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(54) Title: *Nr–CAM* GENE, NUCLEIC ACIDS AND NUCLEIC ACID PRODUCTS FOR THERAPEUTIC AND DIAGNOSTIC USES
FOR TUMORS

(57) Abstract

The present invention relates to the identification of a novel role of *Nr–CAM* in cell transformation and aberrant cellular proliferation.
In particular, the present invention relates to the altered gene expression of *Nr–CAM* in a number of primary tumors and cell lines derived
from tumors, in addition to, the altered gene expression of ligands for *Nr–CAM*. Further, the present invention relates, in part, to the
Applicants' surprising discovery that the inhibition of *Nr–CAM* gene expression or the inhibition of *–CAM* activity in transformed cells
reverses the transformed phenotype.

Nr-CAM GENE, NUCLEIC ACIDS AND NUCLEIC ACID PRODUCTS FOR THERAPEUTIC AND DIAGNOSTIC USES FOR TUMORS

5   The present application is entitled to and claims priority benefits of U.S. Application No. 60/083,152, filed April 27, 1998; and 60/112,098, filed December 14, 1998, the entire disclosures of which are incorporated herein by reference.

1. FIELD OF THE INVENTION

10   The present invention relates to the identification of a novel role for the neuron-glia-related cell adhesion molecule (*Nr-CAM*) gene in tumorigenesis, in particular primary brain tumorigenesis. The present invention is related to the role of *Nr-CAM* nucleic acids and polypeptides as diagnostic tools to indicate a pre-cancerous condition or cancer, and therapeutic agents based thereon to inhibit *Nr-CAM* gene expression and/or activity as a method of treating, inhibiting and/or preventing tumorigenesis.

2. BACKGROUND OF THE INVENTION

2.1. BRAIN TUMORS

20   Brain tumors are among the leading cause of death among young children and adults. A survey by the American Cancer Society has documented that 13,300 people died of brain tumors in 1995 and predicated that over 17,900 would die in 1996 (Parker *et al.*, 1996, CA Cancer J. Clin., 46:5-28). The number of deaths due to brain tumors has been increasing at a significant rate each year. On average, 25,000 Americans are diagnosed with brain cancer yearly. Brain tumors claim the lives of more children than any other form of cancer except leukemia.

30   The increased incidence of brain tumors is not only evident in children but also in adults. It has been documented that a significant increase in mortality has

occurred in adult primary malignant tumors between 1982 and 1996 (Parker et al., 1996). Glioblastomas, astrocytomas and meningiomas are the most common brain tumors that affect adults (Thapar and Laws, 1993, CA Cancer J. Clin., 43:263-271).

The transformation of normal human brain cells into gliomas occurs as a result of the accumulation of a series of cellular and genetic changes (Sehgal, 1998, Cancer Surv., 25:233-275; vonDiemiling et al., 1995, Glia 15:328-338; Furnari et al., 1995, J. Surg. Oncol. 67:234). These genetic alterations include the loss, gain or amplification of different chromosomes. These genetic changes lead to altered expression of proteins that play important roles in the regulation of normal cell proliferation. Several common genetic alternations at the chromosomal level (loss of 17p, 13q, 9p, 19, 10, 22q, 18q and amplification of 7 and 12q) have been observed (Sehgal et al., 1998 J. Surg. Oncol. 67:23; vonDiemiling et al., 1995, Glia 15:328-338; Furnari et al., 1995, Cancer Surv. 25:233-275). These alterations lead to changes in the expression of several genes (p53, RB, INFα/β, CDKN2, MMAC1, DCC, EGFR, PDGF, PDGFr, MDM2, GLI, CDK4 and SAS) during the genesis and progression of human gliomas (Sehgal, 1998, J. Surg. Oncol. 67:234; vonDiemiling et al., 1995, Glia 15:328-338). Recent studies have suggested that altered expression of several other genes (MET, MYC, TGFβ, CD44, VEGF, N-CAML1, p21$^{wafl/Cip1}$, trkA, MMRs, C4-2, D2-2) and proteins (cathepsins, tenascin, matrix metalloproteases, tissue inhibitors of metalloproteases, nitric oxide synthetase, integrins, IL 13 receptor, Connexin 43, uPAR's extracellular matrix proteins and heat shock proteins) are associated with the genesis of human gliomas (Sehgal, 1998, J. Surg. Oncol. 67:234). Taken together these findings point to the fact that accumulation of multiple genetic mutations coupled with extensive changes in gene expression may be a

prerequisite in the etiology of human gliomas. Despite
identification of these genetic alterations, the exact series
of events that leads to the genesis of human gliomas is not
clear.

5   Glioblastoma multiforme are high grade astrocytomas
that grow very rapidly and contain cells that are very
malignant (Thapar and Laws, 1993, CA Cancer J. Clin., 43:263-
271). The molecular basis of glioblastoma multiforme
occurrence may involve systematic events at the chromosomal
level or at a gene expression level. These may include
10 inactivation of tumor suppressor genes, activation of
oncogenes or specific translocations at the chromosomal
level. Some genetic changes at the chromosomal level and
gene expression level have been well documented for other
brain tumors (Furnari et al., 1995, Cancer Surv., 25:233-
15 275). For example, it has been documented that loss of tumor
suppressor(s) genes at chromosome 10, mutations in p53, or
overexpression of epidermal growth factor receptor, may be
major events leading to glioblastoma multiforme. A number of
other genes such as EGFR, CD44, β4 integrins, membrane-type
20 metalloproteinase (MT-MMP), p21, p16, p15, myc, and VEGF have
been shown to be overexpressed in different types of brain
tumors (Faillot et al., 1996, Neurosurgery, 39:478-483; Eibl
et al., 1995, J. Neurooncol., 26:165-170; Previtali et al.,
1996, Neuropathol. Exp. Neurol. 55:456-465; Yamamoto et al.,
25 1996, Cancer Res., 56:384-392; Jung et al., 1995, Oncogene,
11:2021-2028; Tsuzuki et al., 1996, Cancer, 78:287-293; Chen
et al., 1995, Nature Med., 1:638-643; Takano, et al., 1996,
Cancer Res., 56:2185-2190; Bogler et al., 1995, Glia, 15:308-
327). Several cell adhesion molecules (CAMs), such as
integrins, cadherins, IgSF proteins (carcinoembryonic
30 antigen, N-CAM and VCAM-1) or lectins, are thought to be
involved in tumorigenesis (Johnson, 1991, Cancer Metastat.
Rev. 10:11-22). Over-expression of anti-sense to the

secreted glycoprotein SPRAC (secreted protein, acidic and
rich in cysteine), results in suppression of the adhesive and
invasive capacities of melanomas (Ledda et al., 1997, Nature
(Med). 3:171-176). The cell-surface adhesion molecule

5 MCAM(MUC18) when over-expressed may lead to increased
adhesion and metastatic potential of human melanoma cells in
nude mice (Xie et al., 1997, Proc. Nat'l Cancer Conf.
38:522). Expression of N-CAM or ICAM (Intracellular adhesion
molecule) is related inversely to increased metastasis
(Hortsch, 1996, Neuron 17:587-593). Other genes such as p53

10 show mutations in the majority of brain tumors (Bogler et
al., supra). How the interplay of one or more of these genes
leads to tumorigenesis is not known but most likely multiple
steps are required for neoplastic transformation. The exact
series of events that lead to initiation or progression of

15 glioblastoma are not known at present and useful markers for
early detection of brain tumors are lacking.

2.2. CAMs

A subfamily of the immunoglobulin superfamily

20 (IgSF) proteins are termed "cell adhesion molecules" (CAMs)
(Hortsch, 1996, Neuron 17:587-593).

Several CAM family members are implicated in the
process of tumorigenesis including, N-CAM, CEA,
(Cacinoembryonic Antigen), DCC (Deleted in Colon Carcinoma)

25 and L1.

CEA is a cell surface glycoprotein of colon mucosal
cells. High levels of CEA are observed in the serum of tumor
patients (Benchimol, et al., 1989, Cell, 57:327-334). It was
demonstrated that over-expression of CEA in malignant cells
may disturb intercellular adhesion that may in turn cause

30 tissue disruption leading to the metastasis of primary tumor
cells (Albelda, 1993, Lab Invest, 68:4-14; Benchimol et al.,
1989).

L1 is another cell adhesion molecule that belongs
to the Ig superfamily and is expressed in neuroblastomas,
melanomas, lymphocytes and Schwann cells (Izumoto, et al.,
1996, Cancer Res, 56:1440-1444). Antibody neutralization
5 experiments demonstrate that L1 is responsible for the highly
invasive nature of the C6 glioma cells. Mutations in the L1
are known to be associated with a spectrum of neurological
deficiencies including mental retardation (Izumoto et al.,
1996).

10   The neural cell adhesion molecules (N-CAMs) are
predominantly though not exclusively, expressed in developing
peripheral and central nervous systems of a number of
invertebrates and vertebrates. These proteins are generally
present on the cell surface and consist of multiple Ig
domains, multiple fibronectin type III repeats near the cell
15 membrane and either a transmembrane domain or a
glycophosphatidylinositol-linked membrane anchor at the C-
terminus (Hortsch, 1996). The N-CAMs can be grouped into 2
major structural families, one resembling N-CAM and the other
resembling the liver CAM (L-CAM) and its mammalian homologue
20 uvomorulin or E-cadherins. Within N-CAM, 2 major types are
observed, the N-CAM (neuronal CAM) and the neuron-glial CAM
(Ng-CAM) (Grumet et al., 1991, J. Cell Biol. 113:1399-1412).

        N-CAM is expressed in a wide variety of tissues and
is implicated in embryonic development. Over-expression of
25 N-CAM is observed in a variety of tumors including multiple
myelomas, small cell carcinomas and adenoid cystic
carcinomas. Down regulation of N-CAM is observed in
malignant glioma cells (Albelda, 1993; Poley, et al., 1997,
Anticancer Research, 17:3021-3024). In Wilm's tumor of
kidney, the N-CAM exists in h-PSA form that is less adhesive
30 to surrounding cells and fibers. In a recent study it was
demonstrated that high levels of N-CAM were detected in
patients with prostate carcinomas (Lynch, et al., 1997,

Prostate, 32:214-220). *In vivo* studies in nude mice demonstrated that N-CAM may be involved not only in adhesive and motile behavior of cells but also in their growth regulation (Poley *et al.*, 1997; Lynch *et al.*, 1997).

5   DCC is a cell adhesion molecule that belongs to the N-CAM family. DCC was first shown to be expressed in a variety of tumors including the brain and lung but its expression was reduced and mutated in a number of colorectal carcinomas (Fearon, *et al.*, 1990, Cell, 61:759-767). The down regulation or mutation of the DCC molecule lead to the
10 disruption of normal cell-cell adhesion in the intestinal epithelium. This process is known to play an important role in the metastasis of colorectal carcinomas (Albelda, 1993; Fearon *et al.*, 1990).

15   2.2.1.   **Nr-CAM**

          Nr-CAM (neuron-glia related CAM) was cloned from a chicken brain library when Ng-CAM cDNA Clones were being isolated (Grumet et al., 1991, J. Cell Biol. 113:1399-1412). Monoclonal antibodies against E8 tectal surface protein
20 identified a similar molecule that was cloned from a chicken brain library. This protein was designated Bravo/Nr-CAM (Grumet et al., 1991; Lane et al., 1986, Genomics 35:456-465). The Nr-CAM protein contains 6 Ig domains and 5 fibronectin repeats. Numerous studies on chicken Nr-CAM
25 suggested that it may play an important role in cell-cell adhesion during the development of the vertebrate nervous system. The human homologue of the chicken Nr-CAM has been cloned (Lane *et al.*, 1996, Genomics 35:456-465; see Lane et al., Figure 1 at pages 458-9 for nucleotide and deduced amino acid sequences of hNr-CAM, incorporated herein by reference).
30   Sequence analysis of Nr-CAM proteins isolated from human rat, chicken, and mouse showed more than 80% identity. One unique characteristic of hNr-CAM is that the third

fibronectin repeat contains a furin-like cleavage site
(Grumet, 1991, Current Opinion in Neurobiology, 1:370-376;
Suter, et al., 1995, J. Cell Biol., 131:1067-1081). It has
also been reported that the 140KDa protein may exist as a
5 doublet (Grumet, et al., 1991, J. Cell Biol., 113:1399-1412;
Lane, et al., 1996, Genomics, 35:456-465).

The cytoplasmic tail of the hNr-CAM protein is
known to interact with a cytoplasmic protein named ankyrin
(Davis, et al., 1993, J. Cell Biol., 121:121-133; Davis, et
10 al., 1996, J. Cell Biol., 135:1355-1367). This region of the
hNr-CAM protein is highly conserved among other family
members (Ng-CAM, L1, neurofascin and neuroglian protein).
Neurofascin and L1 proteins contain a phosphorylation site
that may modulate its interaction with ankyrin (Davis, 1993;
Davis 1996). Phosphorylation status of hNr-CAM in this
15 region of the protein has not been reported yet. The
cytoplasmic tail of the hNr-CAM contains sequences that have
potential to interact with PSD-95/discs- large/ZO-1 family of
membrane associated proteins (Grumet, et al., 1991, J Cell
Biol, 113:1399-1412).

20   cDNA analysis of rat Nr-CAM revealed two different
forms of Nr-CAM as a result of alternative mRNA splicing.
The alternatively spliced form of Nr-CAM contains 10-amino
acids inserted between the Ig domain and fibronectin domain,
and 15-amino-acids inserted after the fourth fibronectin
25 repeat, and complete deletion of the fifth fibronectin repeat
(Suter et al., 1995). The extracellular portion of the Nr-
CAM protein contains twenty potential sites for N-linked
glycosylation (Kayyem, et al., 1992, J Cell Biol, 118:1259-
1270; Suter et al., 1995; Grumet, 1991; Cell Tissue Res.
290:423-428).
30
          Nr-CAM is expressed in growing neurites and radial
cells of the optic chiasm. Nr-CAM protein family members not
only play an important role in cell adhesion but they can

interact with other proteins, such as FGF-R, by
phosphorylation events to bring about neurite extension. *L1-*
CAM gene mutation may be involved in several neurological
disorders (Hortsch, 1996). A number of transformed cells
from a variety of tissues also express L1-CAM, and the
expression is correlated inversely with the metastatic
capacity of a lymphoma cell line in mice, leading to
speculation that these CAMs may play a role in metastatic
events (Hortsch, 1996). See generally, Albelda, 1993, Lab.
Invest. 68:4-14.

     It has been demonstrated that hNr-CAM is a brain
specific protein and is expressed on neurons, Schwann cells,
Muller cells and transiently in the cells of floor plate
(Davis, *et al.*, 1996, J Cell Biol, 135:1355-1367; Grumet,
Cell Tissue Res, [1991] 290:423-428). The hNr-CAM protein is
expressed preferentially on fiber tracts, in spinal cord,
cerebellum, tectum, and telencephalon. It is also known to
be concentrated in the node of Ranvier, thus demonstrating
its potential role in the formation and maintenance of the
nodes (Suter, *et al.*, 1995, J Cell Biol, 131:1067-1081). The
hNr-CAM is also expressed widely in the retina on cell bodies
and fiber layers and on the ganglion cells (Suter *et al.*,
1995; Davis, *et al.*, 1994, J Biol Chem, 269:27163-27166).

     The hNr-CAM protein is a cell adhesion molecule
that can function as a receptor and as a ligand. It not only
interacts with other hNr-CAM molecules on the cell surface
but also with other CAMs and extracellular matrix proteins.
Some of the proteins that interact with the hNr-CAM include:
contactin/F11, axonin-1 ax-1), neurofascin (Nf), Receptor
protein tyrosine phosphataseβ (RPTPβ), Ng-CAM, chondroitin
sulfate proteoglycans neurocan, and phosphacan (Davis *et al.*,
1994; Grumet *et al.*, 1991; Cell Tissue Res. 290:423-428).
Recent analysis has indicated that the cytoplasmic domain of
hNr-CAM can directly interact with ankyrins, a family of

spectrin-binding proteins (Davis, 1993; Davis, 1996). This interaction may be involved in sending signals from the extracellular domain to the cytoplasm of cells. Antibody binding analysis during the tectal neurite growth has

5 demonstrated that the hNr-CAM can act as a receptor and as a ligand. Some of the major functions of the hNr-CAM are: a) modulation of axonal growth and guidance; b) modulation of the function of non-neuronal glial cells; c) synapse formation and maturation of the central nervous system; and d) as a heterophilic neuronal receptor (Grumet, 1991 Cell

10 Tissue Res. 290:423-428).

Citation of references herein shall not be construed as an admission that such references are prior art to the present invention.

15   3. **SUMMARY OF THE INVENTION**

The present invention relates to the discovery of a novel role for Nr-CAM in the aberrant proliferative behavior of a number of cell types, including numerous primary tumors and derived cell lines. In particular, the present invention

20 relates to the identification of the role of Nr-CAM in cell transformation and tumorigenesis, in particular, brain. The present invention encompasses therapeutic and diagnostic applications based on Nr-CAM proteins, nucleic acids, and agonists and antagonists, for the treatment, inhibition or prevention of tumorigenesis. The present invention further

25 encompasses therapeutic and diagnostic applications based on a ligand of Nr-CAM proteins, nucleic acids, and agonists and antagonists, for the treatment, inhibition or prevention of tumorigenesis. The present invention further encompasses screening assays to identify modulators of Nr-CAM activity

30 and/or expression as potential therapeutic agents for the treatment, inhibition and/or prevention of a transformed phenotype or tumorigenesis.

The present invention is based, in part, on the
Applicants' surprising discovery that the *Nr-CAM* nucleotide
sequence and encoded protein product is expressed at high
levels in glioblastoma multiforme tissue, astrocytomas,
5 gliomas, glioblastoma tumor tissues, as well as, certain
other forms of tumors and cancers.

In one embodiment, the present invention
encompasses nucleotide sequences complementary to the
nucleotide sequence of *Nr-CAM*, such as primers, fragments or
antisense nucleotides which may be used to determine the
10 level of *Nr-CAM* expression in a tissue or cell culture sample
as prognostic of a pre-cancerous or transformed cell
phenotype; or to inhibit *Nr-CAM* expression as a method of
treating, inhibiting or preventing a pre-cancerous or
transformed cell phenotype. In a specific embodiment, the
15 *Nr-CAM* gene is a human gene and the Nr-CAM protein is a human
protein.

The present invention also encompasses inhibitors
of Nr-CAM activities related to cellular transformation. Nr-
CAM is a known protein thought to play a role in cell-cell
20 adhesion, *e.g.* during development of not only the vertebrate
nervous system but also by interaction with other proteins,
such as FGF-R, in neurite extension. The present invention
encompasses peptide fragments or antagonists, antibodies, or
small compounds which may inhibit or compete with ligands
25 binding to Nr-CAM and thus inhibit Nr-CAM activity. The
invention further encompasses peptide fragments (and
derivatives and analogs thereof) which comprise one or more
domains of a Nr-CAM protein which may be used to prevent
ligands binding to Nr-CAM. Antibodies to Nr-CAM, and to Nr-
CAM derivatives and analogs, are additionally provided.
30 Methods of production of the Nr-CAM proteins, derivatives and
analogs, *e.g.*, by recombinant means, are also provided.

The present invention further encompasses screening assays to identify compounds which inhibit *Nr-CAM* nucleic acid expression or nucleic acid product activity as potential therapeutics for the treatment and/or prevention of

5 tumorigenesis. In particular, the present invention encompasses host cell lines or transgenic animals which express Nr-CAM at high levels which have utility as tools for screening assays to identify agents which inhibit Nr-CAM expression and/or activity as potential therapeutic agents for the treatment and prevention of tumorigenesis.

10   The present invention also encompasses therapeutic and diagnostic methods and compositions based on Nr-CAM proteins and nucleic acids. Therapeutic compounds of the invention include but are not limited to Nr-CAM proteins and analogs and derivatives (including fragments) thereof;

15 antibodies thereto; nucleic acids encoding the Nr-CAM proteins, analogs, or derivatives; and Nr-CAM antisense nucleic acids.

The invention provides for treatment of disorders of overproliferation (*e.g.*, tumors, cancer and

20 hyperproliferative disorders) by administering compounds that decrease or antagonize (inhibit) Nr-CAM function (*e.g.*, antibodies, antisense nucleic acids, ribozymes).

The invention also provides methods of treatment of disorders involving deficient cell proliferation (growth) or

25 in which cell proliferation is otherwise desired (*e.g.*, degenerative disorders, growth deficiencies, lesions, physical trauma) by administering compounds that promote Nr-CAM activity (*e.g.*, an agonist of Nr-CAM; nucleic acids that encode Nr-CAM).

30 Animal models, diagnostic methods and screening methods for predisposition to disorders, and methods for identification of Nr-CAM agonists and antagonists, are also provided by the invention.

3.1. DEFINITIONS AND ABBREVIATIONS

As used herein, underscoring or italicizing the name of a gene shall indicate the gene, in contrast to its encoded protein product, which is indicated by the name of
5 the gene in the absence of any underscoring or italicizing. For example, *"Nr-CAM"* shall mean the *Nr-CAM* gene, whereas "Nr-CAM" shall indicate the protein product of the *Nr-CAM* gene.

As used herein, the following terms shall have the
10 meanings indicated.

Nr-CAM nucleotides or coding sequences: DNA sequences encoding Nr-CAM mRNA transcripts, protein, polypeptide or peptide fragments of Nr-CAM protein, and Nr-CAM fusion proteins, and RNA sequences corresponding to Nr-CAM mRNA transcripts and RNA sequences which are
15 complementary to the mRNA transcript, *Nr-CAM* nucleotide sequences encompass RNA, DNA, including genomic DNA (*e.g.* the *Nr-CAM* gene) and cDNA.

Nr-CAM: gene products, *e.g.*, transcripts and the Nr-CAM protein. Polypeptides or peptide fragments of the
20 protein are referred to as Nr-CAM polypeptides or Nr-CAM peptides. Fusions of Nr-CAM protein, polypeptides, or peptide fragments to an unrelated protein are referred to herein as Nr-CAM fusion proteins.

As used herein, the following terms shall have the
25 abbreviations indicated.

CD: cytoplasmic domain
DD-PCR: differential display - polymerase chain reaction
ECD: extracellular domain
30 FNHA: fetal normal human astrocytes
GMTT: glioblastomas multiforme tumor tissue
MTB: multiple tissue blot

MTT: meningioma tumor tissue

NBT: normal brain tissue

ORF: open reading frame

RT-PCR: reverse transcription - polymerase chain

5   reaction

TM: transmembrane domain

UTR: untranslated region

Brain tumor cell lines:

          CCF-STTG1: astrocytoma grade IV

10   D283 Med: medulloblastoma

          DBTRG-05MG: glioblastoma multiforme

          Hs 683: glioma

          IMR-32: neuroblastoma

          PFSK-1: primitive neuroectodermal tumor

15   SW 1783: astrocytoma grade III

4. DESCRIPTION OF THE FIGURES

          Figure 1 is an autoradiogram of DD-PCR gel

illustrating expression of hNr-CAM (designated D4-1) and of a

20 control D1-2 gene in normal brain tissue (N) and brain tumor

(T) tissue, i.e., glioblastoma multiforme (GM).

          Figures 2 (A-D) present the nucleotide and amino

acid sequences of human Nr-CAM has well as the results of

nucleotide sequence analysis as described in Section 6

25 (Figure 2C) and a schematic illustration of the hNr-CAM gene

showing the area used herein for antisense targeting (Figure

2D). Figure 2A presents the nucleotide sequence of human Nr-

CAM. Features of the nucleotide sequence include the

following: Nucleotides 130-3615 encode the extracellular

domain; nucleotides 202-4026 encode product = hBRAVO-Nr-CAM;

30 nucleotides 316-483 encode the Immunoglobulin I domain;

nucleotides 613-768 encode the Immunoglobulin II domain;

nucleotides 988-1134 encode the Immunoglobulin III domain;

nucleotides 1258-1410 encode the Immunoglobulin IV domain;

nucleotides 1540-1719 encode the Immunoglobulin VI domain;

nucleotides 2113-2265 encode the first Fibronectin (Fn)

repeat; nucleotides 2413-2565, the second Fn repeat;

5 nucleotides 2710-2886, the third Fn repeat; nucleotides 3028-

3186 the fourth Fn repeat; nucleotides 3370-3510, the fifth

Fn repeat; nucleotides 2616-3684, the transmembrane region;

nucleotides 3685-4036, the intracellular domain; and

nucleotides 4030-4134 constitute a 3' untranslated region.

10 Figure 2B presents the amino acid sequence of human Nr-CAM.

The hydrophobic signal sequence is underlined. Figures 2A

and 2B are adapted from Lane et al., 1996, Genomics 35:456-

465.

Figure 2C illustrates nucleotide sequence identity

analysis between previously cloned hNr-CAM (Accession Number

15 U55258), rat Nr-CAM (Accession Number U81037) and the

sequence of clone D4-1 obtained by cloning the hNr-CAM

isolated by DD-PCR into pCRII vector (Invitrogen). Sequence

identity analysis was performed using the DNasis program from

Hitachi Software (South San Francisco, CA). Stars (*)

20 indicate presence of identical nucleotides among the

sequences.

