Pro Pro Trp
             100   105   110

Pro Arg Val Ser Pro Ala Cys Ala Gly Arg Phe Gln Ser Pro Val Asp
         115   120   125

Ile Arg Pro Gln Leu Ala Ala Phe Cys Pro Ala Leu Arg Pro Leu Glu
     130   135   140

Leu Leu Gly Phe Gln Leu Pro Pro Leu Pro Glu Leu Arg Leu Arg Asn
145   150   155   160

44

Asn Gly His Ser Val Gln Leu Thr Leu Pro Pro Gly Leu Glu Met Ala
                165   170   175

Leu Gly Pro Gly Arg Glu Tyr Arg Ala Leu Gln Leu His Leu His Trp
            180   185   190

Gly Ala Ala Gly Arg Pro Gly Ser Glu His Thr Val Glu Gly His Arg
        195   200   205

Phe Pro Ala Glu Ile His Val Val His Leu Ser Thr Ala Phe Ala Arg
    210   215   220

Val Asp Glu Ala Leu Gly Arg Pro Gly Gly Leu Ala Val Leu Ala Ala
225   230   235   240

Phe Leu Glu Glu Gly Pro Glu Glu Asn Ser Ala Tyr Glu Gln Leu Leu
            245   250   255

Ser Arg Leu Glu Glu Ile Ala Glu Glu Gly Ser Glu Thr Gln Val Pro
            260   265   270

Gly Leu Asp Ile Ser Ala Leu Leu Pro Ser Asp Phe Ser Arg Tyr Phe
        275   280   285

Gln Tyr Glu Gly Ser Leu Thr Thr Pro Pro Cys Ala Gln Gly Val Ile
    290   295   300

Trp Thr Val Phe Asn Gln Thr Val Met Leu Ser Ala Lys Gln Leu His
305   310   315   320

Thr Leu Ser Asp Thr Leu Trp Gly Pro Gly Asp Ser Arg Leu Gln Leu
            325   330   335

Asn Phe Arg Ala Thr Gln Pro Leu Asn Gly Arg Val Ile Glu Ala Ser
            340   345   350

Phe Pro Ala Gly Val Asp Ser Ser Pro Arg Ala Ala Glu Pro Val Gln
        355   360   365

Leu Asn Ser Cys Leu Ala Ala Gly Asp
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<210> 88
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gcatgctcgt taagagtcat caccaatccc taatctcaag taatcaggga cacaaacact   180

gcggaaggcc gcagggtcct ctgcctagga aaaccagaga cctttgttca cttgtttatc   240

tgaccttccc tccactattg tccatgaccc tgccaaatcc ccctctgtga gaaacaccca   300

agaattatca ataaaaaat aaatttaaaa aaaaataaca aaaaaaaaa aaaaaaaaa   360

aaaagactta cgaatagtta ttgataaatg aatagctatt ggtaaagcca agtaaatgat   420

catattcaaa accagacggc catcatcaca gctcaagtct acctgatttg atctctttat   480

cattgtcatt ctttggattc actagattag tcatcatcct caaaattctc ccccaagttc   540

taattacgtt ccaaacattt aggggttaca tgaagcttga acctactacc ttctttgctt   600

ttgagccatg agttgtagga atgatgagtt tacaccttac atgctgggga ttaatttaaa   660

ctttacctct aagtcagttg ggtagccttt ggcttatttt tgtagctaat tttgtagtta   720

atggatgcac tgtgaatctt gctatgatag ttttcctcca cactttgcca ctaggggtag   780

46

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gtaggtactc agttttcagt aattgcttac ctaagaccct aagccctatt tctcttgtac 840

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tgtttgtttg tttgttttttt tgagacggag tcttgcatct gtcatgccca ggctggagta 960

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cctcagcctc ccgagtagct gggactacag gcgcccgcca ccatgcccgg ctaatttttt 1080

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atcatgatct ttaaagatca ataatataat cctttcaagg attatgtctt tattataata 2100

aagataattt gtctttaaca gaatcaataa tataatccct taaaggatta tatctttgct 2160

gggcgcagtg gctcacacct gtaatcccag cactttgggt ggccaaggtg gaaggatcaa 2220
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47

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ccagagtgca atggtacagt ctcagctcac tgcagcctca accgcctcgg ctcaaaccat 2460

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atttccatgt ccctagtcca tagcccagtg ctggacctat ggtagtacta aataaatatt 2700