Figure 2D presents a schematic of the hNr-CAM gene,

including, in particular, the area used herein for antisense

targeting. The arrow indicates this area. See text, Section

25 7 for details.

Figures 3 (A-F) illustrate differential expression

of Nr-CAM in glioblastoma multiforme tissue (GM) when

compared to normal brain tissue (NT). The technique of in

situ hybridization was used. Figures 3 (A, B and C) are from

one GM tumor while Figures 3 (D, E and F) are from a second

30 GM tumor. Figures 3 (A and D) show tumor regions and one

hybridized with hNr-CAM anti-sense probes. Figures 3 (B and

E) show serial sections of A and D, and are hybridized with

hNr-CAM sense probes. Figures 3 (C and F) show normal brain

regions of serial sections of the same brain as sections A

and D, respectively, and are hybridized with *hNr-CAM* anti-

sense probe. Cells expressing hNr-CAM are indicated by

5 arrows.

Figures 4 (A-B) illustrate differential expression

of *hNr-CAM* in normal and tumor tissues. Figure 4A is a gel

obtained using RT-PCR of total RNA of a variety of tissues.

The upper panel of Figure 4A shows expression of *hNr-CAM* and

the lower panel shows expression of a central, housekeeping

10 gene *D1-2*. Figure 4B is a bar graph showing relative

expression of *hNr-CAM* after correction for gel load based on

D1-2 expression. In both Figures 4A and 4B, the specific

tissue in each of lanes 1-14 is as follows: Lane 1,

glioblastoma IV; 2, recurrent meningioma; 3, meningioma; 4,

15 normal brain (GS); 5, neuroblastoma; 6, recurrent glioma; 7,

glioblastoma multiforme; 8, melanoma; 9, normal breast; 10,

tumor breast; 11, benign prostate; 12, prostate tumor; 13,

normal brain; and 14, glioma III. Recurrent gliomas

represent a tissue from the same patient previously diagnosed

20 with GM.

Figures 5 (A and B) illustrate differential

expression of *hNR-CAM* in normal brain tissue and astrocytoma

tumor tissue. Figure 5A is a gel obtained using RT-PCR of

total RNA of normal or astrocytoma tissue. The upper panel

25 of Figure 5A shows expression of *hNr-CAM*; the middle panel,

EGFR (epidermal growth factor receptor, a known brain tumor

maker) and the lower panel, *D1-2*. Figure 5B is a bar graph

showing the relative expression of *hNr-CAM* and *EGFR* after

normalizing with *D1-2* gel loading. In both Figures 5A and

5B, Lane a is normal brain tissue and Lane b is astrocytoma

30 tumor tissue.

Figures 6 (A and B) illustrate expression of *hNr-*

CAM in normal brain and brain tumor cell lines. Figure 6A is

- 15 -

an autoradiogram of a Southern blot of hNr-CAM expression
(upper panel); and of *D1-2* expression (lower panel) in
various brain cell lines. Figure 6B is a bar graph showing
relative expression of *hNr-CAM* in respective cell lines after
5   correction for gel loading based on *D1-2* expression. In both
Figures 6A and 6B, the specific cell line in each of Lanes 1-
9 is as follows: Lane 1, astrocytoma III; 2, astrocytoma IV;
3, glioblastoma; 4, glioma; 5, neuroectodermal; 6,
medulloblastoma; 7 neuroblastoma; 8, FNHA; and 9, normal
10 brain.

        Figures 7 (A and B) illustrate expression of hNr-
CAM in different regions of the brain. Figure 7A is an
autoradiogram of a Northern blot of *hNr-CAM* expression (upper
panel) and of *β-actin* expression (lower panel) in a variety
of regions (Lanes 1-8) of normal adult brain. β-actin
15 expression serves as an internal control for gel loading for
different regions of the brain. Figure 7B is a bar graph
showing the relative expression of *hNr-CAM* in different brain
regions after correction for gel loading based on *β-actin*
expression. In both Figures 7A and 7B, the specific region
20 of brain in each of Lanes 1-8 is as follows: Lane 1,
cerebellum; 2, cerebral cortex; 3, medulla; 4, spinal cord;
5, occipital pole; 6, frontal lobe; 7, temporal lobe; and 8,
putamen.

        Figures 8 (A and B) illustrate expression of *hNr-
25 CAM* in human cancer cell lines (Lanes a-h). Figure 8A is an
autoradiogram of a Northern blot of *hNr-CAM* expression (upper
panel) and of *β-actin* expression (lower panel). Figure 8B is
a bar graph of the relative expression of *hNr-CAM* after
correction for gel loading based on *β-actin* expression. In
30 both Figures 8A and 8B, the specific human cancer cell line
in each of Lanes a-h is as follows: Lane a, promyelocytic
leukemia (HL-60); b, HeLa cells (S3); c, chronic myelogenous
leukemia (K-562); d, lymphoblastic leukemia (MOLT-4); e,

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Burkitt's lymphoma (Raji); f, colorectal adenocarcinoma (SA 480); g, lung carcinoma (A549); and h, melanoma (G361).

Figure 9 illustrates genomic Southern blot analysis of hNr-CAM in several brain tumor cell lines and in the NIH 3T3 cell line. Upper panel shows an ethidium bromide-stained gel of genomic DNA digested with EcoRI restriction enzyme. Lower panel shows an autoradiogram of the Southern blot. The arrow indicates hNr-CAM. In both panels, the brain tumor cell lines in each of Lanes 1-4 is as follows: Lane 1, NIH 3T3 cell line; 2, astrocytoma III; 3, glioma and 4, glioblastoma cell line.

Figures 10 (A and B) illustrate the effects of antisense *hNr-CAM* expression on the morphology of glioblastoma (GB) cells. Figure 10A shows GB cells transfected with p-CMV-neovector only. Figure 10B shows GB cells transfected with p-CMV-neovector containing antisense hNr-CAM.

Figure 11 is a graph illustrating the effect of anti-sense hNr-CAM expression on the proliferation of glioblastoma cells.

Figure 12 is a bar graph illustrating the effect of anti-sense hNr-CAM on soft agar colony formation of glioblastoma cells. GB represents Glioblastoma cells; GB-PFCS, represents glioblastoma cells with vector alone; GB-Anti-Nr-CAM, represents GB cells expressing Nr-CAM 1/3.

Figure 13 illustrates the effect of overexpression of antisense hNr-CAM (pCMV-1/3 Nr-AS) on native *hNr-CAM* mRNA level. See text Section 7.1.6. for details.

Figures 14 (A and B) schematically illustrate the effect of antisense *hNr-CAM* expression in glioblastoma cells. Figure 14A shows untransfected cells and Figure 14B shows pCMV-1/3Nr-AS transfected cells.

Figures 15 (A-D) illustrate effect of antisense hNr-CAM expression on morphology of glioblastoma (GB) cells.

Figures 15 (A and B) show 5GB cells transfected with pCMV-
neovector only, *i.e.*, control cells (at different
magnifications). Figures 15 (C and D) show 5 GB cells
transfected with pCMV-1/3Nr-AS (at different magnifications).
5 Spindle shaped cells are indicated by arrows. See text
Section 7.2.1. for details.

Figures 16 (A-D) show the effect of serum treatment
on the morphology of the pCMV-1/3Nr-AS transfected 5GB cells.
5GB (pCMVneo or pCMV-Nr-1/3AS transfected) cells were plated
at an approximate density of $3x10^4$. Both cell types were
10 treated with different concentrations of FBS. Figures 15 A,
B, C, and D, respectively, show 5GB cells (pCMV-1/3Nr-*CAM*
transfected) treated with 0.1, 1.0, 2.0 and 5% FBS serum.

Figure 17 shows the effect of antisense *hNr-CAM*
pCMV-1/3Nr-AS) expression on the proliferation of 5GB human
15 glioblastoma cell line.

Figure 18 shows the effect of anti-sense *hNr-CAM*
overexpression on the migration ability of 5GB cells.
Briefly, $1x10^6$ pCMV-neo and pCMV-1/3Nr-AS transfected cells
were plated in triplicates in transwell inserts ($8\mu m$) from
20 Coastar (Cambridge, MA). Inserts were placed in 6 well
tissue culture dishes containing the appropriate growth
medium with serum. Cells that migrated through the inserts
and settled on the tissue culture dish were fixed with 4%
paraformaldehyde. Cell were then stained with hematoxylin
and counted under a dissection microscope. Cells that
25 migrated through the transwell inserts are compared for pCMV-
neo and pCMV-1/3Nr-AS transfected cells.

Figure 19 shows the effect of anti-sense *hNr-CAM*
overexpression on the invasion ability of 5GB cells.
Briefly, $1x10^4$ pCMV-neo and pCMV-1/3Nr-AS transfected cells
30 were plated on transwell inserts ($8\mu m$) coated with 825ng
extracellular matrix (ECM) gel from Coastar (Cambridge, MA).
Inserts were placed in 6 well tissue culture dishes

containing the appropriate growth media with serum. Cells
that migrated through the inserts and settled on the tissue
culture dish were fixed with 4% paraformaldehyde. Cell are
then stained with hematoxylin and counted under a dissection
5 microscope. Cells that migrated through the transwell
inserts are compared for pCMV-neo and pCMV-1/3Nr-AS
transfected cells.

Figures 20 (A-C) show the effect of UV radiation on
antisense hNr-CAM transfected 5GB cells. Figures 20 (A and
B) show pCMV-neo and pCMV-1/3Nr-AS transfected cells analyzed
10
for apoptosis. Arrows indicate cells undergoing apoptosis.
Figure 20C shows % of cells undergoing apoptosis after
treatment with 100 units of UV radiation.

Figure 21 shows the effect of the antisense hNr-CAM
over-expression on the tumor forming ability of 5GB cells in
15 vivo. Lower three mice were injected with pCMV-neo
transfected 5GB cells. Top three mice were injected with
pCMV-1/3Nr-CAM transfected 5GB cells. The tumors and the
site of injection are indicated by arrows.

Figures 22 (A and B) show the effects of intra-
20 tumoral inoculation of plasmid expressing antisense hNr-CAM.
Figure 22 A illustrates results obtained using three athymic
nude mice injected 72 days post-implantation with 5 GB cells
with 50 µg either pCMVneo (control animal (●)) or pCMVneo 1/3
Nr-AS (two animals (■ and Δ). Arrows indicate days of
25 intratumoral injection. See Section 7.2.9. for details.
Figure 22B shows results with four athymic nude mice
implanted 3x3mm pieces of glioblastoma tumor. This tumor was
generated previously by injecting 1x10^7 5GB glioblastoma
cells subcutaneously. 28 days post implantation, 50µg of
either pCMVneo or pCMV1/3Nr-AS plasmids were mixed with DMRIE
30
(liposomes) reagent (Gibco/BRL) and injected twice a week for
four weeks around the tumor site. Tumor size was measured
twice a week with a caliper and tumor volume was determined.

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Arrow indicates the first day of intra-tumoral injection.
See Section 7.2.9. for details.

Figures 23 (A-C) show the effect of antisense *hNr-CAM* on GB1690 glioblastoma cells. The full length clone for *hNr-CAM* was provided by William J. Dreyer (California Institute of Technology). A PCR product of approximately 1360 bases (Nucleotide positions 1410-2746) was generated from the full length *hNr-CAM* clone using specific primers (5'TAGATACAACTAGTCAATGCCTCTAATGAATATGG ATA3' (SEQ. ID. No.: 6); and 5' AGATAGATCCGCGGAATAGTAAA TCCGATA GCCTTGTA3' (SEQ. ID. No.: 7). The PCR product was then cloned into Spel and SacII sites of pCMV-neo vector and was termed as pCMV-2/3Nr-AS. The pCMV-neo or pCMV-2/3Nr-AS were then transfected into GB1690 glioblastoma cell line and selected in G418. Figures 23 (A and B) show cell morphology of cells transfected with pCMV-neo and pCMV-2/3Nr-AS, respectively. Arrows indicate spindle shape cells. Figure 23C shows a comparison of number of soft agar colonies formed by pCMV-neo and pCMV-2/3Nr-AS transfected GB1690 cells respectively.

Figure 24 shows the sequence identity analysis between human and rat *Nr-CAM* nucleotide sequence.

Figures 25 (A and B) schematically show the packaging of infectious, replication-incompetent retroviral particles (Figure 25 A) and a retroviral vector (Figure 25B). The retroviral vector, containing the gene of interest (antisense hNr-CAM), a selection gene (Neor), and psi+, the packaging signal necessary for retrovirus particle formation, are stably integrated or transiently expressed. The packaging cell line provides the other genes necessary for particle formation which have been deleted from the vector: gag (structural proteins), pol (reverse transcriptase, integrase), and env (coat glycoproteins). Virus released from this cell line contains the products of these genes (and is infectious), but lacks the genes themselves, thus

preventing retroviral production from subsequently infected
cell lines. Figure 25B shows pLXSN retroviral vector that
can be used for cloning antisense hNr-CAM gene.

Figures 26 (A and B) show the identification of
differentially expressed genes in 5GB glioblastoma cells
transfected with pCMVneo (Figure 26B) or pCMV1/3Nr-AS (Figure
26A) vectors. Differential hybridization was performed as
described in Section 8.. White arrow in Figure 26A indicates
novel cDNA (accession# H77485) and selectin gene is indicated
by black arrow (See Figure 26B).

5.   DETAILED DESCRIPTION OF THE INVENTION

The present invention relates to the identification
of a novel role of Nr-CAM in cell transformation and aberrant
cellular proliferation. In particular, the present invention
relates to the discovery of altered expression of Nr-CAM in a
number of primary tumors and cell lines derived from tumors,
in addition to, the altered expression of ligands for Nr-CAM.
Further, the present invention relates, in part, to the
Applicants' surprising discovery that the inhibition of *Nr-
CAM* gene expression or the inhibition of Nr-CAM activity in
transformed cells reverses the transformed phenotype.

The present invention encompasses compounds and
methods for the detection of aberrant *Nr-CAM* gene expression
or activity as a diagnostic or prognostic tool to indicate a
transformed, pre-cancerous or cancerous cell phenotype. The
present invention further encompasses compounds and methods
for the detection of aberrant gene expression or activity as
a diagnostic or prognostic tool to indicate a transformed,
pre-cancerous or cancerous cell phenotype. The present
invention also encompasses compounds and methods for the
modulation of *Nr-CAM* gene expression or activity as a method
of treating or preventing a transformed, pre-cancerous or
cancerous cell phenotype. In this regard, the present

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invention provides nucleotide sequences of *Nr-CAM* genes, and
amino acid sequences of their encoded proteins. The
invention further provides fragments and other derivatives,
and analogs, of Nr-CAM proteins. Nucleic acids encoding such
5 fragments or derivatives are also within the scope of the
invention. The invention provides *Nr-CAM* nucleic acids and
their encoded proteins of humans and related genes (homologs)
in other species. In specific embodiments, the *Nr-CAM*
nucleic acids and proteins are from vertebrates, or more
particularly, mammals. In a preferred embodiment of the
10
invention, the *Nr-CAM* nucleic acids and proteins are of human
origin. Production of the foregoing nucleic acids, proteins
and derivatives, *e.g.*, by recombinant methods, is provided.

     Nr-CAM is a gene identified by the method of the
invention, that is expressed at high levels in glioblastoma
15 multiforme tissue as well as certain others forms of tumors
and cancers.

     The invention also provides Nr-CAM derivatives and
analogs of the invention which are functionally active, *i.e.*,
they are capable of displaying one or more functional
20 activities described herein associated with a full-length
(wild-type) Nr-CAM protein. Such functional activities
include, but are not limited to, antigenicity, *i.e.*, ability
to bind (or compete with Nr-CAM for binding) to an anti-Nr-
CAM antibody, immunogenicity, *i.e.*, ability to generate
25 antibody which binds to Nr-CAM, and ability to bind (or
compete with Nr-CAM for binding) to a ligand for Nr-CAM. The
invention further provides fragments (and derivatives and
analogs thereof) of Nr-CAM which comprise one or more domains
of the Nr-CAM protein. Antibodies to Nr-CAM, its derivatives
and analogs, are additionally provided.
30
     The present invention also provides therapeutic and
diagnostic methods and compositions based on Nr-CAM proteins
and nucleic acids and anti-Nr-CAM antibodies. The invention

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provides for treatment of disorders of overproliferation
(e.g., cancer and hyperproliferative disorders) by
administering compounds that decrease Nr-CAM activity (e.g.,
antibodies, Nr-CAM antisense nucleic acids).

5   The invention also provides methods of treatment of
disorders involving deficient cell proliferation or in which
cell proliferation (growth) is otherwise desirable (e.g.,
growth deficiencies, degenerative disorders, lesions,
physical trauma) by administering compounds that promote Nr-
CAM function.
10   The present invention further provides screening
assays to identify novel agents which target Nr-CAM gene or
nucleic acid expression or Nr-CAM protein activity, including
interaction with ligands, and, thus are potential therapeutic
agents for the treatment or prevention of cell
15 transformation, or pre-cancerous or cancerous phenotypes,
i.e., tumorigenesis. The screening assays of the present
invention may function to identify novel exogenous or
endogenous agents that inhibit Nr-CAM expression or inhibit
the interaction between Nr-CAM and its ligand. A variety of
20 protocols and techniques may be used to identify drugs that
inhibit Nr-CAM expression and/or Nr-CAM activity, and as a
result inhibit Nr-CAM participation in aberrant cellular
proliferative activity. Such identified agents have utility
in the treatment of hosts demonstrating a cellular
transformed phenotype or aberrant cellular proliferative
25
behavior, and advantageously would be effective to treat
and/or prevent tumorigenesis.
           The present invention further encompasses
pharmaceutical compositions containing the novel agents
identified by the screening assays described herein. The
30 invention provides therapeutic modalities and pharmaceutical
compositions for the treatment of tumorigenesis and the
prevention of transformed phenotypes. The therapeutic

modalities of the present invention further encompass
combination therapies in which an agent which inhibits Nr-CAM
expression and/or activity, and at least one other
therapeutic agent, *e.g.*, a chemotherapeutic agent, are

5 administered either concurrently, *e.g.*, as an admixture,
separately but simultaneously or concurrently, or
sequentially.

        The novel therapeutic combinations of the present
invention provide a means of treatment which may not only
reduce the effective dose of either drug required for

10
antitransformation or antitumorigenesis, thereby reducing
toxicity, but may improve the absolute therapeutic effect as
a result of attacking aberrant cellular proliferation through
a variety of mechanisms.

        The invention is illustrated by way of examples

15 *infra* which disclose, *inter alia*, the isolation and
characterization of Nr-CAM, and patterns of expression of Nr-
CAM in certain tumors (see Section 6).

        For clarity of disclosure, and not by way of
limitation, the detailed description of the invention is

20 divided into the subsections which follow.

        5.1. **IDENTIFICATION OF ROLE OF Nr-CAM IN TRANSFORMATION**

        The present invention relates to a novel role of
Nr-CAM in the promotion of cell transformation and
tumorigenesis. In particular, the present invention relates

25
to the Applicants' findings that (a) Nr-CAM is highly over-
expressed in glioblastoma multiforme tumor tissue and is
over-expressed a number of other primary tumors; and (b)
over-expression of Nr-CAM in the anti-sense orientation
results in decreased cellular proliferation and colony

30 formation of glioblastoma cells in soft agar.

        The present invention further relates to the
Applicants' findings that *Nr-CAM* is over-expressed in several

brain tumor derived cell lines and primary brain tumor
tissues, including astrocytoma grade IV, glioma, glioblastoma
and neuroectodermal human tumor cell lines compared to NBT.
Low or no expression of *hNr-CAM* was observed in cell lines
5 derived from astrocytoma III, medulloblastoma, neuroblastoma
and NBT. Further, Nr-CAM was found to be expressed at high
levels in melanoma G361, lymphoblastic leukemia cell lines,
and Burkitt's lymphoma cell lines. A low level of hNr-CAM
expression was observed in colorectal adenocarcinoma, lung
carcinoma, pro-myelocytic leukemia HeLa cell S3, and chronic
10 myelogenous leukemia.

     The present invention relates to the role of Nr-CAM
in promotion of cell transformation and tumorigenesis, and
provides methods including the use of *Nr-CAM* nucleic acids
and nucleic acids which hybridize or complement *Nr-CAM*
15 nucleic acids, as diagnostic and prognostic tools for the
detection of transformed, pre-cancerous and cancerous
phenotypes. The present invention provides methods for use
of *Nr-CAM* nucleic acids and those which complement and/or
hybridize to nucleic acid sequences which encode Nr-CAM as
20 therapeutics to treat, inhibit or prevent transformed, pre-
cancerous and cancerous phenotypes. In particular, the
invention provides compositions comprising nucleic acid
sequences which inhibit Nr-CAM expression as therapeutics to
treat or prevent transformed, pre-cancerous, and cancerous
25 phenotypes.

5.2. THE PRODUCTION OF *Nr-CAM* NUCLEIC ACIDS, POLYPEPTIDES AND ANTIBODIES AS DIAGNOSTICS, THERAPEUTICS AND COMPONENTS FOR SCREENING ASSAYS

     The present invention encompasses the use of agents
30 for the detection of aberrant *Nr-CAM* gene expression as
diagnostic or prognostic tools to detect a transformed
phenotype, pre-cancerous or cancerous condition. Diagnostic

or prognostic tools which may be used in accordance with the
present invention include, but are not limited to, (a)
nucleic acids which hybridize or are complementary to the *Nr-CAM* nucleotide sequence; (b) polypeptides, peptide fragments
5 or synthetic molecules which bind to the Nr-CAM ligand
binding domain; and (c) antibodies which bind to Nr-CAM.

         The present invention relates to the use of agents
which inhibit Nr-CAM expression and/or protein activity as
therapeutics for the treatment and/or prevention of a
transformed or pre-cancerous phenotype, or cancer or
10 tumorigenesis. Therapeutic agents which may be used in
accordance with the present invention include, but are not
limited to, (a) nucleic acids which inhibit *Nr-CAM* gene
expression, *e.g.*, antisense molecules, ribozymes or triple
helix molecules complementary to *Nr-CAM*; (b) polypeptides,
15 peptides, antibodies, small organic molecules or synthetic
molecules which inhibit Nr-CAM activity or prevent Nr-CAM
from binding its ligand; and (c) peptides, polypeptides,
antibodies, small organic molecules or synthetic molecules
which act as antagonists of Nr-CAM activity.

20   The present invention provides screening assays for
the identification of agents which inhibit *Nr-CAM* gene
expression and/or activity. In one embodiment of the
invention, an important component of the screening assays of
the present invention are nucleotide coding sequences
25 encoding Nr-CAM proteins, polypeptides and peptides. The
present invention further encompasses (a) DNA vectors that
contain any of the foregoing Nr-CAM encoding sequences and/or
their complements; (b) DNA expression vectors that contain
any of the foregoing Nr-CAM coding sequences operatively
associated with a regulatory element that directs the
30 expression of the coding sequences in the host cell; and (c)
genetically engineered host cells that contain any of the
foregoing Nr-CAM coding sequences operatively associated with

a regulatory element that directs the expression of the

coding sequences in the host cell.

5.2.1.   THE *Nr-CAM* NUCLEIC ACIDS

5   The invention relates to the nucleotide sequences

of *Nr-CAM* nucleic acids.   In an embodiment, the *Nr-CAM*

nucleic acids comprise the nucleotide sequence shown in

Figure 2A, *i.e.*, SEQ. ID. No.: 1 or specific regions thereof.

In specific embodiments, *Nr-CAM* nucleic acids comprise the

10   cDNA sequences of SEQ. ID. NO.:   5, or the coding regions

thereof, or nucleotide sequences acids encoding a Nr-CAM

protein (*e.g.*, a protein having the sequence shown in Figure

2B, *i.e.*, SEQ. ID. NO.: 2) or a fragment thereof.   Nucleic

acids of the present invention can be single or double

stranded.   The invention also relates to nucleic acids

15   hybridizable to or complementary to the foregoing sequences.

In specific aspects, nucleic acids are provided which

comprise a sequence complementary to at least 10, 25, 50,

100, 200, or 250 contiguous nucleotides of a *Nr-CAM* gene.   In

a specific embodiment, a nucleic acid which is hybridizable

20 to a *Nr-CAM* nucleic acid (*e.g.*, having sequence SEQ. ID. NO.:

1), or to a nucleic acid encoding a Nr-CAM derivative, under

conditions of low stringency is provided.

            By way of example and not limitation, procedures

using such conditions of low stringency are as follows (see

25 also Shilo and Weinberg, 1981, Proc. Natl. Acad. Sci. USA

78:6789-6792):   Filters containing DNA are pretreated for 6 h

at 40°C in a solution containing 35% formamide, 5X SSC, 50 mM

Tris-HCl (pH 7.5), 5 mM EDTA, 0.1% PVP, 0.1% Ficoll, 1% BSA,

and 500 µg/ml denatured salmon sperm DNA.   Hybridizations are

carried out in the same solution with the following

30 modifications: 0.02% PVP, 0.02% Ficoll, 0.2% BSA, 100 µg/ml

salmon sperm DNA, 10% (wt/vol) dextran sulfate, and

5-20 X 10^6 cpm ^{32}P-labeled probe is used.   Filters are

incubated in hybridization mixture for 18-20 h at 40°C, and
then washed for 1.5 h at 55°C in a solution containing 2X
SSC, 25 mM Tris-HCl (pH 7.4), 5 mM EDTA, and 0.1% SDS. The
wash solution is replaced with fresh solution and incubated

5 an additional 1.5 h at 60°C. Filters are blotted dry and
exposed for autoradiography. If necessary, filters are
washed for a third time at 65-68°C and re-exposed to film.
Other conditions of low stringency which may be used are well
known in the art (e.g., as employed for cross-species
hybridizations).

10   In another specific embodiment, a nucleic acid
which is hybridizable to a Nr-CAM nucleic acid under
conditions of high stringency is provided. By way of example
and not limitation, procedures using such conditions of high
stringency are as follows: Prehybridization of filters

15 containing DNA is carried out for 8 h to overnight at 65°C in
buffer composed of 6X SSC, 50 mM Tris-HCl (pH 7.5), 1 mM
EDTA, 0.02% PVP, 0.02% Ficoll, 0.02% BSA, and 500 μg/ml
denatured salmon sperm DNA. Filters are hybridized for 48 h
at 65°C in prehybridization mixture containing 100 μg/ml

20 denatured salmon sperm DNA and 5-20 X 10⁶ cpm of ³²P-labeled
probe. Washing of filters is done at 37°C for 1 h in a
solution containing 2X SSC, 0.01% PVP, 0.01% Ficoll, and
0.01% BSA. This is followed by a wash in 0.1X SSC at 50°C
for 45 min before autoradiography. Other conditions of high
stringency which may be used are well known in the art.

25   In another specific embodiment, a nucleic acid
which is hybridizable to a Nr-CAM nucleic acid under
conditions of moderate stringency is provided.

        Various other stringency conditions which promote
nucleic acid hybridization can be used. For example,

30 hybridization in 6x SSC at about 45° C, followed by washing
in 2xSSC at 50° C may be used. Alternatively, the salt
concentration in the wash step can range from low stringency

of about 5xSSC at 50° C, to moderate stringency of about
2xSSC at 50°C, to high stringency of about 0.2x SSC at 50° C.
In addition, the temperature of the wash step can be
increased from low stringency conditions at room temperature,
to moderately stringent conditions at about 42° C, to high
stringency conditions at about 65° C. Other conditions
include, but are not limited to, hybridizing at 68° C in 0.5M
NaHPO$_4$ (pH7.2)/ 1 mM EDTA/ 7% SDS, or hybridization in 50%
formamide/0.25M NaHPO$_4$ (pH 7.2)/.25 M NaCl/1 mM EDTA/7% SDS;
followed by washing in 40 mM NaHPO$_4$ (pH 7.2)/1 mM EDTA/5% SDS
at 42° C or in 40 mM NaHPO$_4$ (pH7.2) 1 mM EDTA/1% SDS at 50° C.
Both temperature and salt may be varied, or alternatively,
one or the other variable may remain constant while the other
is changed.

Low, moderate and high stringency conditions are
well known to those of skill in the art, and will vary
predictably depending on the base composition of the
particular nucleic acid sequence and on the specific organism
from which the nucleic acid sequence is derived. For
guidance regarding such conditions see, for example, Sambrook
et al., 1989, Molecular Cloning, A Laboratory Manual, Second
Edition, Cold Spring Harbor Press, N.Y., pp. 9.47-9.57; and
Ausubel et al., 1989, Current Protocols in Molecular Biology,
Green Publishing Associates and Wiley Interscience, N.Y.

The invention also encompasses nucleic acids having
at least 60%, 70%, 75%, 80%, 90%, 95% or greater sequence
identity when compared to a portion of identical-sized hNr-
CAM sequence shown in Figure 2A or when compared to said
sequence when the alignment or comparison is conducted by a
computer homology programmer search aligorithm known in the
art.

By way of example and not limitation, useful
computer homology programs include the following: Basic
Local Alignment Search Tool (BLAST) (www.ncbi.nlm.nih.gov)

(Altschul et al., 1990, J. of Molec. Biol., 215:403-410, "The

BLAST Algorithm"; FASTA (also TFASTA) (see, Pearson et al.,

1988, Proc. Nat'l Acad. Sci. USA 85:2444-2448) and CLUSTALW

(see, Higgins et al., 1996, Methods Enzymol 266:383-402).

5   Altschul et al., 1997, Nuc. Acids Res.

25:3389-3402) describe BLAST, a heuristic search algorithm

tailored to searching for sequence similarity which ascribes

significance using the statistical methods of Karlin and

Altschul 1990, Proc. Nat'l Acad. Sci. USA, 87:2264-68; 1993,

Proc. Nat'l Acad. Sci. USA 90:5873-77. Five specific BLAST
10
programs perform the following tasks:

          1)   The BLASTP program compares an amino acid

query sequence against a protein sequence database.

          2)   The BLASTN program compares a nucleotide query

sequence against a nucleotide sequence database.

15   3)   The BLASTX program compares the six-frame

conceptual translation products of a nucleotide query

sequence (both strands) against a protein sequence database.

          4)   The TBLASTN program compares a protein query

sequence against a nucleotide sequence database translated in

20 all six reading frames (both strands).

          5)   The TBLASTX program compares the six-frame

translations of a nucleotide query sequence against the

six-frame translations of a nucleotide sequence database.

          As will be understood by those skilled in the art,

the TBLASTN program is particularly useful to identify
25
nucleic acids with a desired percent identity and the BLASTP

program is particularly useful to identify amino acid

sequences with a desired percent identity.

          Smith-Waterman (database: European Bioinformatics

Institute wwwz.ebi.ac.uk/bic_sw/) (Smith-Waterman, 1981, J.

30 of Molec. Biol., 147:195-197) is a mathematically rigorous

algorithm for sequence alignments.

FASTA (see Pearson et al., 1988, Proc. Nat'l Acad.
Sci. USA, 85:2444-2448) is a heuristic approximation to the
Smith-Waterman algorithm. For a general discussion of the
procedure and benefits of the BLAST, Smith-Waterman and FASTA
5 algorithms see Nicholas et al., 1998, "A Tutorial on
Searching Sequence Databases and Sequence Scoring Methods"
(www.psc.edu) and references cited therein.

Nucleic acids encoding derivatives and analogs of
Nr-CAM proteins (see Sections 5.2.2), and Nr-CAM antisense
nucleic acids are additionally provided. As is readily
10 apparent, as used herein, a "nucleic acid encoding a fragment
or portion of a Nr-CAM protein" shall be construed as
referring to a nucleic acid encoding only the recited
fragment or portion of the Nr-CAM protein and not the other
contiguous portions of the Nr-CAM protein as a continuous
15 sequence.

Fragments of *Nr-CAM* nucleic acids comprising
regions conserved between other *Nr-CAM* nucleic acids, of the
same or different species, are also provided. Nucleic acids
encoding one or more Nr-CAM domains are provided.

20 Specific embodiments for the cloning of a *Nr-CAM*
gene, presented as a particular example but not by way of
limitation, follow:

For expression cloning (a technique commonly known
in the art), an expression library is constructed by methods
known in the art. For example, mRNA (e.g., human) is
25 isolated, cDNA is made and ligated into an expression vector
(e.g., a bacteriophage derivative) such that it is capable of
being expressed by the host cell into which it is then
introduced. Various screening assays can then be used to
select for the expressed Nr-CAM product. In one embodiment,
30 anti-Nr-CAM antibodies can be used for selection.

In another embodiment, polymerase chain reaction
(PCR) is used to amplify the desired sequence in a genomic or

cDNA library, prior to selection. Oligonucleotide primers

representing known *Nr-CAM* sequences can be used as primers in

PCR. In a preferred aspect, the oligonucleotide primers

represent at least part of the *Nr-CAM* sequence presented in

5   Figure 2A. The synthetic oligonucleotides may be utilized as

primers to amplify by PCR sequences from a source (RNA or

DNA), preferably a cDNA library, of potential interest. PCR

can be carried out, *e.g.*, by use of a Perkin-Elmer Cetus

thermal cycler and Taq polymerase (Gene Amp""). The DNA being

10 amplified can include mRNA, cDNA, or genomic DNA from any

eukaryotic species. One can choose to synthesize several

different degenerate primers, for use in the PCR reactions.

It is also possible to vary the stringency of hybridization

conditions used in priming the PCR reactions, to allow for

greater or lesser degrees of nucleotide sequence similarity

15 between the known *Nr-CAM* nucleotide sequence and the nucleic

acid homolog being isolated. For cross species

hybridization, low stringency conditions are preferred. For

same species hybridization, moderately stringent conditions

are preferred. After successful amplification of a segment

20 of a *Nr-CAM* homolog, that segment may be molecularly cloned

and sequenced, and utilized as a probe to isolate a complete

cDNA or genomic clone. This, in turn, will permit the

determination of the gene's complete nucleotide sequence, the

analysis of its expression, and the production of its protein

25 product for functional analysis, as described *infra*. In this

fashion, additional genes encoding Nr-CAM proteins and Nr-CAM

analogs may be identified.

The above-methods are not meant to limit the

following general description of methods by which clones of

Nr-CAM may be obtained.

30   Any eukaryotic cell potentially can serve as the

nucleic acid source for the molecular cloning of the *Nr-CAM*

gene. The nucleic acid sequences encoding Nr-CAM can be

isolated from vertebrate sources, including mammalian
sources, such as porcine, bovine, feline, and equine, canine,
human, as well as additional primate sources, avian,
reptilian, amphibian, piscine, etc. sources, non-vertebrate
5 sources such as insects, from plants, etc. The DNA may be
obtained by standard procedures known in the art from cloned
DNA (e.g., a DNA "library"), by chemical synthesis, by cDNA
cloning, or by the cloning of genomic DNA, or fragments
thereof, purified from the desired cell. (See, for example,
Sambrook et al., 1989, Molecular Cloning, A Laboratory
10 Manual, 2d Ed., Cold Spring Harbor Laboratory Press, Cold
Spring Harbor, New York; Glover, D.M. (ed.), 1985, DNA
Cloning: A Practical Approach, MRL Press, Ltd., Oxford, U.K.
Vol. I, II.) Clones derived from genomic DNA may contain
regulatory and intron DNA regions in addition to coding
15 regions; clones derived from cDNA will contain only exon
sequences. Whatever the source, the gene should be
molecularly cloned into a suitable vector for propagation of
the gene.

In the molecular cloning of the gene from genomic
20 DNA, DNA fragments are generated, some of which will encode
the desired gene. The DNA may be cleaved at specific sites
using various restriction enzymes. Alternatively, one may
use DNAse in the presence of manganese to fragment the DNA,
or the DNA can be physically sheared, as for example, by
sonication. The linear DNA fragments can then be separated
25 according to size by standard techniques, including but not
limited to, agarose and polyacrylamide gel electrophoresis
and column chromatography.

Once the DNA fragments are generated,
identification of the specific DNA fragment containing the
30 desired gene may be accomplished in a number of ways. For
example, if an amount of a portion of a Nr-CAM (of any
species) gene or its specific RNA, or a fragment thereof (see

Section 5.6), is available and can be purified and labeled, the generated DNA fragments may be screened by nucleic acid hybridization to the labeled probe (Benton and Davis, 1977, Science 196:180; Grunstein and Hogness, 1975, Proc. Natl.

5 Acad. Sci. U.S.A. 72:3961). Those DNA fragments with substantial homology to the probe will hybridize. It is also possible to identify the appropriate fragment by restriction enzyme digestion(s) and comparison of fragment sizes with those expected according to a known restriction map if such

10 is available. Further selection can be carried out on the basis of the properties of the gene. Alternatively, the presence of the gene may be detected by assays based on the physical, chemical, or immunological properties of its expressed product. For example, cDNA clones, or DNA clones which hybrid-select the proper mRNAs, can be selected which

15 produce a protein that, e.g., has similar or identical electrophoretic migration, isoelectric focusing behavior, proteolytic digestion maps, promotion of cell proliferation activity, substrate binding activity, or antigenic properties of Nr-CAM. If an antibody to Nr-CAM is available, the Nr-CAM

20 protein may be identified by binding of labeled antibody to the putatively Nr-CAM synthesizing clones, in an ELISA (enzyme-linked immunosorbent assay)-type procedure.

The *Nr-CAM* gene can also be identified by mRNA selection by nucleic acid hybridization followed by *in vitro*

25 translation. In this procedure, fragments are used to isolate complementary mRNAs by hybridization. Such DNA fragments may represent available, purified Nr-CAM DNA of another species (e.g., human, mouse, etc.). Immunoprecipitation analysis or functional assays (e.g., aggregation ability *in vitro*; binding to receptor; see *infra*)

30 of the *in vitro* translation products of the isolated products of the isolated mRNAs identifies the mRNA and, therefore, the complementary DNA fragments that contain the desired

sequences. In addition, specific mRNAs may be selected by
adsorption of polysomes isolated from cells to immobilized
antibodies specifically directed against Nr-CAM protein. A
radiolabelled Nr-CAM cDNA can be synthesized using the
5 selected mRNA (from the adsorbed polysomes) as a template.
The radiolabelled mRNA or cDNA may then be used as a probe to
identify the Nr-CAM DNA fragments from among other genomic
DNA fragments.

Alternatives to isolating the Nr-CAM genomic DNA
include, but are not limited to, chemically synthesizing the
10 gene sequence itself from a known sequence or making cDNA to
the mRNA which encodes the Nr-CAM protein. For example, RNA
for cDNA cloning of the Nr-CAM gene can be isolated from
cells which express Nr-CAM. Other methods are possible and
within the scope of the invention.

15 The identified and isolated gene can then be
inserted into an appropriate cloning vector. A large number
of vector-host systems known in the art may be used.
Possible vectors include, but are not limited to, plasmids or
modified viruses, but the vector system must be compatible
20 with the host cell used. Such vectors include, but are not
limited to, bacteriophages such as lambda derivatives, or
plasmids such as PBR322 or pUC plasmid derivatives or the
Bluescript vector (Stratagene). The insertion into a cloning
vector can, for example, be accomplished by ligating the DNA
fragment into a cloning vector which has complementary
25 cohesive termini. However, if the complementary restriction
sites used to fragment the DNA are not present in the cloning
vector, the ends of the DNA molecules may be enzymatically
modified. Alternatively, any site desired may be produced by
ligating nucleotide sequences (linkers) onto the DNA termini;
30 these ligated linkers may comprise specific chemically
synthesized oligonucleotides encoding restriction
endonuclease recognition sequences. In an alternative

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method, the cleaved vector and *Nr-CAM* gene may be modified by
homopolymeric tailing. Recombinant molecules can be
introduced into host cells via transformation, transfection,
infection, electroporation, etc., so that many copies of the
gene sequence are generated.

In an alternative method, the desired gene may be
identified and isolated after insertion into a suitable
cloning vector in a "shot gun" approach. Enrichment for the
desired gene, for example, by size fractionization, can be
done before insertion into the cloning vector.

In specific embodiments, transformation of host
cells with recombinant DNA molecules that incorporate the
isolated *Nr-CAM* gene, cDNA, or synthesized DNA sequence
enables generation of multiple copies of the gene. Thus, the
gene may be obtained in large quantities by growing
transformants, isolating the recombinant DNA molecules from
the transformants and, when necessary, retrieving the
inserted gene from the isolated recombinant DNA.

The *Nr-CAM* sequences provided by the present
invention include those nucleotide sequences encoding
substantially the same amino acid sequences as found in
native Nr-CAM proteins, and those encoded amino acid
sequences with functionally equivalent amino acids, as well
as those encoding other Nr-CAM derivatives or analogs, as
described in Section 5.2.2 *infra* for Nr-CAM derivatives and
analogs.

The *Nr-CAM* sequences provided by the present
invention include those that encode Nr-CAM mutants that are
constitutively expressed.

5.2.2. EXPRESSION OF *Nr-CAM* NUCLEIC ACIDS

The nucleotide sequence coding for a Nr-CAM protein
or a functionally active analog or fragment or other
derivative thereof, can be inserted into an appropriate

expression vector, *i.e.*, a vector which contains the
necessary elements for the transcription and translation of
the inserted protein-coding sequence. The necessary
transcriptional and translational signals can also be
5 supplied by the native *Nr-CAM* gene and/or its flanking
regions. A variety of host-vector systems may be utilized to
express the protein-coding sequence. These include but are
not limited to mammalian cell systems infected with virus
(*e.g.*, vaccinia virus, adenovirus, etc.); insect cell systems
infected with virus (*e.g.*, baculovirus); microorganisms such
10
as yeast containing yeast vectors, or bacteria transformed
with bacteriophage, DNA, plasmid DNA, or cosmid DNA. The
expression elements of vectors vary in their strengths and
specificities. Depending on the host-vector system utilized,
any one of a number of suitable transcription and translation
15 elements may be used. In specific embodiments, the human *Nr-*
CAM gene is expressed, or a sequence encoding a functionally
active portion of human Nr-CAM. In yet another embodiment, a
fragment of Nr-CAM comprising a domain of the Nr-CAM protein
is expressed.

20   Any of the methods previously described for the
insertion of DNA fragments into a vector may be used to
construct expression vectors containing a chimeric gene
consisting of appropriate transcriptional/translational
control signals and the protein coding sequences. These
25 methods may include *in vitro* recombinant DNA and synthetic
techniques and *in vivo* recombinants (genetic recombination).
Expression of nucleic acid sequence encoding a Nr-CAM protein
or peptide fragment may be regulated by a second nucleic acid
sequence so that the Nr-CAM protein or peptide is expressed
in a host transformed with the recombinant DNA molecule. For
30
example, expression of a Nr-CAM protein may be controlled by
any promoter/enhancer element known in the art. Promoters
which may be used to control Nr-CAM expression include, but

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are not limited to, the SV40 early promoter region (Bernoist and Chambon, 1981, Nature 290:304-310), the promoter contained in the 3' long terminal repeat of Rous sarcoma virus (Yamamoto, et al., 1980, Cell 22:787-797), the herpes thymidine kinase promoter (Wagner et al., 1981, Proc. Natl. Acad. Sci. U.S.A. 78:1441-1445), the regulatory sequences of the metallothionein gene (Brinster et al., 1982, Nature 296:39-42); prokaryotic expression vectors such as the β-lactamase promoter (Villa-Kamaroff, et al., 1978, Proc. Natl. Acad. Sci. U.S.A. 75:3727-3731), or the tac promoter (DeBoer, et al., 1983, Proc. Natl. Acad. Sci. U.S.A. 80:21-25); see also "Useful proteins from recombinant bacteria" in Scientific American, 1980, 242:74-94; plant expression vectors comprising the nopaline synthetase promoter region (Herrera-Estrella et al., 1983, Nature 303:209-213) or the cauliflower mosaic virus 35S RNA promoter (Gardner, et al., 1981, Nucl. Acids Res. 9:2871), and the promoter of the photosynthetic enzyme ribulose biphosphate carboxylase (Herrera-Estrella et al., 1984, Nature 310:115-120); promoter elements from yeast or other fungi such as the Gal 4 promoter, the ADC (alcohol dehydrogenase) promoter, PGK (phosphoglycerol kinase) promoter, alkaline phosphatase promoter, and the following animal transcriptional control regions, which exhibit tissue specificity and have been utilized in transgenic animals: elastase I gene control region which is active in pancreatic acinar cells (Swift et al., 1984, Cell 38:639-646; Ornitz et al., 1986, Cold Spring Harbor Symp. Quant. Biol. 50:399-409; MacDonald, 1987, Hepatology 7:425-515); insulin gene control region which is active in pancreatic beta cells (Hanahan, 1985, Nature 315:115-122), immunoglobulin gene control region which is active in lymphoid cells (Grosschedl et al., 1984, Cell 38:647-658; Adames et al., 1985, Nature 318:533-538; Alexander et al., 1987, Mol. Cell. Biol. 7:1436-1444), mouse

mammary tumor virus control region which is active in testicular, breast, lymphoid and mast cells (Leder et al., 1986, Cell 45:485-495), albumin gene control region which is active in liver (Pinkert et al., 1987, Genes and Devel. 1:268-276), alpha-fetoprotein gene control region which is active in liver (Krumlauf et al., 1985, Mol. Cell. Biol. 5:1639-1648; Hammer et al., 1987, Science 235:53-58; alpha 1-antitrypsin gene control region which is active in the liver (Kelsey et al., 1987, Genes and Devel. 1:161-171), beta-globin gene control region which is active in myeloid cells (Mogram et al., 1985, Nature 315:338-340; Kollias et al., 1986, Cell 46:89-94; myelin basic protein gene control region which is active in oligodendrocyte cells in the brain (Readhead et al., 1987, Cell 48:703-712); myosin light chain-2 gene control region which is active in skeletal muscle (Sani, 1985, Nature 314:283-286), and gonadotropic releasing hormone gene control region which is active in the hypothalamus (Mason et al., 1986, Science 234:1372-1378).

In a specific embodiment, a vector is used that comprises a promoter operably linked to a Nr-CAM-encoding nucleic acid, one or more origins of replication, and, optionally, one or more selectable markers (e.g., an antibiotic resistance gene).

In a specific embodiment, an expression construct is made by subcloning a Nr-CAM coding sequence into the EcoRI restriction site of each of the three pGEX vectors (Glutathione S-Transferase expression vectors; Smith and Johnson, 1988, Gene 7:31-40). This allows for the expression of the Nr-CAM protein product from the subclone in the correct reading frame.

In another specific embodiment, the promoter that is operably linked to the hNr-CAM gene is not the native hNr-CAM gene promoter (i.e., it is a heterologous promoter).

Expression vectors containing *Nr-CAM* gene inserts
can be identified by three general approaches: (a) nucleic
acid hybridization, (b) presence or absence of "marker" gene
functions, and (c) expression of inserted sequences. In the
first approach, the presence of a *Nr-CAM* gene inserted in an
expression vector can be detected by nucleic acid
hybridization using probes comprising sequences that are
homologous to an inserted *Nr-CAM* gene. In the second
approach, the recombinant vector/host system can be
identified and selected based upon the presence or absence of
certain "marker" gene functions (*e.g.*, thymidine kinase
activity, resistance to antibiotics, transformation
phenotype, occlusion body formation in baculovirus, etc.)
caused by the insertion of a *Nr-CAM* gene in the vector. For
example, if the *Nr-CAM* gene is inserted within the marker
gene sequence of the vector, recombinants containing the Nr-
CAM insert can be identified by the absence of the marker
gene function. In the third approach, recombinant expression
vectors can be identified by assaying the Nr-CAM product
expressed by the recombinant. Such assays can be based, for
example, on the physical or functional properties of the Nr-
CAM protein in *in vitro* assay systems, *e.g.*, binding with
anti-Nr-CAM antibody, promotion of cell proliferation.

Once a particular recombinant DNA molecule is
identified and isolated, several methods known in the art may
be used to propagate it. Once a suitable host system and
growth conditions are established, recombinant expression
vectors can be propagated and prepared in quantity. As
previously explained, the expression vectors which can be
used include, but are not limited to, the following vectors
or their derivatives: human or animal viruses such as
vaccinia virus or adenovirus; insect viruses such as
baculovirus; yeast vectors; bacteriophage vectors (*e.g.*,

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lambda), and plasmid and cosmid DNA vectors, to name but a
few.

In addition, a host cell strain may be chosen which
modulates the expression of the inserted sequences, or
5 modifies and processes the gene product in the specific
fashion desired. Expression from certain promoters can be
elevated in the presence of certain inducers; thus,
expression of the genetically engineered Nr-CAM protein may
be controlled. Furthermore, different host cells have
characteristic and specific mechanisms for the translational
10 and post-translational processing and modification (e.g.,
glycosylation, phosphorylation of proteins. Appropriate cell
lines or host systems can be chosen to ensure the desired
modification and processing of the foreign protein expressed.
For example, expression in a bacterial system can be used to
15 produce an unglycosylated core protein product. Expression
in yeast will produce a glycosylated product. Expression in
mammalian cells can be used to ensure "native" glycosylation
of a heterologous protein. Furthermore, different
vector/host expression systems may effect processing
20 reactions to different extents.

In other specific embodiments, the Nr-CAM protein,
fragment, analog, or derivative may be expressed as a fusion,
or chimeric protein product (comprising the protein,
fragment, analog, or derivative joined via a peptide bond to
a heterologous protein sequence (of a different protein)).
25 Such a chimeric product can be made by ligating the
appropriate nucleic acid sequences encoding the desired amino
acid sequences to each other by methods known in the art, in
the proper coding frame, and expressing the chimeric product
by methods commonly known in the art. Alternatively, such a
30 chimeric product may be made by protein synthetic techniques,
e.g., by use of a peptide synthesizer.