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ccccgataac cttctgcctg tgcacacacc tgcccctcac tccacccccа tcctagcttt 3360

ggtatggggg agagggcaca gggccagaca aacctgtgag actttggctc catctctgca 3420

aaagggcgct ctgtgagtca gcctgctccc ctccaggctt gctcctcccc cacccagctc 3480

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48

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ccctccaggc ttgctcctcc cccacccagc tctcgtttcc aatgcacgta cagcccgtac 180

acaccgtgtg ctgggacacc ccac   204

<210> 92
<211> 132
<212> DNA
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<400> 92
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ccactcaggg ttaaatggat taagggcggt gcaagatgtg ctttgttaaa cagatgcttg 120

aaggcagcat gc   132

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acagggccag acaaacctgt gagactttgg ctccatctct gcaaaagggc gctctgtgag 180

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acagcccgta cacaccgtgt gctgggacac cccac   275

<210> 94
<211> 89
<212> DNA
<213> HUMAN

<400> 94
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ccgtacacac cgtgtgctgg gacacccca   89

<210> 95
<211> 61
<212> DNA
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<210> 96
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<212> DNA
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acaaacctgt gagactttgg ctccatctct gcaaaagggc gctctgtgag tcagcc   116

<210> 97
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<400> 97
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            20   25   30

Glu Asp Leu Pro
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<210> 98
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<210> 99
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<213> HUMAN

<400> 99
Glu Glu Asp Leu
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<210> 100
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<213> HUMAN

<400> 100
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<210> 101
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<400> 101
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<210> 102
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<400> 102
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<210> 103
<211> 6
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<212> PRT
<213> HUMAN


<400> 103
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<210> 104
<211> 22
<212> PRT
<213> HUMAN


<400> 104
Gly Gly Ser Ser Gly Glu Asp Asp Pro Leu Gly Glu Glu Asp Leu Pro
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Ser Glu Glu Asp Ser Pro
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<210> 105
<211> 25
<212> PRT
<213> HUMAN


<400> 105
Gly Glu Glu Asp Leu Pro Ser Glu Glu Asp Ser Pro Arg Glu Glu Asp
  1               5                   10                  15

Pro Pro Gly Glu Glu Asp Leu Pro Gly
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<210> 106
<211> 24
<212> PRT
<213> HUMAN


<400> 106
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  1               5                   10                  15

Gly Glu Glu Asp Leu Pro Glu Val
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<210> 107
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<211> 7
<212> PRT
<213> HUMAN

<400> 107
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<210> 108
<211> 7
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<400> 108
Gly Glu Thr Arg Glu Pro Leu
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<210> 109
<211> 7
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<400> 109
Gly Gln Thr Arg Ser Pro Leu
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<210> 110
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<212> DNA
<213> HUMAN

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<222> (1)..(1247)

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gtaagaggga tgattcaggt gaatctgaca ctaagaaact cccctacctg aggtctgaga 240

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53

ttcttaagca agatatgcta aagttttgtg agcctttttc cagagagagg tctcatatct 360

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tgttattgga tatcatcatt ggcccacgct ttctgacctt ggaaacaatt aagggttcat 540

aatctcaatt ctgtcagaat tggtacaaga aatagctgct atgtttcttg acattccact 600

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ttccttctta ctgtgttaaa aaaaagtatg atcttgctct gagaggtgag gcattcttaa 720

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agataatttg tctttaacag aatcaataat ataatccctt aaaggattat atctttgctg 840

ggcgcagtgg ctcacacctg taatcccagc actttgggtg gccaaggtgg aaggatcaaa 900

tttgcctact tctatattat cttctaaagc agaattcatc tctcttccct caatatgatg 960

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<213> HUMAN

<400> 111
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<210> 112
<211> 23
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<400> 112
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<210> 113
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<400> 113
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<210> 114
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<400> 114
cactccaccc ccatcctagc   20

<210> 115
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<400> 115
gggagagggc acaggccag acaaac   26

<210> 116
<211> 20
<212> PRT
<213> HUMAN

<400> 116
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 1   5   10   15

Gly Gly Gly Ser
          20

<210> 117
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<212> DNA
<213> HUMAN

<400> 117
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<210> 118
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<400> 118
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  1   5   10   15

<210> 119
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<212> PRT
<213> HUMAN

<400> 119
Gly Glu Glu Asp Leu Pro Gly Glu Glu Asp Leu Pro
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<210> 120
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  1   5   10

<210> 121
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<212> PRT
<213> HUMAN

<400> 121
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  1   5   10

<210> 122
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56

<213> HUMAN

<400> 122
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