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Both cDNA and genomic sequences can be cloned and
expressed.

5.2.3.   IDENTIFICATION AND PURIFICATION
OF THE *Nr-CAM* PRODUCTS

In particular aspects, the invention provides amino
acid sequences of Nr-CAM, preferably human Nr-CAM, and
fragments and derivatives thereof which comprise an antigenic
determinant (*i.e.*, can be recognized by an antibody) or which
are otherwise functionally active, as well as nucleic acid
sequences encoding the foregoing. "Functionally active" Nr-
CAM material as used herein refers to that material
displaying one or more functional activities associated with
a full-length (wild-type) Nr-CAM protein, *e.g.*, promotion of
cell proliferation, binding to a Nr-CAM substrate or Nr-CAM
binding partner, antigenicity (binding to an anti-Nr-CAM
antibody), immunogenicity, etc.

In other specific embodiments, the invention
provides fragments of a Nr-CAM protein consisting of at least
6 amino acids, 10 amino acids, 50 amino acids, or of at least
75 amino acids of SEQ. ID. No.: 2. In other embodiments, the
invention provides proteins comprising, having, or consisting
essentially of a sequence of amino acids 100% identical with
SEQ. ID. NO.: 2, SEQ. ID. NO.: 2 or a protein encoded by SEQ.
ID. NO.: 1, or any combination of the foregoing. Fragments
or proteins comprising such sequences are particularly
advantageously used for immunotherapy as described below.
Fragments, or proteins comprising fragments, lacking some or
all of the foregoing regions of a Nr-CAM protein are also
provided. Nucleic acids encoding the foregoing are provided.
In specific embodiments, the foregoing proteins or fragments
are not more than 25, 50 or 100 contiguous amino acids.

Once a recombinant which expresses the *Nr-CAM* gene
sequence is identified, the gene product can be analyzed.

This is achieved by assays based on the physical or
functional properties of the product, including radioactive
labelling of the product followed by analysis by gel
electrophoresis, immunoassay, etc.

5   Once the Nr-CAM protein is identified, it may be
isolated and/or purified by standard methods including
chromatography (e.g., ion exchange, affinity, and sizing
column chromatography), centrifugation, differential
solubility, or by any other standard technique for the
purification of proteins. The functional properties may be
10   evaluated using any suitable assay (see Section 5.3).

         Alternatively, once a Nr-CAM protein produced by a
recombinant is identified, the amino acid sequence of the
protein can be deduced from the nucleotide sequence of the
chimeric gene contained in the recombinant. As a result, the
15   protein can be synthesized by standard chemical methods known
in the art (e.g., see Hunkapiller, M., et al., 1984, Nature
310:105-111).

         In another alternate embodiment, native Nr-CAM
proteins can be purified from natural sources, by standard
20   methods such as those described above (e.g., immunoaffinity
purification).

         In a specific embodiment of the present invention,
such Nr-CAM proteins, whether produced by recombinant DNA
techniques or by chemical synthetic methods or by
purification of native proteins, include but are not limited
25   to those containing, as a primary amino acid sequence, all or
part of the amino acid sequence substantially, as well as
fragments and other derivatives, and analogs as shown in
Figure 2B (SEQ. ID. NO.: 2) thereof, including proteins
homologous thereto.
30

5.2.4. ANTIBODIES AND IMMUNE CELLS TO Nr-CAM

5.2.4.1. GENERATION OF ANTIBODIES TO Nr-CAM PROTEINS AND DERIVATIVES THEREOF

According to the invention, Nr-CAM protein, its
fragments or other derivatives, or analogs thereof, may be
used as an immunogen to generate antibodies which
immunospecifically bind such an immunogen. Such antibodies
include but are not limited to polyclonal, monoclonal,
chimeric, single chain, Fab fragments, and an Fab expression
library. In a specific embodiment, antibodies to a human Nr-
CAM protein are produced. In another embodiment, antibodies
to a domain of a Nr-CAM protein are produced. In a specific
embodiment, fragments of a Nr-CAM protein identified as
hydrophilic are used as immunogens for antibody production.

In another specific embodiment, the antibody to a
human Nr-CAM protein is a bispecific antibody (see generally,
e.g. Fanger and Drakeman, 1995, *Drug News and Perspectives* 8:
133-137). Such a bispecific antibody is genetically
engineered to recognize both (1) a human Nr-CAM epitope and
(2) one of a variety of "trigger" molecules, e.g. Fc
receptors on myeloid cells, and CD3 and CD2 on T cells, that
have been identified as being able to cause a cytotoxic T-
cell to destroy a particular target. Such bispecific
antibodies can be prepared either by chemical conjugation,
hybridoma, or recombinant molecular biology techniques known
to the skilled artisan.

Various procedures known in the art may be used for
the production of polyclonal antibodies to a Nr-CAM protein
or derivative or analog. In a particular embodiment, rabbit
polyclonal antibodies to an epitope of a Nr-CAM protein, or a
subsequence thereof, can be obtained. For the production of
antibody, various host animals can be immunized by injection
with the native Nr-CAM protein, or a synthetic version, or
derivative (e.g., fragment) thereof, including but not

limited to rabbits, mice, rats, etc. Various adjuvants may
be used to increase the immunological response, depending on
the host species, and including but not limited to Freund's
(complete and incomplete), mineral gels such as aluminum
5 hydroxide, surface active substances such as lysolecithin,
pluronic polyols, polyanions, peptides, oil emulsions,
keyhole limpet hemocyanins, dinitrophenol, and potentially
useful human adjuvants such as BCG (bacille Calmette-Guerin)
and corynebacterium parvum.

10   For preparation of monoclonal antibodies directed
toward a Nr-CAM protein sequence or analog thereof, any
technique which provides for the production of antibody
molecules by continuous cell lines in culture may be used.
For example, the hybridoma technique originally developed by
Kohler and Milstein (1975, Nature 256:495-497), as well as
15 the trioma technique, the human B-cell hybridoma technique
(Kozbor et al., 1983, Immunology Today 4:72), and the EBV-
hybridoma technique to produce human monoclonal antibodies
(Cole et al., 1985, in Monoclonal Antibodies and Cancer
Therapy, Alan R. Liss, Inc., pp. 77-96). In an additional
20 embodiment of the invention, monoclonal antibodies can be
produced in germ-free animals utilizing technology described
in PCT/US90/02545. According to the invention, human
antibodies may be used and can be obtained by using human
hybridomas (Cote et al., 1983, Proc. Natl. Acad. Sci. U.S.A.
80:2026-2030) or by transforming human B cells with EBV virus
25 in vitro (Cole et al., 1985, in Monoclonal Antibodies and
Cancer Therapy, Alan R. Liss, pp. 77-96). In fact, according
to the invention, techniques developed for the production of
"chimeric antibodies" (Morrison et al., 1984, PROC. NATL.
ACAD. SCI. U.S.A. 81:6851-6855; Neuberger et al., 1984,
30 Nature 312:604-608; Takeda et al., 1985, Nature 314:452-454)
by splicing the genes from a mouse antibody molecule specific
for Nr-CAM together with genes from a human antibody molecule

of appropriate biological activity can be used; such
antibodies are within the scope of this invention.

        According to the invention, techniques described
for the production of single chain antibodies (U.S. Patent
5 4,946,778) can be adapted to produce Nr-CAM-specific single
chain antibodies. An additional embodiment of the invention
utilizes the techniques described for the construction of Fab
expression libraries (Huse *et al.*, 1989, Science 246:1275-
1281) to allow rapid and easy identification of monoclonal
Fab fragments with the desired specificity for Nr-CAM
10 proteins, derivatives, or analogs.

        Antibody fragments which contain the idiotype of
the molecule can be generated by known techniques. For
example, such fragments include but are not limited to: the
$F(ab')_2$ fragment which can be produced by pepsin digestion of
15 the antibody molecule; the Fab' fragments which can be
generated by reducing the disulfide bridges of the $F(ab')_2$
fragment, the Fab fragments which can be generated by
treating the antibody molecule with papain and a reducing
agent, and Fv fragments.

20   In the production of antibodies, screening for the
desired antibody can be accomplished by techniques known in
the art, *e.g.* ELISA (enzyme-linked immunosorbent assay). For
example, to select antibodies which recognize a specific
domain of a Nr-CAM protein, one may assay generated
hybridomas for a product which binds to a Nr-CAM fragment
25 containing such domain. For selection of an antibody that
specifically binds a first Nr-CAM homolog but which does not
specifically bind a different Nr-CAM homolog, one can select
on the basis of positive binding to the first Nr-CAM homolog
and a lack of binding to the second Nr-CAM homolog.
30   Antibodies specific to a domain of a Nr-CAM protein
are also provided.

The foregoing antibodies can be used in methods known in the art relating to the localization and activity of the Nr-CAM protein sequences of the invention, *e.g.*, for imaging these proteins, measuring levels thereof in

5 appropriate physiological samples, in diagnostic methods, etc.

In another embodiment of the invention (see *infra*), anti-Nr-CAM antibodies and fragments thereof containing the binding domain are Therapeutics.

10

Antibodies and antigen-binding antibody fragments may also be conjugated to a heterologous protein or peptide by chemical conjugation or recombinant DNA technology. The resultant chimeric protein possesses the antigen-binding specificity of the antibody and the function of the

15 heterologous protein. For example, a polynucleotide encoding the antigen binding region of an antibody specific for the extracellular domain of Nr-CAM can be genetically fused to a coding sequence for the zeta chain of the T cell receptor. After expressing this construct in T cells, the T cells are

20 expanded *ex vivo* and infused into a brain cancer patient. T cells expressing this chimeric protein are specifically directed to tumors that express Nr-CAM as a result of the antibody binding specificity and cause tumor cell killing. Alternatively, an antibody is fused to a protein which

25 induces migration of leukocytes or has an affinity to attract other compounds to a tumor cite. A specific protein of this type is streptavidin. The binding of a streptavidin conjugated antibody to a tumor cell can be followed by the addition of a biotinylated drug, toxin or radioisotope to cause tumor specific killing.

30 Kits for use with such *in vitro* tumor localization and therapy methods containing the monoclonal antibodies (or fragments thereof) conjugated to any of the above types of

substances can be prepared. The components of the kits can
be packaged either in aqueous medium or in lyophilized form.
When the monoclonal antibodies (or fragments thereof) are
used in the kits in the form of conjugates in which a label
5 or a therapeutic moiety is attached, such as a radioactive
metal ion or a therapeutic drug moiety, the components of
such conjugates can be supplied either in fully conjugated
form, in the form of intermediates or as separate moieties to
be conjugated by the user of the kit.

10
5.2.5. Nr-CAM PROTEINS, DERIVATIVES AND ANALOGS
The invention further encompasses compositions
comprising Nr-CAM proteins, and derivatives (including but
not limited to fragments) and analogs of Nr-CAM proteins, in
particular, those derivatives which act as antagonists of Nr-
15 CAM activity. Nucleic acids encoding Nr-CAM protein
derivatives and protein analogs are also provided. In one
embodiment, the Nr-CAM proteins are encoded by the *Nr-CAM*
nucleic acids described in Section 5.2.1. *supra*. In
particular aspects, the proteins, derivatives, or analogs are
20 of Nr-CAM proteins of animals, *e.g.*, fly, frog, mouse, rat,
pig, cow, dog, monkey, human, or of plants.
The production and use of derivatives and analogs
related to Nr-CAM are within the scope of the present
invention. In a specific embodiment, the derivative or
25 analog is functionally active, *i.e.*, capable of exhibiting
one or more functional activities associated with a full-
length, wild-type Nr-CAM protein. As one example, such
derivatives or analogs which have the desired immunogenicity
or antigenicity can be used, for example, in immunoassays,
for immunization, for inhibition of Nr-CAM activity, etc.
30 Derivatives or analogs that retain, or alternatively lack or
inhibit, a desired Nr-CAM property of interest (*e.g.*, binding
to Nr-CAM binding partner, promotion of cell proliferation),

can be used as inducers, or inhibitors, respectively, of such
property and its physiological correlates. A specific
embodiment relates to a Nr-CAM fragment that can be bound by
an anti-Nr-CAM antibody. Derivatives or analogs of Nr-CAM

5 can be tested for the desired activity by procedures known in
the art, including but not limited to the assays described in
Sections 5.3 and 5.5.

In particular, Nr-CAM derivatives can be made by
altering Nr-CAM sequences by substitutions, additions or
deletions that provide for functionally equivalent molecules.

10 Due to the degeneracy of nucleotide coding sequences, other
DNA sequences which encode substantially the same amino acid
sequence as a *Nr-CAM* gene may be used in the practice of the
present invention. These include but are not limited to
nucleotide sequences comprising all or portions of *Nr-CAM*

15 genes which are altered by the substitution of different
codons that encode a functionally equivalent amino acid
residue within the sequence, thus producing a silent change.
Likewise, the Nr-CAM derivatives of the invention include,
but are not limited to, those containing, as a primary amino

20 acid sequence, all or part of the amino acid sequence of a
Nr-CAM protein including altered sequences in which
functionally equivalent amino acid residues are substituted
for residues within the sequence resulting in a silent
change. For example, one or more amino acid residues within
the sequence can be substituted by another amino acid of a

25 similar polarity which acts as a functional equivalent,
resulting in a silent alteration. Substitutes for an amino
acid within the sequence may be selected from other members
of the class to which the amino acid belongs. For example,
the nonpolar (hydrophobic) amino acids include alanine,

30 leucine, isoleucine, valine, proline, phenylalanine,
tryptophan and methionine. The polar neutral amino acids
include glycine, serine, threonine, cysteine, tyrosine,

asparagine, and glutamine. The positively charged (basic)
amino acids include arginine, lysine and histidine. The
negatively charged (acidic) amino acids include aspartic acid
and glutamic acid.

5   In a specific embodiment of the invention, proteins
consisting of or comprising a fragment of a Nr-CAM protein
consisting of at least 10 (continuous) amino acids of the Nr-
CAM protein is provided. In other embodiments, the fragment
consists of at least 20 or 50 amino acids of the Nr-CAM
protein. In specific embodiments, such fragments are not
10 larger than 35, 100 or 200 amino acids. Derivatives or
analogs of Nr-CAM include but are not limited to those
molecules comprising regions that are substantially
homologous to Nr-CAM or fragments thereof (*e.g.*, in various
embodiments, at least 60% or 70% or 80% or 90% or 95%
15 identity over an amino acid sequence of identical size or
when compared to an aligned sequence in which the alignment
is done by a computer homology program known in the art) or
whose encoding nucleic acid is capable of hybridizing to a
coding Nr-CAM sequence, under stringent, moderately
20 stringent, or nonstringent conditions. See, *supra* Section
5.2.1. for useful computer programs for sequence comparisons.

          The Nr-CAM derivatives and analogs of the invention
can be produced by various methods known in the art. The
manipulations which result in their production can occur at
the gene or protein level. For example, the cloned *Nr-CAM*
25 gene sequence can be modified by any of numerous strategies
known in the art (Sambrook *et al.*, 1989, Molecular Cloning, A
Laboratory Manual, 2d Ed., Cold Spring Harbor Laboratory
Press, Cold Spring Harbor, New York). The sequence can be
cleaved at appropriate sites with restriction
30 endonuclease(s), followed by further enzymatic modification
if desired, isolated, and ligated *in vitro*. In the
production of the gene encoding a derivative or analog of Nr-

CAM, care should be taken to ensure that the modified gene
remains within the same translational reading frame as Nr-
CAM, uninterrupted by translational stop signals, in the gene
region where the desired Nr-CAM activity is encoded.

5   Additionally, the Nr-CAM-encoding nucleic acid
sequence can be mutated *in vitro* or *in vivo*, to create and/or
destroy translation, initiation, and/or termination
sequences, or to create variations in coding regions and/or
form new restriction endonuclease sites or destroy
preexisting ones, to facilitate further *in vitro*
10 modification. Any technique for mutagenesis known in the art
can be used, including but not limited to, chemical
mutagenesis, *in vitro* site-directed mutagenesis (Hutchinson,
C., *et al.*, 1978, J. Biol. Chem 253:6551), use of TAB®
linkers (Pharmacia), etc.

15   Manipulations of the Nr-CAM sequence may also be
made at the protein level. Included within the scope of the
invention are Nr-CAM protein fragments or other derivatives
or analogs which are differentially modified during or after
translation, *e.g.*, by glycosylation, acetylation,
20 phosphorylation, amidation, derivatization by known
protecting/blocking groups, proteolytic cleavage, linkage to
an antibody molecule or other cellular ligand, etc. Any of
numerous chemical modifications may be carried out by known
techniques, including but not limited to specific chemical
25 cleavage by cyanogen bromide, trypsin, chymotrypsin, papain,
V8 protease, $NaBH_4$; acetylation, formylation, oxidation,
reduction; metabolic synthesis in the presence of
tunicamycin; etc.

          In addition, analogs and derivatives of Nr-CAM can
be chemically synthesized. For example, a peptide
30 corresponding to a portion of a Nr-CAM protein which
comprises the desired domain, or which mediates the desired
activity *in vitro*, can be synthesized by use of a peptide

synthesizer. Furthermore, if desired, nonclassical amino
acids or chemical amino acid analogs can be introduced as a
substitution or addition into the Nr-CAM sequence. Non-
classical amino acids include but are not limited to the D-
isomers of the common amino acids, α-amino isobutyric acid,
4-aminobutyric acid, Abu, 2-amino butyric acid, γ-Abu, ε-Ahx,
6-amino hexanoic acid, Aib, 2-amino isobutyric acid, 3-amino
propionic acid, ornithine, norleucine, norvaline,
hydroxyproline, sarcosine, citrulline, cysteic acid, t-
butylglycine, t-butylalanine, phenylglycine,
cyclohexylalanine, β-alanine, fluoro-amino acids, designer
amino acids such as β-methyl amino acids, Cα-methyl amino
acids, Nα-methyl amino acids, and amino acid analogs in
general. Furthermore, the amino acid can be D (dextrorotary)
or L (levorotary).

In a specific embodiment, the Nr-CAM derivative is
a chimeric, or fusion, protein comprising a Nr-CAM protein or
fragment thereof (preferably consisting of at least a domain
or motif of the Nr-CAM protein, or at least 10 amino acids of
the Nr-CAM protein) joined at its amino- or carboxy-terminus
via a peptide bond to an amino acid sequence of a different
protein. In one embodiment, such a chimeric protein is
produced by recombinant expression of a nucleic acid encoding
the protein (comprising a Nr-CAM-coding sequence joined in-
frame to a coding sequence for a different protein). Such a
chimeric product can be made by ligating the appropriate
nucleic acid sequences encoding the desired amino acid
sequences to each other by methods known in the art, in the
proper coding frame, and expressing the chimeric product by
methods commonly known in the art. Alternatively, such a
chimeric product may be made by protein synthetic techniques,
e.g., by use of a peptide synthesizer. Chimeric genes
comprising portions of Nr-CAM fused to any heterologous
protein-encoding sequences may be constructed. A specific

embodiment relates to a chimeric protein comprising a
fragment of Nr-CAM of at least six amino acids.

In another specific embodiment, the Nr-CAM
derivative is a molecule comprising a region of homology with
5 a Nr-CAM protein. By way of example, in various embodiments,
a first protein region can be considered "homologous" to a
second protein region when the amino acid sequence of the
first region is at least 30%, 40%, 50%, 60%, 70%, 75%, 80%,
90%, or 95% identical, when compared to any sequence in the
second region of an equal number of amino acids as the number
10 contained in the first region or when compared to an aligned
sequence of the second region that has been aligned by a
computer homology program known in the art. For example, a
molecule can comprise one or more regions homologous to a Nr-
CAM domain or a portion thereof.
15 Other specific embodiments of derivatives and
analogs are described in the subsections below and examples
sections *infra*.

5.3. ASSAYS OF Nr-CAM PROTEINS, DERIVATIVES AND ANALOGS

20 The functional activity of Nr-CAM proteins,
derivatives and analogs can be assayed by various methods.

For example, in one embodiment, where one is
assaying for the ability to bind or compete with wild-type
Nr-CAM for binding to anti-Nr-CAM antibody, various
25 immunoassays known in the art can be used, including but not
limited to competitive and non-competitive assay systems
using techniques such as radioimmunoassays, ELISA (enzyme
linked immunosorbent assay), "sandwich" immunoassays,
immunoradiometric assays, gel diffusion precipitin reactions,
30 immunodiffusion assays, *in situ* immunoassays (using colloidal
gold, enzyme or radioisotope labels, for example), western
blots, precipitation reactions, agglutination assays (*e.g.*,

gel agglutination assays, hemagglutination assays),
complement fixation assays, immunofluorescence assays,
protein A assays, and immunoelectrophoresis assays, etc. In
one embodiment, antibody binding is detected by detecting a
5 label on the primary antibody. In another embodiment, the
primary antibody is detected by detecting binding of a
secondary antibody or reagent to the primary antibody. In a
further embodiment, the secondary antibody is labelled. Many
means are known in the art for detecting binding in an
immunoassay and are within the scope of the present
10 invention.

In another embodiment, where a Nr-CAM-binding
protein is identified, the binding can be assayed, e.g., by
means well-known in the art. In another embodiment,
physiological correlates of Nr-CAM binding to its substrates
15 (signal transduction) can be assayed.

In addition, assays that can be used to detect or
measure the ability to inhibit, or alternatively promote,
cell proliferation are described in Section 5.4.

Other methods will be known to the skilled artisan
20 and are within the scope of the invention.

5.4. DIAGNOSIS AND SCREENING

Nr-CAM proteins, analogs, derivatives, and
subsequences thereof, Nr-CAM nucleic acids (and sequences
complementary thereto), anti-Nr-CAM antibodies, have uses in
25
diagnostics. Such molecules can be used in assays, such as
immunoassays, to detect, prognose, diagnose, or monitor
various conditions, diseases, and disorders affecting Nr-CAM
expression, or monitor the treatment thereof. In particular,
such an immunoassay is carried out by a method comprising
30 contacting a sample derived from a patient with an anti-Nr-
CAM antibody under conditions such that immunospecific
binding can occur, and detecting or measuring the amount of

any immunospecific binding by the antibody. In a specific
aspect, such binding of antibody, in tissue sections, can be
used to detect aberrant Nr-CAM localization or aberrant
(e.g., high, low or absent) levels of Nr-CAM. In a specific
5 embodiment, antibody to Nr-CAM can be used to assay in a
patient tissue or serum sample for the presence of Nr-CAM
where an aberrant level of Nr-CAM is an indication of a
diseased condition. By "aberrant levels," is meant increased
or decreased levels relative to that present, or a standard
level representing that present, in an analogous sample from
10 a portion of the body or from a subject not having the
disorder. In a specific embodiment, antibody to Nr-CAM can
be used to assay and screen tissues or bodily fluids
including but not limited to spinal fluid and brain tissue
for elevated levels of Nr-CAM expression indicative of a
15 tumor.

        The immunoassays which can be used include but are
not limited to competitive and non-competitive assay systems
using techniques such as western blots, radioimmunoassays,
ELISA (enzyme linked immunosorbent assay), "sandwich"
20 immunoassays, immunoprecipitation assays, precipitin
reactions, gel diffusion precipitin reactions,
immunodiffusion assays, agglutination assays, complement-
fixation assays, immunoradiometric assays, fluorescent
immunoassays, protein A immunoassays, to name but a few.

        Nr-CAM genes and related nucleic acid sequences and
25 subsequences, including complementary sequences, can also be
used in hybridization assays. Nr-CAM nucleic acid sequences,
or subsequences thereof comprising about at least 8
nucleotides, can be used as hybridization probes.
Hybridization assays can be used to detect, prognose,
30 diagnose, or monitor conditions, disorders, or disease states
associated with aberrant changes in Nr-CAM expression and/or
activity as described supra. In particular, such a

hybridization assay is carried out by a method comprising
contacting a sample containing nucleic acid with a nucleic
acid probe capable of hybridizing to Nr-CAM DNA or RNA, under
conditions such that hybridization can occur, and detecting
5 or measuring any resulting hybridization.

In specific embodiments, diseases and disorders
involving overproliferation of cells can be diagnosed, or
their suspected presence can be screened for, or a
predisposition to develop such disorders can be detected, by
detecting increased levels of Nr-CAM protein, Nr-CAM RNA, or
10 Nr-CAM functional activity or by detecting mutations in Nr-
CAM RNA, DNA or protein (e.g., translocations in Nr-CAM
nucleic acids, truncations in the Nr-CAM gene or protein,
changes in nucleotide or amino acid sequence relative to
wild-type Nr-CAM) that cause increased expression or activity
15 of Nr-CAM. Such diseases and disorders include but are not
limited to those tumors or tissue types mentioned in Section
6 in which Nr-CAM is overexpressed. By way of example,
levels of Nr-CAM protein can be detected by immunoassay,
levels of Nr-CAM RNA can be detected by hybridization assays
20 (e.g., Northern blots, dot blots), translocations and point
mutations in Nr-CAM nucleic acids can be detected by Southern
blotting, RFLP analysis, PCR using primers that preferably
generate a fragment spanning at least most of the Nr-CAM
gene, sequencing of the Nr-CAM genomic DNA or cDNA obtained
25 from the patient, etc.

In a preferred embodiment, levels of Nr-CAM mRNA or
protein in a patient sample are detected or measured, in
which increased levels indicate that the subject has, or has
a predisposition to developing, a malignancy or
hyperproliferative disorder; in which the increased levels
30 are relative to the levels present in an analogous sample
from a portion of the body or from a subject not having the

malignancy or hyperproliferative disorder, as the case may be.

In another specific embodiment, diseases and disorders involving a deficiency in cell proliferation or in which cell proliferation is desirable for treatment, are diagnosed, or their suspected presence can be screened for, or a predisposition to develop such disorders can be detected, by detecting decreased levels of Nr-CAM protein, Nr-CAM RNA, or Nr-CAM functional activity, or by detecting mutations in Nr-CAM RNA, DNA or protein (e.g., translocations in *Nr-CAM* nucleic acids, truncations in the gene or protein, changes in nucleotide or amino acid sequence relative to wild-type Nr-CAM) that cause decreased expression or activity of Nr-CAM. Such diseases and disorders include but are not limited to those tumors and tissue types mentioned in Section 6 and its subsections in which Nr-CAM is overexpressed. By way of example, levels of Nr-CAM protein, levels of Nr-CAM RNA, Nr-CAM binding activity, and the presence of translocations or point mutations can be determined as described above.

In a specific embodiment, levels of Nr-CAM mRNA or protein in a patient sample are detected or measured, in which decreased levels indicate that the subject has, or has a predisposition to developing, a malignancy or hyperproliferative disorder; in which the decreased levels are relative to the levels present in an analogous sample from a portion of the body or from a subject not having the malignancy or hyperproliferative disorder, as the case may be.

Kits for diagnostic use are also provided, that comprise, in one or more containers, an anti-Nr-CAM antibody, and, optionally, a labeled binding partner to the antibody. Alternatively, the anti-Nr-CAM antibody can be labeled (with a detectable marker, e.g., a chemiluminescent, enzymatic,

fluorescent, or radioactive moiety). A kit is also provided
that comprises, in one or more containers, a nucleic acid
probe capable of hybridizing to Nr-CAM RNA. In a specific
embodiment, a kit can comprise in one or more containers a

5 pair of primers (e.g., each in the size range of 6-30
nucleotides) that are capable of priming amplification [e.g.,
by polymerase chain reaction (see e.g., Innis et al., 1990,
PCR Protocols, Academic Press, Inc., San Diego, CA), ligase
chain reaction (see EP 320,308) use of Qβ replicase, cyclic

10 probe reaction, or other methods known in the art] under
appropriate reaction conditions of at least a portion of a
Nr-CAM nucleic acid. A kit can optionally further comprise,
in a container, a predetermined amount of a purified Nr-CAM
protein or nucleic acid, e.g., for use as a standard or
control.

15

5.5. **THERAPEUTIC USES**

          The invention provides for treatment, inhibition or
prevention of various diseases and disorders by
administration of a therapeutic compound (termed herein

20 "Therapeutic"). Such "Therapeutics" include but are not
limited to: Nr-CAM proteins and analogs and derivatives
(including fragments) thereof (e.g., as described
hereinabove); antibodies thereto (as described hereinabove);
nucleic acids encoding the Nr-CAM proteins, analogs, or

25 derivatives (e.g., as described hereinabove); Nr-CAM
antisense nucleic acids, and Nr-CAM agonists and antagonists.
Disorders involving tumorigenesis or cell overproliferation
are treated or prevented by administration of a Therapeutic
that antagonizes Nr-CAM function. Disorders in which cell
proliferation is deficient or is desired are treated or

30 prevented by administration of a Therapeutic that promotes
Nr-CAM function. See details in the subsections below.

Generally, it is preferred to administer a product
of a species origin or species reactivity (in the case of
antibodies) that is the same as that of the recipient. Thus,
in a preferred embodiment, a human Nr-CAM protein,

5 derivative, or analog, or nucleic acid, or an antibody to a
human Nr-CAM protein, is therapeutically or prophylactically
administered to a human patient.

Additional descriptions and sources of Therapeutics
that can be used according to the invention are found in
Sections 5.1 through 5.7 herein.

10

5.5.1.   TREATMENT, INHIBITION AND PREVENTION OF DISORDERS INVOLVING OVERPROLIFERATION OF CELLS

Diseases and disorders involving cell
overproliferation are treated, inhibited or prevented by

15

administration of a Therapeutic that antagonizes (*i.e.*,
inhibits) Nr-CAM function. Examples of such a Therapeutic
include but are not limited to Nr-CAM antibodies, Nr-CAM
antisense nucleic acids, derivatives, or analogs that are
functionally active, particularly that are active in

20 inhibiting cell proliferation (*e.g.*, as demonstrated in *in
vitro* assays or in animal models or in *Drosophila*). Other
Therapeutics that can be used, *e.g.*, Nr-CAM antagonists, can
be identified using *in vitro* assays or animal models,
examples of which are described *infra*.

25   In specific embodiments, Therapeutics that inhibit
Nr-CAM function are administered therapeutically (including
prophylactically): (1) in diseases or disorders involving an
increased (relative to normal or desired) level of Nr-CAM
protein or function, for example, in patients where Nr-CAM

30 protein is overexpressed, genetically defective, or
biologically hyperactive; or (2) in diseases or disorders
wherein *in vitro* (or *in vivo*) assays (see *infra*) indicate the

utility of Nr-CAM antagonist administration. The increased
level in Nr-CAM protein or function can be readily detected,
e.g., by obtaining a patient tissue sample (e.g., from biopsy
tissue) and assaying it in vitro for RNA or protein levels,
5 structure and/or activity of the expressed Nr-CAM RNA or
protein. Many methods standard in the art can be thus
employed, including but not limited to immunoassays to detect
and/or visualize Nr-CAM protein (e.g., Western blot,
immunoprecipitation followed by sodium dodecyl sulfate
10 polyacrylamide gel electrophoresis, immunocytochemistry,
etc.) and/or hybridization assays to detect Nr-CAM expression
by detecting and/or visualizing Nr-CAM mRNA (e.g., Northern
assays, dot blots, in situ hybridization, etc.), etc.

    Diseases and disorders involving cell
overproliferation that can be treated, inhibited or prevented
15 include but are not limited to malignancies, premalignant
conditions (e.g., hyperplasia, metaplasia, dysplasia), benign
tumors, hyperproliferative disorders, benign dysproliferative
disorders, etc. Examples of these are detailed below.

20   5.5.1.1. **MALIGNANCIES**

    Malignancies and related disorders that can be
treated or prevented by administration of a Therapeutic that
inhibits Nr-CAM function include but are not limited to those
listed in Table 1 (for a review of such disorders, see
25 Fishman et al., 1985, Medicine, 2d Ed., J.B. Lippincott Co.,
Philadelphia).

30

TABLE 1
MALIGNANCIES AND RELATED DISORDERS

Leukemia
    acute leukemia
        acute lymphocytic leukemia
        acute lymphoblastic leukemia
        acute myelocytic leukemia
            myeloblastic
            myelogenous
            promyelocytic
            myelomonocytic
            monocytic
            erythroleukemia
    chronic leukemia
        chronic myelocytic (granulocytic) leukemia
        chronic myelogenous leukemia
        chronic lymphocytic leukemia
Polycythemia vera
Lymphoma
    Hodgkin's disease
    non-Hodgkin's disease
Multiple myeloma
Waldenström's macroglobulinemia
Heavy chain disease
Solid tumors
    sarcomas and carcinomas
        adenocarcinoma
        fibrosarcoma
        myxosarcoma
        liposarcoma
        chondrosarcoma
        osteogenic sarcoma
        chordoma
        angiosarcoma
        endotheliosarcoma
        lymphangiosarcoma
        lymphangioendotheliosarcoma
        synovioma
        mesothelioma
        Ewing's tumor
        leiomyosarcoma
        rhabdomyosarcoma
        colon carcinoma
        colorectal adenocarcinoma
        colon tumor metastatic to brain
        lung carcinoma
        pancreatic cancer
        breast cancer

ovarian cancer
prostate cancer
squamous cell carcinoma
basal cell carcinoma
adenocarcinoma
sweat gland carcinoma
sebaceous gland carcinoma
papillary carcinoma
papillary adenocarcinomas
cystadenocarcinoma
medullary carcinoma
bronchogenic carcinoma
renal cell carcinoma
hepatoma
bile duct carcinoma
choriocarcinoma
seminoma
embryonal carcinoma
Wilms' tumor
cervical cancer
uterine cancer
testicular tumor
lung carcinoma
small cell lung carcinoma
bladder carcinoma
epithelial carcinoma
glioblastoma
glioma
astrocytoma
medulloblastoma
craniopharyngioma
ependymoma
pinealoma
hemangioblastoma
acoustic neuroma
oligodendroglioma
meningioma
melanoma
neuroblastoma
retinoblastoma

In specific embodiments, malignancy or
dysproliferative changes (such as metaplasias and
dysplasias), or hyperproliferative disorders, are treated,
inhibited or prevented in the brain. In other specific

embodiments, carcinoma, melanoma, or leukemia is treated,
inhibited or prevented.

5.5.1.2. PREMALIGNANT CONDITIONS

5   The Therapeutics of the invention that antagonize
Nr-CAM activity can also be administered to treat, inhibit
premalignant conditions and to inhibit or prevent progression
to a neoplastic or malignant state, including but not limited
to those disorders listed in Table 1. Such prophylactic or
therapeutic use is indicated in conditions known or suspected
10   of preceding progression to neoplasia or cancer, in
particular, where non-neoplastic cell growth consisting of
hyperplasia, metaplasia, or most particularly, dysplasia has
occurred (for review of such abnormal growth conditions, see
Robbins and Angell, 1976, Basic Pathology, 2d Ed., W.B.
15 Saunders Co., Philadelphia, pp. 68-79.) Hyperplasia is a
form of controlled cell proliferation involving an increase
in cell number in a tissue or organ, without significant
alteration in structure or function. As but one example,
endometrial hyperplasia often precedes endometrial cancer.
20 Metaplasia is a form of controlled cell growth in which one
type of adult or fully differentiated cell substitutes for
another type of adult cell. Metaplasia can occur in
epithelial or connective tissue cells. Atypical metaplasia
involves a somewhat disorderly metaplastic epithelium.
25 Dysplasia is frequently a forerunner of cancer, and is found
mainly in the epithelia; it is the most disorderly form of
non-neoplastic cell growth, involving a loss in individual
cell uniformity and in the architectural orientation of
cells. Dysplastic cells often have abnormally large, deeply
stained nuclei, and exhibit pleomorphism. Dysplasia
30 characteristically occurs where there exists chronic
irritation or inflammation, and is often found in the cervix,
respiratory passages, oral cavity, and gall bladder.

        Alternatively or in addition to the presence of
abnormal cell growth characterized as hyperplasia,
metaplasia, or dysplasia, the presence of one or more
characteristics of a transformed phenotype, or of a malignant
5 phenotype, displayed *in vivo* or displayed *in vitro* by a cell
sample from a patient, can indicate the desirability of
prophylactic/therapeutic administration of a Therapeutic that
inhibits Nr-CAM function. As mentioned *supra*, such
characteristics of a transformed phenotype include morphology
   changes, looser substratum attachment, loss of contact
10
   inhibition, loss of anchorage dependence, protease release,
increased sugar transport, decreased serum requirement,
expression of fetal antigens, disappearance of the 250,000
dalton cell surface protein, etc. (see also *id.*, at pp. 84-90
for characteristics associated with a transformed or
15 malignant phenotype).

        In a specific embodiment, leukoplakia, a benign-
appearing hyperplastic or dysplastic lesion of the
epithelium, or Bowen's disease, a carcinoma *in situ*, are pre-
neoplastic lesions indicative of the desirability of
20 prophylactic intervention.

        In another embodiment, fibrocystic disease (cystic
hyperplasia, mammary dysplasia, particularly adenosis (benign
epithelial hyperplasia)) is indicative of the desirability of
prophylactic intervention.

        In other embodiments, a patient which exhibits one
25
   or more of the following predisposing factors for malignancy
is treated by administration of an effective amount of a
Therapeutic: a chromosomal translocation associated with a
malignancy (*e.g.*, the Philadelphia chromosome for chronic
myelogenous leukemia, t(14;18) for follicular lymphoma,
30 etc.), familial polyposis or Gardner's syndrome (possible
forerunners of colon cancer), benign monoclonal gammopathy (a
possible forerunner of multiple myeloma), and a first degree

kinship with persons having a cancer or precancerous disease
showing a Mendelian (genetic) inheritance pattern (*e.g.*,
familial polyposis of the colon, Gardner's syndrome,
hereditary exostosis, polyendocrine adenomatosis, medullary
thyroid carcinoma with amyloid production and
pheochromocytoma, Peutz-Jeghers syndrome, neurofibromatosis
of Von Recklinghausen, retinoblastoma, carotid body tumor,
cutaneous melanocarcinoma, intraocular melanocarcinoma,
xeroderma pigmentosum, ataxia telangiectasia, Chediak-Higashi
syndrome, albinism, Fanconi's aplastic anemia, and Bloom's
syndrome; *see* Robbins and Angell, 1976, Basic Pathology, 2d
Ed., W.B. Saunders Co., Philadelphia, pp. 112-113) etc.)

In another specific embodiment, a Therapeutic of
the invention is administered to a human patient to prevent
progression to brain, breast, colon, prostate, lung, or skin.
In other specific embodiments, carcinoma, melanoma, or
leukemia is treated or prevented.

5.5.1.3.   GENE THERAPY

In a specific embodiment, anti-sense nucleic acids
complementary to a sequence encoding a Nr-CAM protein or
functional derivative thereof, are administered to inhibit
Nr-CAM function, by way of gene therapy. Gene therapy refers
to therapy performed by the administration of a nucleic acid
to a subject. In this embodiment of the invention, the
antisense nucleic acid mediates a therapeutic effect by
inhibiting Nr-CAM transcription and translation.

Any of the methods for gene therapy available in
the art can be used according to the present invention.
Exemplary methods are described below.

For general reviews of the methods of gene therapy,
see Goldspiel *et al.*, 1993, Clinical Pharmacy 12:488-505; Wu
and Wu, 1991, Biotherapy 3:87-95; Tolstoshev, 1993, Ann. Rev.
Pharmacol. Toxicol. 32:573-596; Mulligan, 1993, Science

260:926-932; and Morgan and Anderson, 1993, Ann. Rev.
Biochem. 62:191-217; May, 1993, TIBTECH 11(5):155-215).
Methods commonly known in the art of recombinant DNA
technology which can be used are described in Ausubel *et al.*,
5 (eds.), 1993, Current Protocols in Molecular Biology, John
Wiley & Sons, NY; and Kriegler, 1990, Gene Transfer and
Expression, A Laboratory Manual, Stockton Press, NY.

In one embodiment, the Therapeutic comprises an Nr-
CAM sense or antisense nucleic acid that is part of an
expression vector that expresses a Nr-CAM protein or fragment
10 or chimeric protein thereof in a suitable host. In
particular, such a nucleic acid has a promoter operably
linked to the Nr-CAM coding region, said promoter being
inducible or constitutive, and, optionally, tissue-specific.
In another particular embodiment, a nucleic acid molecule is
15 used in which the Nr-CAM coding sequences and any other
desired sequences are flanked by regions that promote
homologous recombination at a desired site in the genome,
thus providing for intrachromosomal expression of the *Nr-CAM*
nucleic acid (Koller and Smithies, 1989, Proc. Natl. Acad.
20 Sci. USA 86:8932-8935; Zijlstra *et al.*, 1989, Nature 342:435-
438).

Delivery of the nucleic acid into a patient may be
either direct, in which case the patient is directly exposed
to the nucleic acid or nucleic acid-carrying vector, or
indirect, in which case, cells are first transformed with the
25 nucleic acid *in vitro*, then transplanted into the patient.
These two approaches are known, respectively, as *in vivo* or
ex vivo gene therapy.

In a specific embodiment, the nucleic acid is
directly administered *in vivo*, where it is expressed to
30 produce the encoded product. This can be accomplished by any
of numerous methods known in the art, *e.g.*, by constructing
it as part of an appropriate nucleic acid expression vector

and administering it so that it becomes intracellular, *e.g.*,
by infection using a defective or attenuated retroviral or
other viral vector (see U.S. Patent No. 4,980,286), or by
direct injection of naked DNA, or by use of microparticle
bombardment (*e.g.*, a gene gun; Biolistic, Dupont), or coating
with lipids or cell-surface receptors or transfecting agents,
encapsulation in liposomes, microparticles, or microcapsules,
or by administering it in linkage to a peptide which is known
to enter the nucleus, by administering it in linkage to a
ligand subject to receptor-mediated endocytosis (see *e.g.*, Wu
and Wu, 1987, *J. Biol. Chem.* 262:4429-4432) (which can be
used to target cell types specifically expressing the
receptors), etc. In another embodiment, a nucleic acid-
ligand complex can be formed in which the ligand comprises a
fusogenic viral peptide to disrupt endosomes, allowing the
nucleic acid to avoid lysosomal degradation. In yet another
embodiment, the nucleic acid can be targeted *in vivo* for cell
specific uptake and expression, by targeting a specific
receptor (see, *e.g.*, PCT Publications WO 92/06180 dated April
16, 1992 (Wu *et al.*); WO 92/22635 dated December 23, 1992
(Wilson *et al.*); WO92/20316 dated November 26, 1992 (Findeis
et al.); WO93/14188 dated July 22, 1993 (Clarke *et al.*), WO
93/20221 dated October 14, 1993 (Young)). Alternatively, the
nucleic acid can be introduced intracellularly and
incorporated within host cell DNA for expression, by
homologous recombination (Koller and Smithies, 1989, Proc.
Natl. Acad. Sci. USA 86:8932-8935; Zijlstra *et al.*, 1989,
Nature 342:435-438).

In a specific embodiment, a viral vector that
contains the *Nr-CAM* nucleic acid is used. For example, a
retroviral vector can be used (see Miller *et al.*, 1993, Meth.
Enzymol. 217:581-599; Kondo, *et al.*, 1998, *Cancer Res*,
68:962-967; Boviatsis, *et al.*, 1994, *Human Gene Therapy*,
5:183-191. These retroviral vectors have been modified to

delete retroviral sequences that are not necessary for
packaging of the viral genome and integration into host cell
DNA. The *Nr-CAM* nucleic acid to be used in gene therapy is
cloned into the vector, which facilitates delivery of the
gene into a patient. More detail about retroviral vectors
can be found in Boesen *et al.*, 1994, Biotherapy 6:291-302,
which describes the use of a retroviral vector to deliver the
mdr1 gene to hematopoietic stem cells in order to make the
stem cells more resistant to chemotherapy. Other references
illustrating the use of retroviral vectors in gene therapy
are: Clowes *et al.*, 1994, J. Clin. Invest. 93:644-651; Kiem
et al., 1994, Blood 83:1467-1473; Salmons and Gunzberg, 1993,
Human Gene Therapy 4:129-141; and Grossman and Wilson, 1993,
Curr. Opin. in Genetics and Devel. 3:110-114.

Adenoviruses are other viral vectors that can be
used in gene therapy. Adenoviruses are especially attractive
vehicles for delivering genes to respiratory epithelia.
Adenoviruses naturally infect respiratory epithelia where
they cause a mild disease. Other targets for adenovirus-
based delivery systems are liver, the central nervous system,
endothelial cells, and muscle. Adenoviruses have the
advantage of being capable of infecting non-dividing cells.
Kozarsky and Wilson, 1993, Current Opinion in Genetics and
Development 3:499-503 present a review of adenovirus-based
gene therapy. Bout *et al.*, 1994, Human Gene Therapy 5:3-10
demonstrated the use of adenovirus vectors to transfer genes
to the respiratory epithelia of rhesus monkeys. Other
instances of the use of adenoviruses in gene therapy can be
found in Rosenfeld *et al.*, 1991, Science 252:431-434;
Rosenfeld *et al.*, 1992, Cell 68:143-155; and Mastrangeli *et
al.*, 1993, J. Clin. Invest. 91:225-234.

Adeno-associated virus (AAV) has also been proposed
for use in gene therapy (Walsh *et al.*, 1993, Proc. Soc. Exp.
Biol. Med. 204:289-300.

Another approach to gene therapy involves
transferring a gene to cells in tissue culture by such
methods as electroporation, lipofection, calcium phosphate
mediated transfection, or viral infection. Usually, the
method of transfer includes the transfer of a selectable
marker to the cells. The cells are then placed under
selection to isolate those cells that have taken up and are
expressing the transferred gene. Those cells are then
delivered to a patient.

In this embodiment, the nucleic acid is introduced
into a cell prior to administration *in vivo* of the resulting
recombinant cell. Such introduction can be carried out by
any method known in the art, including but not limited to
transfection, electroporation, microinjection, infection with
a viral or bacteriophage vector containing the nucleic acid
sequences, cell fusion, chromosome-mediated gene transfer,
microcell-mediated gene transfer, spheroplast fusion, etc.
Numerous techniques are known in the art for the introduction
of foreign genes into cells (see *e.g.*, Loeffler and Behr,
1993, Meth. Enzymol. 217:599-618; Cohen *et al.*, 1993, Meth.
Enzymol. 217:618-644; Cline, 1985, Pharmac. Ther. 29:69-92)
and may be used in accordance with the present invention,
provided that the necessary developmental and physiological
functions of the recipient cells are not disrupted. The
technique should provide for the stable transfer of the
nucleic acid to the cell, so that the nucleic acid is
expressible by the cell and preferably heritable and
expressible by its cell progeny.

The resulting recombinant cells can be delivered to
a patient by various methods known in the art. In a
preferred embodiment, epithelial cells are injected, *e.g.*,
subcutaneously. In another embodiment, recombinant skin
cells may be applied as a skin graft onto the patient.
Recombinant blood cells (*e.g.*, hematopoietic stem or

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progenitor cells) are preferably administered intravenously.
The amount of cells envisioned for use depends on the desired
effect, patient state, etc., and can be determined by one
skilled in the art.

5   Cells into which a nucleic acid can be introduced
for purposes of gene therapy encompass any desired, available
cell type, and include but are not limited to epithelial
cells, endothelial cells, keratinocytes, fibroblasts, muscle
cells, hepatocytes; blood cells such as T lymphocytes,
B lymphocytes, monocytes, macrophages, neutrophils,
10 eosinophils, megakaryocytes, granulocytes; various stem or
progenitor cells, in particular hematopoietic stem or
progenitor cells, e.g., as obtained from bone marrow,
umbilical cord blood, peripheral blood, fetal liver, etc.

          In a preferred embodiment, the cell used for gene
15 therapy is autologous to the patient.

          In an embodiment in which recombinant cells are
used in gene therapy, a *Nr-CAM* nucleic acid is introduced
into the cells such that it is expressible by the cells or
their progeny, and the recombinant cells are then
20 administered *in vivo* for therapeutic effect. In a specific
embodiment, stem or progenitor cells are used. Any stem
and/or progenitor cells which can be isolated and maintained
in vitro can potentially be used in accordance with this
embodiment of the present invention. Such stem cells include
but are not limited to hematopoietic stem cells (HSC), stem
25 cells of epithelial tissues such as the skin and the lining
of the gut, embryonic heart muscle cells, liver stem cells
(PCT Publication WO 94/08598, dated April 28, 1994), and
neural stem cells (Stemple and Anderson, 1992, Cell 71:973-
985).
30   Epithelial stem cells (ESCs) or keratinocytes can
be obtained from tissues such as the skin and the lining of
the gut by known procedures (Rheinwald, 1980, Meth. Cell Bio.

<u>21A</u>:229). In stratified epithelial tissue such as the skin, renewal occurs by mitosis of stem cells within the germinal layer, the layer closest to the basal lamina. Stem cells within the lining of the gut provide for a rapid renewal rate

5 of this tissue. ESCs or keratinocytes obtained from the skin or lining of the gut of a patient or donor can be grown in tissue culture (Rheinwald, 1980, Meth. Cell Bio. <u>21A</u>:229; Pittelkow and Scott, 1986, Mayo Clinic Proc. <u>61</u>:771). If the ESCs are provided by a donor, a method for suppression of host versus graft reactivity (*e.g.*, irradiation, drug or

10 antibody administration to promote moderate immunosuppression) can also be used.

With respect to hematopoietic stem cells (HSC), any technique which provides for the isolation, propagation, and maintenance *in vitro* of HSC can be used in this embodiment of

15 the invention. Techniques by which this may be accomplished include (a) the isolation and establishment of HSC cultures from bone marrow cells isolated from the future host, or a donor, or (b) the use of previously established long-term HSC cultures, which may be allogeneic or xenogeneic. Non-

20 autologous HSC are used preferably in conjunction with a method of suppressing transplantation immune reactions of the future host/patient. In a particular embodiment of the present invention, human bone marrow cells can be obtained from the posterior iliac crest by needle aspiration (see,

25 *e.g.*, Kodo *et al.*, 1984, J. Clin. Invest. <u>73</u>:1377-1384). In a preferred embodiment of the present invention, the HSCs can be made highly enriched or in substantially pure form. This enrichment can be accomplished before, during, or after long-term culturing, and can be done by any techniques known in the art. Long-term cultures of bone marrow cells can be

30 established and maintained by using, for example, modified Dexter cell culture techniques (Dexter *et al.*, 1977, J. Cell

Physiol. 91:335) or Witlock-Witte culture techniques (Witlock
and Witte, 1982, Proc. Natl. Acad. Sci. USA 79:3608-3612).

In a specific embodiment, the nucleic acid to be
introduced for purposes of gene therapy comprises an
5 inducible promoter operably linked to the coding region, such
that expression of the nucleic acid is controllable by
controlling the presence or absence of the appropriate
inducer of transcription.

Additional methods that can be adapted for use to
deliver a nucleic acid encoding a Nr-CAM protein or
10 functional derivative thereof are described in Section
5.5.2.2.2., infra.

5.5.2.   TREATMENT, INHIBITION AND PREVENTION OF HYPERPROLIFERATIVE AND DYSPROLIFERATIVE DISORDERS
15

Diseases and disorders involving an increase in
cell proliferation (growth) or in which cell proliferation is
otherwise undesirable, are treated, inhibited or prevented by
administration of a Therapeutic that antagonizes (inhibits)
20 Nr-CAM function. Therapeutics that can be used include but
are not limited to anti-Nr-CAM antibodies (and fragments and
derivatives thereof containing the binding region thereof),
Nr-CAM antisense nucleic acids, and Nr-CAM nucleic acids that
are dysfunctional (e.g., due to a heterologous (non-Nr-CAM
25 sequence) insertion within the Nr-CAM coding sequence) that
are used to "knockout" endogenous Nr-CAM function by
homologous recombination (see, e.g., Capecchi, 1989, Science
244:1288-1292). In a specific embodiment of the invention, a
nucleic acid containing a portion of a Nr-CAM gene in which
Nr-CAM sequences flank (are both 5' and 3' to) a different
30 gene sequence, is used, as a Nr-CAM antagonist, to promote
Nr-CAM inactivation by homologous recombination (see also
Koller and Smithies, 1989, Proc. Natl. Acad. Sci. USA

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<u>86</u>:8932-8935; Zijlstra *et al.*, 1989, Nature <u>342</u>:435-438).
Other Therapeutics that inhibit Nr-CAM function can be
identified by use of known convenient *in vitro* assays, *e.g.*,
based on their ability to inhibit binding of Nr-CAM to

5 another protein or inhibit any known Nr-CAM function, as
preferably assayed *in vitro* or in cell culture, although
genetic assays in *Drosophila* or another species may also be
employed. Preferably, suitable *in vitro* or *in vivo* assays,
are utilized to determine the effect of a specific

10 Therapeutic and whether its administration is indicated for
treatment of the affected tissue.

In specific embodiments, Therapeutics that inhibit
Nr-CAM function are administered therapeutically (including
prophylactically): (1) in diseases or disorders involving an
increased (relative to normal or desired) level of Nr-CAM

15 protein or function, for example, in patients where Nr-CAM
protein is overactive or overexpressed; or (2) in diseases or
disorders wherein *in vitro* (or *in vivo*) assays (see *infra*)
indicate the utility of Nr-CAM antagonist administration.
The increased levels in Nr-CAM protein or function can be

20 readily detected, *e.g.*, by quantifying protein and/or RNA, by
obtaining a patient tissue sample (*e.g.*, from biopsy tissue)
and assaying it *in vitro* for RNA or protein levels, structure
and/or activity of the expressed Nr-CAM RNA or protein. Many
methods standard in the art can be thus employed, including

25 but not limited to immunoassays to detect and/or visualize
Nr-CAM protein (*e.g.*, Western blot, immunoprecipitation
followed by sodium dodecyl sulfate polyacrylamide gel
electrophoresis, immunocytochemistry, etc.) and/or
hybridization assays to detect Nr-CAM expression by detecting
and/or visualizing respectively Nr-CAM mRNA (*e.g.*, Northern

30 assays, dot blots, *in situ* hybridization, etc.), etc.

In other embodiments, chemical mutagenesis, or
homologous recombination with an insertionally inactivated

Nr-CAM gene (see Capecchi, 1989, Science <u>244</u>:1288-1292 and
Section 5.14 *infra*) can be carried out to reduce or destroy
endogenous Nr-CAM function, in order to decrease cell
proliferation. Suitable methods, modes of administration and
5 compositions, that can be used to inhibit Nr-CAM function are
described in Sections 5.8.2 through 5.8.2.1.2, above.

In an embodiment of the invention, a Therapeutic
that inhibits Nr-CAM activity is used to treat, inhibit or
prevent hyperproliferative or benign dysproliferative
disorders. Specific embodiments are directed to treatment,
10
inhibition or prevention of cirrhosis of the liver (a
condition in which scarring has overtaken normal liver
regeneration processes), treatment or inhibition of keloid
(hypertrophic scar) formation (disfiguring of the skin in
which the scarring process interferes with normal renewal),
15 psoriasis (a common skin condition characterized by excessive
proliferation of the skin and delay in proper cell fate
determination), benign tumors, fibrocystic conditions, and
tissue hypertrophy (*e.g.*, prostatic hyperplasia).

20   **5.5.2.1. <u>ANTISENSE REGULATION OF *Nr-CAM* EXPRESSION</u>**
In a specific embodiment, Nr-CAM function is
inhibited by use of Nr-CAM antisense nucleic acids. The
present invention provides the therapeutic or prophylactic
use of nucleic acids of at least six nucleotides that are
antisense to a gene or cDNA encoding Nr-CAM or a portion
25
thereof. A Nr-CAM "antisense" nucleic acid as used herein
refers to a nucleic acid capable of hybridizing to a portion
of a Nr-CAM RNA (preferably mRNA) by virtue of some sequence
complementarity. The antisense nucleic acid may be
complementary to a coding and/or noncoding region of a Nr-CAM
30
mRNA. Such antisense nucleic acids have utility as
Therapeutics that inhibits Nr-CAM function, and can be used

in the treatment or prevention of disorders as described
supra in Section 5.5.2 and its subsections.

The antisense nucleic acids of the invention can be
oligonucleotides that are double-stranded or single-stranded,
RNA or DNA or a modification or derivative thereof, which can
be directly administered to a cell, or which can be produced
intracellularly by transcription of exogenous, introduced
sequences.

In a specific embodiment, the Nr-CAM antisense
nucleic acids provided by the present invention can be used
to inhibit or prevent tumors or other forms of aberrant cell
proliferation.

The invention further provides pharmaceutical
compositions comprising an effective amount of the Nr-CAM
antisense nucleic acids of the invention in a
pharmaceutically acceptable carrier, as described *infra*.

In another embodiment, the invention is directed to
methods for inhibiting the expression of a *Nr-CAM* nucleic
acid sequence in a prokaryotic or eukaryotic cell comprising
providing the cell with an effective amount of a composition
comprising an Nr-CAM antisense nucleic acid of the invention.

Nr-CAM antisense nucleic acids and their uses are
described in detail below.

5.5.2.1.1.   *Nr-CAM* ANTISENSE NUCLEIC ACIDS

The Nr-CAM antisense nucleic acids are of at least
six nucleotides and are preferably oligonucleotides (ranging
from 6 to about 50 oligonucleotides). In specific aspects,
the oligonucleotide is at least 10 nucleotides, at least 15
nucleotides, at least 100 nucleotides, or at least 200
nucleotides. The oligonucleotides can be DNA or RNA or
chimeric mixtures or derivatives or modified versions
thereof, single-stranded or double-stranded. In a specific
embodiment, the antisense nucleic acids of the invention are

double-stranded RNA (see, Fire et al., 1998, Nature 391:806-
811). The oligonucleotide can be modified at the base
moiety, sugar moiety, or phosphate backbone. The
oligonucleotide may include other appending groups such as
5 peptides, or agents facilitating transport across the cell
membrane (see, e.g., Letsinger et al., 1989, Proc. Natl.
Acad. Sci. U.S.A. 86:6553-6556; Lemaitre et al., 1987, Proc.
Natl. Acad. Sci. 84:648-652; PCT Publication No. WO 88/09810,
published December 15, 1988) or blood-brain barrier (see,
10 e.g., PCT Publication No. WO 89/10134, published April 25,
1988), hybridization-triggered cleavage agents (see, e.g.,
Krol et al., 1988, BioTechniques 6:958-976) or intercalating
agents (see, e.g., Zon, 1988, Pharm. Res. 5:539-549).

In a preferred aspect of the invention, a Nr-CAM
antisense oligonucleotide is provided, preferably of single-
15 stranded DNA. The oligonucleotide may be modified at any
position on its structure with substituents generally known
in the art.

The Nr-CAM antisense oligonucleotide may comprise
at least one modified base moiety which is selected from the
20 group including but not limited to 5-fluorouracil,
5-bromouracil, 5-chlorouracil, 5-iodouracil, hypoxanthine,
xanthine, 4-acetylcytosine, 5-(carboxyhydroxylmethyl) uracil,
5-carboxymethylaminomethyl-2-thiouridine,
5-carboxymethylaminomethyluracil, dihydrouracil, beta-D-
25 galactosylqueosine, inosine, N6-isopentenyladenine,
1-methylguanine, 1-methylinosine, 2,2-dimethylguanine,
2-methyladenine, 2-methylguanine, 3-methylcytosine,
5-methylcytosine, N6-adenine, 7-methylguanine,
5-methylaminomethyluracil, 5-methoxyaminomethyl-2-thiouracil,
beta-D-mannosylqueosine, 5'-methoxycarboxymethyluracil,
30 5-methoxyuracil, 2-methylthio-N6-isopentenyladenine,
uracil-5-oxyacetic acid (v), wybutoxosine, pseudouracil,
queosine, 2-thiocytosine, 5-methyl-2-thiouracil,

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2-thiouracil, 4-thiouracil, 5-methyluracil, uracil-
5-oxyacetic acid methylester, uracil-5-oxyacetic acid (v),
5-methyl-2-thiouracil, 3-(3-amino-3-N-2-carboxypropyl)
uracil, (acp3)w, and 2,6-diaminopurine.

5   In another embodiment, the oligonucleotide
comprises at least one modified sugar moiety selected from
the group including but not limited to arabinose,
2-fluoroarabinose, xylulose, and hexose.

           In yet another embodiment, the oligonucleotide
comprises at least one modified phosphate backbone selected
10   from the group consisting of a phosphorothioate, a
phosphorodithioate, a phosphoramidothioate, a
phosphoramidate, a phosphordiamidate, a methylphosphonate, an
alkyl phosphotriester, and a formacetal or analog thereof.

           In yet another embodiment, the oligonucleotide is
15   an α-anomeric oligonucleotide. An α-anomeric oligonucleotide
forms specific double-stranded hybrids with complementary RNA
in which, contrary to the usual β-units, the strands run
parallel to each other (Gautier et al., 1987, Nucl. Acids
Res. 15:6625-6641).

20   The oligonucleotide may be conjugated to another
molecule, e.g., a peptide, hybridization triggered cross-
linking agent, transport agent, hybridization-triggered
cleavage agent, etc.

           Oligonucleotides of the invention may be
25   synthesized by standard methods known in the art, e.g. by use
of an automated DNA synthesizer (such as are commercially
available from Biosearch, Applied Biosystems, etc.). As
examples, phosphorothioate oligonucleotides may be
synthesized by the method of Stein et al. (1988, Nucl. Acids
Res. 16:3209), methylphosphonate oligonucleotides can be
30   prepared by use of controlled pore glass polymer supports
(Sarin et al., 1988, Proc. Natl. Acad. Sci. U.S.A. 85:7448-
7451), etc.

In a specific embodiment, the Nr-CAM antisense
oligonucleotide comprises catalytic RNA, or a ribozyme (see,
e.g., PCT International Publication WO 90/11364, published
October 4, 1990; Sarver et al., 1990, Science 247:1222-1225).
In another embodiment, the oligonucleotide is a 2'-0-
methylribonucleotide (Inoue et al., 1987, Nucl. Acids Res.
15:6131-6148), or a chimeric RNA-DNA analog (Inoue et al.,
1987, FEBS Lett. 215:327-330).

In an alternative embodiment, the Nr-CAM antisense
nucleic acid of the invention is produced intracellularly by
transcription from an exogenous sequence. For example, a
vector can be introduced in vivo such that it is taken up by
a cell, within which cell the vector or a portion thereof is
transcribed, producing an antisense nucleic acid (RNA) of the
invention. Such a vector would contain a sequence encoding
the Nr-CAM antisense nucleic acid. Such a vector can remain
episomal or become chromosomally integrated, as long as it
can be transcribed to produce the desired antisense RNA.
Such vectors can be constructed by recombinant DNA technology
methods standard in the art. Vectors can be plasmid, viral,
or others known in the art, used for replication and
expression in mammalian cells. Expression of the sequence
encoding the Nr-CAM antisense RNA can be by any promoter
known in the art to act in mammalian, preferably human,
cells. Such promoters can be inducible or constitutive.
Such promoters include but are not limited to: the SV40 early
promoter region (Bernoist and Chambon, 1981, Nature 290:304-
310), the promoter contained in the 3' long terminal repeat of
Rous sarcoma virus (Yamamoto et al., 1980, Cell 22:787-797),
the herpes thymidine kinase promoter (Wagner et al., 1981,
Proc. Natl. Acad. Sci. U.S.A. 78:1441-1445), the regulatory
sequences of the metallothionein gene (Brinster et al., 1982,
Nature 296:39-42), etc.

The antisense nucleic acids of the invention comprise a sequence complementary to at least a portion of an RNA transcript of a *Nr-CAM* nucleic acid or *Nr-CAM* gene, preferably a human *Nr-CAM* gene. However, absolute

5 complementarily, although preferred, is not required. A sequence "complementary to at least a portion of an RNA," as referred to herein, means a sequence having sufficient complementarity to be able to hybridize with the RNA, forming a stable duplex; in the case of double-stranded Nr-CAM antisense nucleic acids, a single strand of the duplex DNA

10 may thus be tested, or triplex formation may be assayed. The ability to hybridize will depend on both the degree of complementarity and the length of the antisense nucleic acid. Generally, the longer the hybridizing nucleic acid, the more base mismatches with a Nr-CAM RNA it may contain and still

15 form a stable duplex (or triplex, as the case may be). One skilled in the art can ascertain a tolerable degree of mismatch by use of standard procedures to determine the melting point of the hybridized complex.

20   5.5.2.1.2.   **THERAPEUTIC USE OF *Nr-CAM***
                             ANTISENSE NUCLEIC ACIDS

The Nr-CAM antisense nucleic acids can be used to treat, inhibit (or prevent) disorders of a cell type that expresses, or preferably overexpresses, Nr-CAM. In a specific embodiment, such a disorder is a hyperproliferative

25 disorder, e.g. tumorigenesis. In a preferred embodiment, a single-stranded DNA antisense Nr-CAM oligonucleotide is used.

Cell types which express or overexpress Nr-CAM RNA can be identified by various methods known in the art. Such methods include but are not limited to hybridization with a

30 Nr-CAM-specific nucleic acid (*e.g.* by Northern hybridization, dot blot hybridization, *in situ* hybridization), observing the ability of RNA from the cell type to be translated *in vitro*

into Nr-CAM, immunoassay, etc. In a preferred aspect, primary tissue from a patient can be assayed for Nr-CAM expression prior to treatment, *e.g.*, by immunocytochemistry or *in situ* hybridization.

5   Pharmaceutical compositions of the invention (see Section 5.10), comprising an effective amount of a Nr-CAM antisense nucleic acid in a pharmaceutically acceptable carrier, can be administered to a patient having a disease or disorder which is of a type that expresses or overexpresses Nr-CAM RNA or protein.

10   The amount of Nr-CAM antisense nucleic acid which will be effective in the treatment of a particular disorder or condition will depend on the nature of the disorder or condition, and can be determined by standard clinical techniques. Where possible, it is desirable to determine the

15 antisense cytotoxicity of the tumor type to be treated *in vitro*, and then in useful animal model systems prior to testing and use in humans.

        In a specific embodiment, pharmaceutical compositions comprising Nr-CAM antisense nucleic acids are

20 administered via liposomes, microparticles, or microcapsules. In various embodiments of the invention, it may be useful to use such compositions to achieve sustained release of the Nr-CAM antisense nucleic acids. In a specific embodiment, it may be desirable to utilize liposomes targeted via antibodies

25 to specific identifiable tumor antigens (Leonetti *et al.*, 1990, Proc. Natl. Acad. Sci. U.S.A. <u>87</u>:2448-2451; Renneisen *et al.*, 1990, J. Biol. Chem. <u>265</u>:16337-16342).

        Additional methods that can be adapted for use to deliver a Nr-CAM antisense nucleic acid are described in Section 5.9.1.4.

30

5.6.   DEMONSTRATION OF THERAPEUTIC
OR PROPHYLACTIC UTILITY

The Therapeutics of the invention are preferably
tested *in vitro*, and then *in vivo* for the desired therapeutic
or prophylactic activity, prior to use in humans.

For example, *in vitro* assays which can be used to
determine whether administration of a specific Therapeutic is
indicated, include *in vitro* cell culture assays in which a
patient tissue sample is grown in culture, and exposed to or
otherwise administered a Therapeutic, and the effect of such
Therapeutic upon the tissue sample is observed. In one
embodiment, where the patient has a malignancy, a sample of
cells from such malignancy is plated out or grown in culture,
and the cells are then exposed to a Therapeutic. A
Therapeutic which inhibits survival or growth of the
malignant cells is selected for therapeutic use *in vivo*.
Many assays standard in the art can be used to assess such
survival and/or growth; for example, cell proliferation can
be assayed by measuring ^{3}H-thymidine incorporation, by direct
cell count, by detecting changes in transcriptional activity
of known genes such as proto-oncogenes (*e.g.*, *fos*, *myc*) or
cell cycle markers; cell viability can be assessed by trypan
blue staining, differentiation can be assessed visually based
on changes in morphology, etc.

In another embodiment, a Therapeutic is indicated
for use which exhibits the desired effect, inhibition or
promotion of cell growth, upon a patient cell sample from
tissue having or suspected of having a hyper- or
hypoproliferative disorder, respectively. Such hyper- or
hypoproliferative disorders include but are not limited to
those described herein.

In another specific embodiment, a Therapeutic is
indicated for use in treating or inhibiting cell injury or a

degenerative disorder which exhibits *in vitro* promotion of growth/proliferation of cells of the affected patient type.

In various specific embodiments, *in vitro* assays can be carried out with representative cells of cell types involved in a patient's disorder, to determine if a Therapeutic has a desired effect upon such cell types.

In another embodiment, cells of a patient tissue sample suspected of being pre-neoplastic are similarly plated out or grown *in vitro*, and exposed to a Therapeutic. The Therapeutic which results in a cell phenotype that is more normal (*i.e.*, less representative of a pre-neoplastic state, neoplastic state, malignant state, or transformed phenotype) is selected for therapeutic use. Many assays standard in the art can be used to assess whether a pre-neoplastic state, neoplastic state, or a transformed or malignant phenotype, is present. For example, characteristics associated with a transformed phenotype (a set of *in vitro* characteristics associated with a tumorigenic ability *in vivo*) include a more rounded cell morphology, looser substratum attachment, loss of contact inhibition, loss of anchorage dependence, release of proteases such as plasminogen activator, increased sugar transport, decreased serum requirement, expression of fetal antigens, disappearance of the 250,000 dalton surface protein, etc. (see Luria *et al.*, 1978, General Virology, 3d Ed., John Wiley & Sons, New York pp. 436-446).

In other specific embodiments, the *in vitro* assays described *supra* can be carried out using a cell line, rather than a cell sample derived from the specific patient to be treated, in which the cell line is derived from or displays characteristic(s) associated with the malignant, neoplastic or pre-neoplastic disorder desired to be treated or prevented, or is derived from the cell type upon which an effect is desired, according to the present invention.

Compounds for use in therapy can be tested in
suitable animal model systems prior to testing in humans,
including but not limited to rats, mice, chicken, cows,
monkeys, rabbits, etc. For *in vivo* testing, prior to
5 administration to humans, any animal model system known in
the art may be used.

5.7. THERAPEUTIC/PROPHYLACTIC
ADMINISTRATION AND COMPOSITIONS

The invention provides methods of treatment (and
10 prophylaxis) by administration to a subject of an effective
amount of a Therapeutic of the invention. In a preferred
aspect, the Therapeutic is isolated, purified or
substantially purified. The subject is preferably an animal,
including but not limited to animals such as cows, pigs,
15 horses, chickens, cats, dogs, etc., and is preferably a
mammal, and most preferably human. In a specific embodiment,
a non-human mammal is the subject.

Formulations and methods of administration that can
be employed when the Therapeutic comprises a nucleic acid are
described above; additional appropriate formulations and
20 routes of administration can be selected from among those
described hereinbelow.

Various delivery systems are known and can be used
to administer a Therapeutic of the invention, e.g.,
encapsulation in liposomes, microparticles, microcapsules,
25 recombinant cells capable of expressing the Therapeutic,
receptor-mediated endocytosis (see, e.g., Wu and Wu, 1987, J.
Biol. Chem. 262:4429-4432), construction of a Therapeutic
nucleic acid as part of a retroviral or other vector, etc.
Methods of introduction include but are not limited to
30 intradermal, intramuscular, intraperitoneal, intravenous,
subcutaneous, intranasal, epidural, and oral routes. The
compounds may be administered by any convenient route, for

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example by infusion or bolus injection, by absorption through epithelial or mucocutaneous linings (*e.g.*, oral mucosa, rectal and intestinal mucosa, etc.) and may be administered together with other biologically active agents.

5 Administration can be systemic or local.

In addition, it may be desirable to introduce a Therapeutic of the invention into the central nervous system by any suitable route, including, but not limited to intraventricular and intrathecal injection. Intraventricular injection may be facilitated by an intraventricular catheter,

10 for example, attached to a reservoir, such as an Ommaya reservoir. Agents which enhance the delivery of chemotherapeutics to brain tumors, such as agonists which activate specific receptors on endothelial cells which regulate permeability, including, *e.g.*, bradykinin agonists

15 (see, *e.g.*, Elliott, *et al.*, 1996, Cancer Research 56:3998-4005) tumor angiogenesis factors (Cserr and Knopf, 1992, Immunol Today 12:507-512) etc. can be used in formulations and methods of administration when the Therapeutic is intended for delivery to a tumor of the central nervous

20 system.

In a specific embodiment, injection into spinal fluid, and/or procedures utilizing an Ommaya reservoir, can be used to introduce a therapeutic of the invention such as an anti-Nr-CAM antibody, *e.g.* a bispecific anti-Nr-CAM

25 antibody, directly into the central nervous system for immunotherapy of a tumor.

In yet another specific embodiment, an anti-Nr-CAM antibody, *e.g.* a bispecific anti-Nr-CAM antibody, is employed as a Therapeutic in an immunotherapeutic treatment of a non-brain tumor and is infused into a recipient intravenously.

30 Immune cells, *e.g.* dendritic cells or cytotoxic T-cells, can cross the blood-brain barrier and have access to brain tissue, especially in the presence of tumor

angiogenesis factors (Cserr and Knopf, 1992, Immunol. Today,
12:507-512). In a preferred embodiment, activated dendritic
cells (HLA-matched to the recipient) (see generally, Tjoa et
al., 1996, Prostate 28:65-69) that have been exposed to a Nr-
5 CAM protein, analog or derivative thereof are infused into a
recipient under conditions that permit their crossing the
blood-brain barrier, e.g. in the presence of tumor
angiogenesis factors. In another preferred embodiment,
activated cytotoxic T-cells (HLA-matched to the recipient)
(see generally, Tjoa et al., 1996, Prostate 28:65-69) that
10
have been exposed ex vivo (i.e. in vitro) to a Nr-CAM
protein, analog, or derivative thereof are infused into a
recipient under conditions that permit their crossing the
blood-brain barrier.

        In yet another specific embodiment, a Therapeutic
15 of the invention; e.g., activated dendritic cells that have
been exposed to a Nr-CAM protein, analog or derivative
thereof, or activated cytotoxic T-cells that have been
exposed ex vivo dendritic cells that have been exposed to a
Nr-CAM protein, analog, or derivative thereof, is
20 administered for the treatment of a non-brain tumor.

        Pulmonary administration of a Therapeutic can also
be employed, e.g., by use of an inhaler or nebulizer, and
formulation with an aerosolizing agent.

        In a specific embodiment, it may be desirable to
25 administer the Therapeutic of the invention locally to the
area in need of treatment; this may be achieved by, for
example, and not by way of limitation, local infusion during
surgery, topical application, e.g., in conjunction with a
wound dressing after surgery, by injection, by means of a
catheter, by means of a suppository, or by means of an
30
implant, said implant being of a porous, non-porous, or
gelatinous material, including membranes, such as sialastic
membranes, or fibers. In one embodiment, administration can

be by direct injection at the site (or former site) of a
malignant tumor or neoplastic or pre-neoplastic tissue.

In another embodiment, the Therapeutic can be
delivered in a vesicle, in particular a liposome (see Langer,
5 1990 Science 249:1527-1533; Treat et al., in Liposomes in the
Therapy of Infectious Disease and Cancer, Lopez-Berestein and
Fidler (eds.), Liss, New York, pp. 353-365 (1989);
Lopez-Berestein, ibid., pp. 317-327; see generally ibid.)

In yet another embodiment, the Therapeutic can be
delivered in a controlled release system. In one embodiment,
10
a pump may be used (see Langer, supra; Sefton, CRC Crit. Ref.
Biomed. Eng. 14:201 (1987); Buchwald et al., Surgery 88:507
(1980); Saudek et al., N. Engl. J. Med. 321:574 (1989)). In
another embodiment, polymeric materials can be used (see
Medical Applications of Controlled Release, Langer and Wise
15 (eds.), CRC Pres., Boca Raton, Florida (1974); Controlled
Drug Bioavailability, Drug Product Design and Performance,
Smolen and Ball (eds.), Wiley, New York (1984); Ranger and
Peppas, 1983, J. Macromol. Sci. Rev. Macromol. Chem. 23:61;
see also Levy et al., 1985 Science 228:190; During et al.,
20 1989 Ann. Neurol. 25:351; Howard et al., 1989 J. Neurosurg.
71:105). In yet another embodiment, a controlled release
system can be placed in proximity of the therapeutic target,
i.e., the brain, thus requiring only a fraction of the
systemic dose (see, e.g., Goodson, in Medical Applications of
25 Controlled Release, supra, vol. 2, pp. 115-138 (1984)).

Other controlled release systems are discussed in
the review by Langer (Science 249:1527-1533 (1990)).

In a specific embodiment where the Therapeutic is a
nucleic acid encoding a protein Therapeutic, the nucleic acid
can be administered in vivo to promote expression of its
30
encoded protein, by constructing it as part of an appropriate
nucleic acid expression vector and administering it so that
it becomes intracellular, e.g., by use of a retroviral vector

(see U.S. Patent No. 4,980,286), or by direct injection, or
by use of microparticle bombardment (e.g., a gene gun;
Biolistic, Dupont), or coating with lipids or cell-surface
receptors or transfecting agents, or by administering it in
5 linkage to a homeobox-like peptide which is known to enter
the nucleus (see e.g., Joliot et al., 1991, Proc. Natl. Acad.
Sci. USA 88:1864-1868), etc. Alternatively, a nucleic acid
Therapeutic can be introduced intracellularly and
incorporated within host cell DNA for expression, by
homologous recombination.
10
          The present invention also provides pharmaceutical
compositions. Such compositions comprise a therapeutically
effective amount of a Therapeutic, and a pharmaceutically
acceptable carrier. In a specific embodiment, the term
"pharmaceutically acceptable" means approved by a regulatory
15 agency of the Federal or a state government or listed in the
U.S. Pharmacopeia or other generally recognized pharmacopeia
for use in animals, and more particularly in humans. The
term "carrier" refers to a diluent, adjuvant, excipient, or
vehicle with which the therapeutic is administered. Such
20 pharmaceutical carriers can be sterile liquids, such as water
and oils, including those of petroleum, animal, vegetable or
synthetic origin, such as peanut oil, soybean oil, mineral
oil, sesame oil and the like. Water is a preferred carrier
when the pharmaceutical composition is administered
intravenously. Saline solutions and aqueous dextrose and
25
glycerol solutions can also be employed as liquid carriers,
particularly for injectable solutions. Suitable
pharmaceutical excipients include starch, glucose, lactose,
sucrose, gelatin, malt, rice, flour, chalk, silica gel,
sodium stearate, glycerol monostearate, talc, sodium
30 chloride, dried skim milk, glycerol, propylene, glycol,
water, ethanol and the like. The composition, if desired,
can also contain minor amounts of wetting or emulsifying

- 87 -

agents, or pH buffering agents. These compositions can take
the form of solutions, suspensions, emulsion, tablets, pills,
capsules, powders, sustained-release formulations and the
like. The composition can be formulated as a suppository,
with traditional binders and carriers such as triglycerides.
Oral formulation can include standard carriers such as
pharmaceutical grades of mannitol, lactose, starch, magnesium
stearate, sodium saccharine, cellulose, magnesium carbonate,
etc. Examples of suitable pharmaceutical carriers are
described in "Remington's Pharmaceutical Sciences" by E.W.
Martin. Such compositions will contain a therapeutically
effective amount of the Therapeutic, preferably in purified
form, together with a suitable amount of carrier so as to
provide the form for proper administration to the patient.
The formulation should suit the mode of administration.

In a preferred embodiment, the composition is
formulated in accordance with routine procedures as a
pharmaceutical composition adapted for intravenous
administration to human beings. Typically, compositions for
intravenous administration are solutions in sterile isotonic
aqueous buffer. Where necessary, the composition may also
include a solubilizing agent and a local anesthetic such as
lignocaine to ease pain at the site of the injection.
Generally, the ingredients are supplied either separately or
mixed together in unit dosage form, for example, as a dry
lyophilized powder or water free concentrate in a
hermetically sealed container such as an ampoule or sachette
indicating the quantity of active agent. Where the
composition is to be administered by infusion, it can be
dispensed with an infusion bottle containing sterile
pharmaceutical grade water or saline. Where the composition
is administered by injection, an ampoule of sterile water for
injection or saline can be provided so that the ingredients
may be mixed prior to administration.

The Therapeutics of the invention can be formulated as neutral or salt forms. Pharmaceutically acceptable salts include those formed with free amino groups such as those derived from hydrochloric, phosphoric, acetic, oxalic,
5 tartaric acids, etc., and those formed with free carboxyl groups such as those derived from sodium, potassium, ammonium, calcium, ferric hydroxides, isopropylamine, triethylamine, 2-ethylamino ethanol, histidine, procaine, etc.

The amount of the Therapeutic of the invention
10 which will be effective in the treatment of a particular disorder or condition will depend on the nature of the disorder or condition, and can be determined by standard clinical techniques. In addition, *in vitro* assays may optionally be employed to help identify optimal dosage
15 ranges. The precise dose to be employed in the formulation will also depend on the route of administration, and the seriousness of the disease or disorder, and should be decided according to the judgment of the practitioner and each patient's circumstances. However, suitable dosage ranges for
20 intravenous administration are generally about 20-500 micrograms of active compound per kilogram body weight. Suitable dosage ranges for intranasal administration are generally about 0.01 pg/kg body weight to 1 mg/kg body weight. Effective doses may be extrapolated from dose-response curves derived from *in vitro* or animal model test
25 systems.

Suppositories generally contain active ingredient in the range of 0.5% to 10% by weight; oral formulations preferably contain 10% to 95% active ingredient.

The invention also provides a pharmaceutical pack
30 or kit comprising one or more containers filled with one or more of the ingredients of the pharmaceutical compositions of the invention. Optionally associated with such container(s)

can be a notice in the form prescribed by a governmental
agency regulating the manufacture, use or sale of
pharmaceuticals or biological products, which notice reflects
approval by the agency of manufacture, use or sale for human
5 administration.

5.7.1.   TREATMENT AND PREVENTION OF HYPOPROLIFERATIVE DISORDERS

        Diseases and disorders involving decreased cell
proliferation or in which cell proliferation is desired for
10
treatment or prevention, and that can be treated or prevented
by promoting Nr-CAM function, include but are not limited to
degenerative disorders, growth deficiencies,
hypoproliferative disorders, physical trauma, lesions, and
wounds; for example, to promote wound healing, or to promote
15 regeneration in degenerated, lesioned or injured tissues,
etc.   In a specific embodiment, nervous system disorders are
treated.   In another specific embodiment, a disorder that is
not of the nervous system is treated.

        Lesions which may be treated according to the
20 present invention include but are not limited to the
following lesions:

    (i)   traumatic lesions, including lesions caused by
           physical injury or associated with surgery;

    (ii)   ischemic lesions, in which a lack of oxygen
           results in cell injury or death, e.g.,
25
           myocardial or cerebral infarction or ischemia,
           or spinal cord infarction or ischemia;

    (iii) malignant lesions, in which cells are
           destroyed or injured by malignant tissue;

    (iv)   infectious lesions, in which tissue is
30
           destroyed or injured as a result of infection,
           for example, by an abscess or associated with
           infection by human immunodeficiency virus,

herpes zoster, or herpes simplex virus or with
Lyme disease, tuberculosis, syphilis;

(v)   degenerative lesions, in which tissue is
destroyed or injured as a result of a
degenerative process, including but not
limited to nervous system degeneration
associated with Parkinson's disease,
Alzheimer's disease, Huntington's chorea, or
amyotrophic lateral sclerosis;

(vi)   lesions associated with nutritional diseases
or disorders, in which tissue is destroyed or
injured by a nutritional disorder or disorder
of metabolism including but not limited to,
vitamin B12 deficiency, folic acid deficiency,
Wernicke disease, tobacco-alcohol amblyopia,
Marchiafava-Bignami disease (primary
degeneration of the corpus callosum), and
alcoholic cerebellar degeneration;

(vii)   lesions associated with systemic diseases
including but not limited to diabetes or
systemic lupus erythematosus;

(viii) lesions caused by toxic substances including
alcohol, lead, or other toxins; and

(ix)   demyelinated lesions of the nervous system, in
which a portion of the nervous system is
destroyed or injured by a demyelinating
disease including but not limited to multiple
sclerosis, human immunodeficiency virus-
associated myelopathy, transverse myelopathy
or various etiologies, progressive multifocal
leukoencephalopathy, and central pontine
myelinolysis.

    Nervous system lesions which may be treated in a
patient (including human and non-human mammalian patients)

according to the invention include but are not limited to the
lesions of either the central (including spinal cord, brain)
or peripheral nervous systems.

Therapeutics which are useful according to this
embodiment of the invention for treatment of a disorder may
be selected by testing for biological activity in promoting
the survival or differentiation of cells (see also Section
5.9). For example, in a specific embodiment relating to
therapy of the nervous system, a Therapeutic which elicits
one of the following effects may be useful according to the
invention:

(i)   increased sprouting of neurons in culture or
in vivo;

(ii)   increased production of a neuron-associated
molecule in culture or in vivo, e.g., choline
acetyltransferase or acetylcholinesterase with
respect to motor neurons; or

(iii)   decreased symptoms of neuron dysfunction in
vivo.

Such effects may be measured by any method known in the art.
In preferred, non-limiting embodiments, increased sprouting
of neurons may be detected by methods set forth in Pestronk
et al. (1980, Exp. Neurol. 70:65-82) or Brown et al. (1981,
Ann. Rev. Neurosci. 4:17-42); and increased production of
neuron-associated molecules may be measured by bioassay,
enzymatic assay, antibody binding, Northern blot assay, etc.,
depending on the molecule to be measured.

5.8. ADDITIONAL USE OF INCREASED Nr-CAM FUNCTION TO PROMOTE INCREASED GROWTH

Promotion of Nr-CAM function (e.g., by
administering a compound that promotes Nr-CAM function as
described above), has utility that is not limited to
therapeutic or prophylactic applications. For example, Nr-

CAM function can be promoted in order to increase growth of
animals (e.g., cows, horses, pigs, goats, deer, chickens) and
plants (particularly edible plants, e.g., tomatoes, melons,
lettuce, carrots, potatoes, and other vegetables),
5 particularly those that are food or material sources. In an
embodiment in which a Nr-CAM nucleic acid is under the
control of a tissue-specific promoter, the invention can be
used in plants or animals to increase growth where desired
(e.g., in the fruit or muscle). For example, a Nr-CAM
10 nucleic acid under the control of a temperature-sensitive
promoter can be administered to a plant or animal, and the
desired portion of the (or the entire) plant or animal can be
subjected to heat in order to induce Nr-CAM nucleic acid
production, resulting in increased Nr-CAM expression, and
resulting cell proliferation. Methods to make plants
15 recombinant are commonly known in the art and can be used.
Regarding methods of plant transformation (e.g., for
transformation with a Nr-CAM antisense nucleic acid), see
e.g., Valvekens et al., 1988, Proc. Natl. Acad. Sci. USA
85:5536-5540. Regarding methods of targeted gene
20 inactivation in plants (e.g., to inactivate Nr-CAM), see
e.g., Miao and Lam, 1995, The Plant J. 7:359-365.

        Promotion of Nr-CAM function can also have uses in
vitro, e.g., to expand cells in vitro, including but not
limited to stem cells, progenitor cells, muscle cells,
25 fibroblasts, liver cells, etc., e.g., to grow cells/tissue in
vitro prior to administration to a patient (preferably a
patient from which the cells were derived), etc.

        5.9. SCREENING FOR Nr-CAM AGONISTS AND ANTAGONISTS
30   Nr-CAM nucleic acids, proteins, and derivatives
also have uses in screening assays to detect molecules that
specifically bind to Nr-CAM nucleic acids, proteins, or
derivatives and thus have potential use as agonists or

antagonists of Nr-CAM, in particular, molecules that thus affect cell proliferation. In a preferred embodiment, such assays are performed to screen for molecules with potential utility as anti-cancer drugs or lead compounds for drug

5 development. The invention thus provides assays to detect molecules that specifically bind to *Nr-CAM* nucleic acids, proteins, or derivatives. For example, recombinant cells expressing *Nr-CAM* nucleic acids can be used to recombinantly produce Nr-CAM proteins in these assays, to screen for molecules that bind to a Nr-CAM protein. Molecules (*e.g.*,

10 putative binding partners of Nr-CAM) are contacted with the Nr-CAM protein (or fragment thereof) under conditions conducive to binding, and then molecules that specifically bind to the Nr-CAM protein are identified. Similar methods can be used to screen for molecules that bind to Nr-CAM

15 derivatives or nucleic acids. Methods that can be used to carry out the foregoing are commonly known in the art.

By way of example, diversity libraries, such as random or combinatorial peptide or nonpeptide libraries can be screened for molecules that specifically bind to Nr-CAM.

20 Many libraries are known in the art that can be used, *e.g.*, chemically synthesized libraries, recombinant (*e.g.*, phage display libraries), and *in vitro* translation-based libraries.

Examples of chemically synthesized libraries are described in Fodor *et al.*, 1991, Science 251:767-773;

25 Houghten *et al.*, 1991, Nature 354:84-86; Lam *et al.*, 1991, Nature 354:82-84; Medynski, 1994, Bio/Technology 12:709-710; Gallop *et al.*, 1994, J. Medicinal Chemistry 37(9):1233-1251; Ohlmeyer *et al.*, 1993, Proc. Natl. Acad. Sci. USA 90:10922-10926; Erb *et al.*, 1994, Proc. Natl. Acad. Sci. USA 91:11422-11426; Houghten *et al.*, 1992, Biotechniques 13:412;

30 Jayawickreme *et al.*, 1994, Proc. Natl. Acad. Sci. USA 91:1614-1618; Salmon *et al.*, 1993, Proc. Natl. Acad. Sci. USA

90:11708-11712; PCT Publication No. WO 93/20242; and Brenner
and Lerner, 1992, Proc. Natl. Acad. Sci. USA 89:5381-5383.

       Examples of phage display libraries are described
in Scott and Smith, 1990, Science 249:386-390; Devlin *et al.*,
5 1990, Science, 249:404-406; Christian, R.B., *et al.*, 1992, J.
Mol. Biol. 227:711-718); Lenstra, 1992, J. Immunol. Meth.
152:149-157; Kay *et al.*, 1993, Gene 128:59-65; and PCT
Publication No. WO 94/18318 dated August 18, 1994.

       In vitro translation-based libraries include but
are not limited to those described in PCT Publication No.
10
WO 91/05058 dated April 18, 1991; and Mattheakis *et al.*,
1994, Proc. Natl. Acad. Sci. USA 91:9022-9026.

       By way of examples of nonpeptide libraries, a
benzodiazepine library (*see e.g.*, Bunin *et al.*, 1994, Proc.
Natl. Acad. Sci. USA 91:4708-4712) can be adapted for use.
15 Peptoid libraries (Simon *et al.*, 1992, Proc. Natl. Acad. Sci.
USA 89:9367-9371) can also be used. Another example of a
library that can be used, in which the amide functionalities
in peptides have been permethylated to generate a chemically
transformed combinatorial library, is described by Ostresh *et
al.* (1994, Proc. Natl. Acad. Sci. USA 91:11138-11142).

       Screening the libraries can be accomplished by any
of a variety of commonly known methods. See, *e.g.*, the
following references, which disclose screening of peptide
libraries: Parmley and Smith, 1989, Adv. Exp. Med. Biol.
25 251:215-218; Scott and Smith, 1990, Science 249:386-390;
Fowlkes *et al.*, 1992; BioTechniques 13:422-427; Oldenburg *et
al.*, 1992, Proc. Natl. Acad. Sci. USA 89:5393-5397; Yu *et
al.*, 1994, Cell 76:933-945; Staudt *et al.*, 1988, Science
241:577-580; Bock *et al.*, 1992, Nature 355:564-566; Tuerk *et
al.*, 1992, Proc. Natl. Acad. Sci. USA 89:6988-6992; Ellington
30
et al., 1992, Nature 355:850-852; U.S. Patent No. 5,096,815,
U.S. Patent No. 5,223,409, and U.S. Patent No. 5,198,346, all

to Ladner *et al.*; Rebar and Pabo, 1993, Science 263:671-673; and PCT Publication No. WO 94/18318.

In a specific embodiment, screening can be conducted out by contacting the library members with a Nr-CAM protein (or nucleic acid or derivative) immobilized on a solid phase and harvesting those library members that bind to the protein (or nucleic acid or derivative). Examples of such screening methods, termed "panning" techniques are described by way of example in Parmley and Smith, 1988, Gene 73:305-318; Fowlkes *et al.*, 1992, BioTechniques 13:422-427; PCT Publication No. WO 94/18318; and in references cited hereinabove.

In another embodiment, the two-hybrid system for selecting interacting proteins in yeast (Fields and Song, 1989, Nature 340:245-246; Chien *et al.*, 1991, Proc. Natl. Acad. Sci. USA 88:9578-9582) can be used to identify molecules that specifically bind to a Nr-CAM protein or derivative.

5.10. ANIMAL MODELS

The invention also provides animal models. In one embodiment, animal models for diseases and disorders involving cell hypoproliferation (*e.g.*, as described in Section 5.8.1) are provided. Such an animal can be initially produced by promoting homologous recombination between a *Nr-CAM* gene in its chromosome and an exogenous *Nr-CAM* gene that has been rendered biologically inactive (preferably by insertion of a heterologous sequence, *e.g.*, an antibiotic resistance gene). In a preferred aspect, this homologous recombination is carried out by transforming embryo-derived stem (ES) cells with a vector containing the insertionally inactivated *Nr-CAM* gene, such that homologous recombination occurs, followed by injecting the ES cells into a blastocyst, and implanting the blastocyst into a foster mother, followed

by the birth of the chimeric animal ("knockout animal") in
which a *Nr-CAM* gene has been inactivated (see Capecchi, 1989,
Science <u>244</u>:1288-1292). The chimeric animal can be bred to
produce additional knockout animals. Such animals can be
5 mice, hamsters, sheep, pigs, cattle, etc., and are preferably
non-human mammals. In a specific embodiment, a knockout
mouse is produced.

Such knockout animals are expected to develop or be
predisposed to developing diseases or disorders involving
cell hypoproliferation. Such animals can be used to screen
10 for or test molecules for the ability to promote
proliferation and thus treat or prevent such diseases and
disorders.

In a different embodiment of the invention,
transgenic animals that have incorporated and express a
15 functional *Nr-CAM* gene have use as animal models of diseases
and disorders involving cell hyperproliferation or
malignancy. Such animals are expected to develop or be
predisposed to developing diseases or disorders involving
cell hyperproliferation (*e.g.*, malignancy) and thus can have
20 use as animal models of such diseases and disorders, *e.g.*, to
screen for or test molecules (*e.g.*, potential anti-cancer
therapeutics) for the ability to inhibit overproliferation
(*e.g.*, tumor formation) and thus treat or prevent such
diseases or disorders.

25 The following examples are provided for the
purposes of illustration only and are intended to limit the
scope of the invention in any manner.

30

6.   EXAMPLE: ISOLATION OF THE Nr-CAM GENE FROM AND
                 CHARACTERIZATION OF ITS EXPRESSION IN
                 HUMAN GLIOBLASTOMA MULTIFORME TUMOR
                 TISSUE

In this study, the role of Nr-CAM, in brain

5 tumorigenesis was characterized.

6.1.   MATERIALS AND METHODS

6.1.1.   HUMAN TISSUES AND CELL LINES

Tissue samples of brain and non-brain tumors were

10 procured from the tissue bank maintained by Pacific Northwest
Cancer Foundation, Northwest Hospital, and from resources at
the Mayo Clinic (Rochester, MN). Brain tumor cell lines
astrocytoma grade IV (CCF-STTG1), astrocytoma grade III (SW
1738), neuroblastoma (IMR-32), medulloblastoma (D283 Med),
glioma (Hs 683), neuroectodermal (PFSK-1), GM(DBTRG-05MG)

15 were purchased from the ATCC (Rockville, MD). Fetal normal
human astrocytes (FNHAs) were purchased from Clonetics (San
Diego, CA). All cell lines were cultured under the
conditions recommended by the ATCC or Clonetics.

20   6.1.2.   DIFFERENTIAL DISPLAY POLYMERASE
                       CHAIN REACTION (DD-PCR)

In order to isolate and clone genes differentially
expressed in normal brain tissue (NBT) and glioblastoma
multiforme tissue (GMT), the technique of Different Display-
PCR (DD-PCR) was utilized. (Examples of protocols of DD-PCR

25 may be found in Sehgal et al., 1997, J. Surg. Oncol. 64:102-
108; Sehgal et al., 1997, J. Surg. Oncol. 65:249-257; Sehgal
et al., 1997, Int. J. Cancer 71:565-572 (Sehgal, 1997b);
Sehgal et al., 1996, Exp. Lung. Res. 22:419-434).

NBT and GMT were obtained from the same region of

30 the brain. Total RNA was isolated and first strand cDNA
synthesis was carried out using the first strand cDNA
synthesis kit from Clontech (Palo Alto, CA) using BT3-2

primer (5'T [T] 18NG3'). Approximately 125 ng of first strand
cDNA synthesis product were used for carrying out PCR. DD-
PCR was carried out using (λ P^{32}) end-labeled BT3-2 primer and
BT10 (5-NGCTGCTCTCA TACT-3') primer using cDNA from NBT or GMT

5 tissue in duplicate under the conditions described previously
(Sehgal et al., 1997a). PCR products were run on a 6%
sequencing gel. Bands that showed differential expressions
were cut out, and DNA was eluted and cloned into a PCRII
vector Invitrogen, San Diego, CA). Positive clones were
screened by PCR and sequenced using the Sequenase version 2.0

10 sequencing kit from Amersham/USB (Cleveland, OH).

6.1.3. <u>GENE SPECIFIC RT-PCR</u>

To confirm differential expression of clones
isolated by DD-PCR, gene-specific RT-PCR technique was

15 carried out as described previously (Sehgal et al., 1997b).
hNr-CAM specific primers (5'-AACATATGGGTAGAGAGTATATTT-3' (SEQ.
ID No. 9); and 5'-CTTTGCATTCCAGTTCATATTAA-3' (SEQ. ID. NO. 10)
were used for PCR. This PCR results in a 250 bp product at
the 3' end of the hNr-CAM gene. For EGFR, gene-specific

20 primers (5'-TGTGGTGACAGATCACGGCT-3' (SEQ. ID. No. 11) and 5'-
CAGCTCAAACCTGTGATTTCC-3') (SEQ. ID. No. 12) were used for PCR
and an internal primer (5'-
AATAGGTATTGGTGAATTTAAAGACTCACTCTCCATAAATGC
TACGAATATTAAACACTT-3') (SEQ. ID. No. 13) for Southern blot

25 analysis. As a control for PCR, D1-2 (mitochondrial
Cytochrome C oxidase subunit 1 gene, Accession Number
D38112), a housekeeping gene, which is expressed in both NBT
and GMT, was used. PCR was carried out using D1-2-specific
primers (5'-CGGAGCAATATGAAATGATCT-3' (SEQ. ID. NO.: 14) and
5'-GCAAATACAGCTCCTATTG-3') (SEQ. ID. NO.: 15), resulting in a

30 200 bp product. PCR for all 3 genes was carried out using
Taq DNA polymerase under the conditions recommended by Qiagen
(Chatsworth, CA). PCR product was then run on a 2% agarose

gel and transferred onto a Hybond N+ nylon membrane using
standard Southern blotting conditions, as described
previously (Sehgal et al., 1997a). Hybridization was done at
42°C using hNr-CAM, EGFR and D1-2-specific probes. EGFR,

5 hNr-CAM and D1-2-specific probes were prepared by multiprime
labeling (Amersham, Arlington Heights, IL) of hNr-CAM-
specific primers (5'-
GCTGTATGTTAGTATTATGAGAATAGTTACAGCAAAAACATAA CTCAGT-3') (SEQ.
ID. No.: 16) or D1-2-specific primer (5'-
TAGGCCTGACTGGCATTGTATTAGCAAACTCATCACTAGA-3') (SEQ. ID. No.:

10 17). These primers are internal to the primers used for
PCR, and they do not carry any of the primer sequences used
in the PCR. Primer sequences were checked for homologous
sequences using the DNA BLAST program of NCBI (National
center for Biotechnology Information, Bethesda, MD) prior to

15 usage. Quantitation of the signal on Southern blot was
carried out using the ImageQuaNT program of Molecular
Dynamics (Sunnyvale, CA). This protocol was used to
quantitate expression of EGFR, D4-1 or D1-2 in brain tumor
cell lines, FNHA and selected tumor tissues. We have

20 demonstrated previously that this gene-specific RT-PCR
technique is semi-quantitative (Sehgal et al., 1997a, b).

6.1.4.   NORTHERN BLOT ANALYSIS

        Multiple Normal Human tissue blots (MNHTB) were

25 purchased from Clontech (Palo Alto, CA). These blots
contained 2μg of pure polyA+ mRNA. MNHTBs were prehybridized
in express hybridization buffer solution (Clontech) for 3-4
hours. Hybridization was done with multiprime labeled 179 bp
D4-1 probe. After autoradiographic exposure, the probe was
washed from the blot and then hybridized with human β actin

30 probe (Clontech). Quantification of expression of hNr-CAM
and β actin was done using the ImageQuaNT program.

Expression of Nr-CAM in different regions of normal brain and
cell lines formed from tumor tissues was assessed.

6.1.5. QUANTITATION OF NORTHERN AND SOUTHERN BLOTS

5   Quantitation of Northern and Southern blots also
was performed using the ImageQuaNT volume quantitation
program. Volume quantitation calculates the volume under the
surface created by a 3-D plot of pixel locations and pixel
values. We quantitated the volume (the integrated intensity
of all pixels in the spot excluding background) of D1-2 bands
10 in Northern or Southern blots. These pixel values are then
normalized with pixel values in the bands of housekeeping
genes (D1-2 or β actin) and are referred to as "relative
expression" in the figures. The subjective terms "low",
"medium" and "high" refer to relative expression and are
15 based on *hNr-CAM* expression in normal brain as "low" and in
tumor brain as "high".

6.1.6. IN SITU HYBRIDIZATION

           The technique of *In situ* hybridization was done as
20 described previously (Wilkinson, 1992 In Situ Hybridization,
A practical approach. NY: Oxford University Press).
Briefly, 6μm formalin fixed, paraffin embedded human brain
tumor sections were deparaffinized by 2 washes in xylene,
followed by rehydration through graded concentrations of
25 ethanol from 100% to 70%. These were then washed in PBS and
treated with Proteinase K (25 mg/ml for 10 minutes), followed
by fixation in 4% paraformaldehyde. After incubation in
0.25% acetic anhydride/0.1 M TEA (Tri-Ethyl Acetic acid),
sections were dehydrated through graded concentrations of
ethanol from 70% to 100% and prehybridized for 2 hours at
30 55°C in 50% formamide, 5xSSC pH 4.5, 50μg/ml tRNA, 50 μg/ml
heparin, and 1% SDS. Sections were hybridized with 1 μg/ml

DIG-(Digoxygenin) labeled antisense or sense probes for 18
hours at 55°C.

Probes, sense and anti-sense, were synthesized with
the Genius 4 kit (Boehringer Mannheim, Indianapolis, IN)
5 using the T3 and T7 promoters of a PCR template derived from
human Bravo/Nr-CAM sequences corresponding to bases 3731-3754
and 4101-4114. See Figure 2A in which BT180 represents the 5'
primer for the probe corresponding to nucleotides 3731-3754
and BT181, the 3' primer for the probe corresponding to
nucleotides 4104-4114. Following hybridization, slides were
10 washed in 50% formamide, 2xSSC pH 4.5, 1 % SDS at 50°C,
treated with 5 μg/ml RNase A for 30 minutes at 37°C, and
washed in 50% formamide, 2xSSC pH 4.5 at 50°C. Sections were
pre-blocked in 10% normal sheep serum (Sigma, St. Louis, MO)
and incubated with a 1:2000 dilution of alkaline phosphate
15 conjugated anti-dioxigenin Fab fragments (Boehringer
Mannheim) 18 hours at 4°C. For detection, slides were
incubated with NBT/BCIP (5-Bromo-4-chloro-3-indilyl-
phosphate, 4-toluidine salt) in the dark for 46 hours. After
counter staining with eosin Y, slides were mounted with
20 Permount and visualized using an Axioskop (Carl Zeiss,
Thornwood, NY) routine microscope.

6.1.7. GENOMIC SOUTHERN BLOT

NIH3T3, astrocytoma III, glioma and glioblastoma
25 cells were grown in 100 mm diameter plates until 90%
confluent under the conditions recommended by the ATCC.
Genomic DNA was isolated using a DNA isolation kit from
Puragen (Research Triangle Park, NC); 10 μg of DNA were cut
with 50 U of EcoRI restriction enzyme from GIBCO BRL
(Gaithersburg, MD) and run on a 1% agarose gel. DNA was
30 transferred onto a Hybond N+ nylon membrane (Amersham) using
the protocol recommended by Puragen. After incubating the
membrane in pre-hybridization solution, hybridization was

conducted using a 375 bp *hNr-CAM* probe (nucleotide position
3731-4126) (Lane *et al.*, 1996, Genomics <u>35</u>:456-465) labeled
(1 X 104 CPM/ml) using the multiprime labeling kit from
Amersham. Membrane was washed in 0.1 X SSC/0.1% SDS at 24°C
5 for 30 mins and then exposed to Kodak X-ray film.

6.2. RESULTS

6.2.1.   ISOLATION OF HUMAN Nr-CAM FROM
         AND DIFFERENTIAL EXPRESSION OF
         Nr-CAM IN GLIOBLASTOMA

10
        Using the modified technique of DD-PCR as described
in Section 6.1.2., a cDNA fragment, designated D4-1, was
identified which is over-expressed in glioblastoma multiform
tissue (GMT) as compared with normal brain tissue (NBT).
Figure 1 demonstrates that D4-1 is over-expressed in GMT.
15 The band designated C in Figure 1 is the cDNA of the D1-2
gene, a control gene present in both GMT and NBT.

        The D4-1 band was isolated from the gel and cloned
into the pCRII vector from Invitrogen as described in Section
6.1.2. Sequence identity analysis indicated that this cDNA
20 is identical to the last 38 bases at the 3' end of the
previously isolated *hNr-CAM* gene. Figure 2C presents the
results of sequence identity analysis together with a
comparison of the sequence of the isolated *hNr-CAM* with that
of the rat *Nr-CAM*. The isolated D4-1 cDNA has 99.2% sequence
identity (homology) to the rat *Nr-CAM*. Thus, the sequence
25
identity analysis demonstrates that clone D4-2 is *hNr-CAM*.

        In situ hybridization conducted as described in
Section 6.1.6 using an anti-sense Nr-CAM confirmed
differential expression of *hNr-CAM* in glioblastoma as
compared to normal brain tissue. Results are presented in
30 Figures 3(a-f).

        As shown in Figures 3a and d, strong expression of
hNr-CAM is observed in a number of cells from 2 GMT samples,

using the anti-sense *hNr-CAM* probe. Low or no signal was observed when *hNr-CAM* sense probe was used on serial sections (Fig. 3b,e). The NBTs did not show any signal with the *hNr-CAM* anti-sense probe (Fig. 3c,f). This experiment indicates that *hNr-CAM* is differentially over-expressed in GMT as compared to NBT.

6.2.2. EXPRESSION OF *Nr-CAM* IN HUMAN TUMOR TISSUES

As shown in Figures 4 (A and B), *hNr-CAM* is expressed at high levels in glioblastoma IV and glioma tissue as compared to NBT using RT-PCR. Low or no expression of *hNr-CAM* was observed in recurrent meningioma, meningioma, neuroblastoma, recurrent malignant glioma, melanoma, breast tumor, benign prostate tissue or NBT. In one of the GMT samples studied, no *hNr-CAM* expression was observed. High expression of *hNr-CAM* was observed in NBT undergoing extensive gliosis. This is an unusual observation, and the reason for it is not known at present. D1-2 is a housekeeping gene that has been used previously as an internal control in RT-PCR (Sehgal *et al.*, 1997a,b).

The expression of *hNr-CAM* in NBT and astrocytoma tumor tissue was evaluated using RT-PCR. As shown in Figures 5 (A and B), a high level of *hNr-CAM* is expressed in astrocytoma as compared to NBT. The expression pattern of EGFR, a known brain tumor marker, was investigated from this set of cDNA. As shown in the middle and bottom panels of Figures 5A and in 5B, expression of *hNr-CAM* is higher in tumor than is *EGFR*.

6.2.3. EXPRESSION OF *Nr-CAM* IN BRAIN TUMOR CELL LINES

Expression of *hNr-CAM* in cell lines derived from several different kinds of human brain tumor was investigated. As shown in Figures 6 (A and B), *Nr-CAM* is expressed at high levels in astrocytoma IV, glioma,

glioblastoma and neuroectodermal tumor cell lines as compared
to NBT. Low or no expression of *hNr-CAM* was observed in cell
lines derived from astrocytoma III, medulloblastoma,
neuroblastoma and NBT. The *hNr-CAM* transcript was not
detected in FNHAs or NIH3T3 cells (data not shown).

6.2.4. EXPRESSION OF *Nr-CAM* IN NORMAL HUMAN BRAIN

Seven different regions of the brain and spinal
cord were studied for *hNr-CAM* expression. As shown in
Figures 7 (A and B), a natural transcript of *hNr-CAM* (7.5 kb)
was observed in cerebellum, occipital lobe, cerebral cortex
and frontal lobe at a higher level as compared to spinal
cord, medulla, temporal lobe or putamen. Expression of *hNr-CAM* detected is low as compared to tumor cell lines (Figures
8 (A and B) or the other tumor-associated gene (D2-2)
previously studied. Expression of *hNr-CAM* is highest in
kidney, and no significant difference in expression between
fetal and adult tissues was observed (data not shown).

6.2.5. EXPRESSION OF *Nr-CAM* IN HUMAN TUMOR CELL LINES

As shown in Figures 8 (A and)B, *hNr-CAM* was
expressed at high levels in melanoma G361, lymphoblastic
leukemia (MOLT-4) and Burkitt's lymphoma Raji cell lines. A
low level of *hNr-CAM* expression was observed in promyelocytic
leukemia (HL-60), HeLa cell S3, chronic myelogenous leukemia
(K-562), colorectal adrenocarcinoma (SW480) and lung
carcinoma (A549). All of the cell lines studied herein
expressed hNr-CAM mRNAs that are 1.4 kb as compared to the
7.5 kb transcript expressed in normal brain (Figures 5 (A and
B). HeLa cells S3 express low levels of both transcripts.
Melanoma G361 express high levels of the 7.5 kb and low
levels of the 1.4 kb transcript, suggesting alternative
splicing of hNr-CAM mRNA during tumorigenesis.

Using Northern blot analysis as described in
Section 6.1.4, expression of Nr-CAM was investigated in 8
different human tumor cell lines.

5   **6.2.6.   EXPRESSION OF *Nr-CAM* IN HUMAN BRAIN
                        TUMORS USING *IN SITU* HYBRIDIZATION**

In situ hybridization as described above was
performed on a panel of 20 different brain tumors using anti-
sense and sense *hNr-CAM*-specific probes. Results from this
study are shown in Table 2.
10

15

20

25

30

TABLE 2

EXPRESSION OF HNR-CAM IN HUMAN BRAIN TUMORS: PRESENCE OR ABSENCE OF HNR-CAM IS SHOWN BY POSITIVE OR NEGATIVE SIGNS

TISSUE	hNr-CAM
Glioblastoma multiforme	+
	+
	+
	+
	−
	−
	−
	−
Genicystic anaplastic astrocytoma	−
Meningotheliomatous meningioma	+
Normal brain	−
	−
	−
	−
Glioma III/IV	+
Pilocystic astrocytoma	+
Malignant glioma	−
Lipidized meningioma	+
Meningioma syncytial type	+
Meningioma	−
Fibroblastic meningioma	−
Meningotheliomatous meningioma, grade I	+
Genicystic astrocytoma	−
Oligodendroglioma, grade II	+

Eleven of the 20 tumors (55%) showed positive signal for *hNr-CAM*. Four NBT samples did not show any *hNr-*

CAM expression. None of the early grade astrocytomas and
only 50% of the highly anaplastic astrocytomas showed *Nr-CAM*
expression. Similar *hNr-CAM* expression was observed in brain
tumor cell lines (Figures 6 (A and B)). *hNr-CAM* expression

5 was observed in 50% of meningiomas tested and one
oligodendroglioma. These results demonstrate that expression
of *hNr-CAM* is prevalent in malignant glioma tissue.

6.2.7. GENE AMPLIFICATION

10   Genomic Southern blot was performed as described in
Section 6.1.7 on 3 brain tumor cell lines (astrocytoma III,
glioma and glioblastoma) and the NIH3T3 cell line. As shown
in Figures 9 (A and B), no change in the genetic level of
hNr-CAM was observed in the 4 cell lines tested.

15   These results indicate that the over-expression of
hNr-CAM in brain tumors is not due to gene amplification.

      A 1.4 kb transcript of *hNr-CAM* was observed
presently in tumor cell lines as compared to a 7.5 kb
transcript in normal brain (Lane *et al.*, 1996). This may
indicate that the 7.5 kb transcript for *hNr-CAM* generates a

20 1.4 kb transcript that could translate into a small version
of the hNr-CAM protein, which may be tumor-specific. On the
basis of data presented in this study, we conclude that *hNr-
CAM* is over-expressed in human malignant brain tumors and
that it is useful to serve as a marker for detection and for

25 therapy.

7.   EXAMPLE: EFFECT OF REGULATING Nr-CAM EXPRESSION IN GLIOBLASTOMA

      In order to assess the functional role of *Nr-CAM* in
brain tumorigenesis, the effects of over-expressing *Nr-CAM* in

30 the anti-sense direction were examined in a glioblastoma cell
line.

7.1.　MATERIALS AND METHODS

7.1.1.　CLONING OF ANTISENSE Nr-CAM

The full length clone for Nr-CAM was provided by William Dryer (CalTech). Three different portions of the

5 *hNr-CAM* gene were cloned in the antisense direction.

To obtain antisense "Nr-CAM 1/3 clone", Nr-CAM 1/3 (corresponding to nucleotides beginning at nucleotide 119 and ending at nucleotide 1434 of Figure 2A) was amplified using primers BT306 (5' TAGATACAACTAGTCTAATGCAGCTTAAAATAATGCC 3')

10 (SEQ.ID. No.: 18) and BT307 (5' AGATAGATCCGCGGATATCCATATTCATTAGAGGCATTG 3') (SEQ. ID. No. 19) (see Figure 2A) and cloned into precut pCMVneo vector cut with SACII and SPEI restriction enzymes. PCR amplification was carried out for 1 cycle at 94°C 3 min, 61°C 1 min, 72°C 4 min, then for 30 cycles at 94°C 1 min, 61°C 1 min, 72°C 4

15 min followed by 1 cycle at 94°C 1 min, 61°C 1 min, 72°C 10 min. The PCR product was cut with Spel and Sacil, and cloned in the antisense direction into the pCMV-neo vector precut with SacII and Spel enzymes. Orientation of the *hNr-CAM* gene was confirmed by restriction digestion of specific enzymes.

20 This clone was termed "pCMV-1/3Nr-AS".

A 1.3Kb fragment of the *hNr-CAM* gene (spanning the first 1/3 part of the gene) was cloned in the antisense direction into precut pCMVneo vector (See Figure 2D). This vector contains a constitutively active cytomegalovirus

25 promoter and has been used in the past to over-express genes in cells (Huang, *et al.*, 1997, *Int J Cancer*, 72:102-109). This clone was also termed "pCMV-1/3Nr-AS". The pCMV-neo or pCMV-1/3Nr-AS were then transfected into the 5GB glioblastoma cell line (ATCC# 2020-CRL), and selected in G418.

30 To obtain anti-sense Nr-CAM 2/3 clone, Nr-CAM 2/3 (corresponding to nucleotides 1410-2746 of Figure 2A) was amplified using primers BT308 (5' TAGATACAACTAGTCAATGCCTCTAATGAATATGGATA 3') SEQ. ID. No.: 20);

and BT309 (5' AGATAGATCCGCGGAATAGTAAATCCGATAGCCTTGTA 3') (SEQ.
ID. No.: 21) cut with Spe I and SacII, and cloned into precut
pCMVneo vector cut with Sac II and Spe I enzymes.

7.1.2. TRANSFECTION OF GLIOBLASTOMA (GB)

GB cells were plated at an approximate density of 3
x 10^4. 24 hours after plating the cells were washed with
serum-free media and transfected with lipofectamine reagent
plus plasmid DNA diluted in 1 ml total of serum-free media.
Cells were incubated at 37°C for hours after which the
reagent was replaced with media containing 10% FBS. Cells
were incubated at 37°C for 72 hours. Media was changed
adding 1000 μg/ml G418 a selective media to select for
resistance to neomycynin (GIBCO-BRL). Cells were incubated
at 37°C for 72 hours. Media was changed adding 1000 μg/ml
G418. Cells were incubated at 37°C for 96 hours. Media was
changed adding 1000 μg/ml G418. Cells were incubated at 37°C
for 72 hours. At this point all the cells in the control
plate were dead. At this point, media was changed, adding
400 μg/ml G418. Cultures were maintained at 400 μg/ml G418
indefinitely, changing media every 72-96 hours.

7.1.3. CELL MORPHOLOGY

During maintenance, cells transfected as described
in Section 7.1.2 above, were split at a ratio of 1:2-1:3.
After 72-96 hours, photographs of the cells were taken to
compare the morphology.

7.1.4. GROWTH ASSAY

On Day 0, 5GB, 5GBpCMV neo, 5GBNr-CAM 1/3, 5GBNr-
CAM 2/3 cells were trypsinized, counted on a Coulter Counter
and plated at a density of 1 x 10^4 cells/60 mm dish. 12
plates of each condition were plated. At each time point 3

plates of each condition were counted on the Coulter Counter and the counts averaged.

7.1.5. SOFT AGAR ASSAY

Soft agar assay, a common *in vitro* phenotype of transformation, was performed as described previously (Huang *et al.*, 1995, Cancer Research 55:5054-5062). Briefly, 5GB cells that were transfected with vector alone and with Nr-CAM in anti-sense direction were trypsinized. Approximately, $1X10^5$ cell were mixed with 0.26% agar. Cells were then plated on top of a layer of 0.65% agar in 60mm petri dishes and incubated 37°C for 2 weeks. Cells were fed with one ml media containing 10% FBS after 1 week. Colonies were stained and counted under the inverted light microscope.

7.1.6. NORTHERN BLOT ANALYSIS

PolyA+ mRNA was prepared from 5GB glioblastoma cells transfected with pCMVneo or pCMV-1/3Nr-AS cells using the Quick Prep mRNA Purification Kit (Pharmacia Biotech, Piscataway NJ). $2\mu g$ of polyA+ RNA was run in two lanes. Approximately $1x10^6$ cpm/ml of labeled *hNr-CAM* probe was added to the blot and hybridized for 18 hours at 68°C. After washing in 0.1% SSC, 0.1% SDS, the blot was visualized using a Phosphor Imager (Molecular Dynamics) and the signal quantified using ImageQuant software (Molecular Dynamics). After quantification, the blot was stripped by incubating at 90°C for 4 minutes in 0.1% SDS solution. The membrane was then prehybridized for 6 hours at 68°C and then treated with a probe for *β-actin* at a concentration of $5x10^5$ cpm/ml for 18 hours at 68°C. After washing, the blot was analyzed using the Phosphor Imager as described above. The volumes of the images for each probe were compared and normalized for *β-actin* signal.

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7.2.   RESULTS

7.2.1.   EFFECT OF EXPRESSION OF ANTI-SENSE Nr-CAM ON MORPHOLOGY

To study the role of the *Nr-CAM* gene in cell
transformation, Nr-CAM was over-expressed in 5GB cells in the
anti-sense direction. The Nr-CAM antisense construct
designated "Nr-1/3AS" was used. Approximately 10μg of pure
DNA was transfected onto two 60mm-diameter petri-dishes
containing 10,000 cells using lipofectamine (Gibco/BRL).
Transfected cells were selected in G418 (1000 μg/ml) for 2
weeks. After 3 weeks, cells were maintained in 400μg/ml
G418. Cell morphology was observed under the inverted light
microscope and cell proliferation properties of transfected
cell were analyzed by counting cell number at various
intervals. Results are presented in Figures 10A and B.
Figure 10A shows 5GB pCMVneo cells 96 hours after media
change. Figure 10B shows 5GBNr-CAM 1/3 cells 96 hours after
media change. No change in cell growth and morphology was
observed in glioblastoma cells transfected with pCMV-neo
vector (control) but cells transfected with Nr-CAM 1/3 in
anti-sense direction (pCMV-neoCA) showed a change in cell
morphology and slower cell proliferation (see Figure 10B).
Our results indicate that Nr-CAM over-expression in the
antisense direction blocked Nr-CAM gene further in the 5GB
glioblastoma cell line. This result strongly suggests that
Nr-CAM expression is required for continuous proliferation of
5GB cells.

In another set of experiments, the expression of
antisense expression of Nr-CAM on GB morphology was evaluated
in 5GB cells using the pCMV-1/3 Nr-AS construct.

5GB glioblastoma cells were plated at an
approximate density of $3 \times 10^4 \bullet$ 24 hours after plating the
cells were washed with serum-free media and transfected with
lipofectamine reagent plus plasmid DNA diluted in 1 ml total

of serum-free media. Cells were incubated at 37°C for 5
hours after which the reagent was replaced with media
containing 10% FBS. Cells were incubated at 37°C for 72
hours. Media was changed to the one containing 1000 μg/ml

5 G418. Cells were incubated at 37°C for 7 days. At this
point, media was changed to the one containing 200 μg/ml
G418. Cultures were maintained at 200 μg/ml G418
indefinitely, changing media every 72-96 hours. 96 hours
after plating cells, photographs were taken to compare the
morphology and are presented in Figures 15 (A-D).

10
       After transfection of antisense hNr-CAM (pCMV-
1/3Nr-AS), glioblastoma cells were selected in G418 media for
two weeks (1000μg/ml). Untransfected 5GB cells (PCMV-neo)
were used as controls. Cell morphology was compared between
pCMV-neo or pCMV-1/3Nr-AS transfected cells after four weeks

15 of selection. The glioblastoma cells transfected with
antisense hNr-CAM became spindle shaped and showed neurite
outgrowth (compare Figures 15 A and B with Figures 15 C and
D). 5GB cells transfected with pCMV-1/3Nr-AS were grown in
culture for 3 weeks and they demonstrated lack of density

20 dependent inhibition of cell proliferation.
       One unique observation in maintaining the hNr-CAM
antisense transfected cells is that when fresh media
containing 10% fetal bovine serum (FBS) is added to these
cells, their morphology changes temporarily to one that is
similar to pCMV-neo transfected 5GB cells. Both pCMV-neo and

25
pCMV-1/3Nr-AS transfected cells were treated with different
concentrations of serum (0.1, 1, 2 and 5% FBS). As shown in
Figure 16, 2% FBS is sufficient to cause a change in pCMV-
1/3Nr-AS transfected 5GB cells similar to pCMV-neo
transfected cells. No change in cell morphology was observed

30 in cells transfected with pCMV-neo. This result suggests that
one or a combination of more than one growth factors in the

serum transiently reverses the morphology enforced by the
anti-sense *hNr-CAM*.

7.2.2.   EFFECT OF EXPRESSION OF ANTI-SENSE
Nr-CAM ON CELL PROLIFERATION

The effect of Nr-CAM expression in the anti-sense
direction on glioblastoma proliferation was evaluated as
described above in Section 7.1 using anti-sense Nr-CAM 1/3 or
pCMV-1/3Nr-AS. Results are illustrated in Figure 11 and
Figure 17.

As shown in Figure 11, expression of anti-sense Nr-
CAM inhibited proliferation of GB cells compared to GB cells
containing vector only (GB/pFCS).

As shown in Figure 17, 5GB cells transfected with
pCMV-1/3Nr-AS proliferate slowly as compared to pCMV-neo
transfected cells. This result clearly demonstrates that
hNr-CAM is required for continuous proliferation of cells.
Even though 5GB cells transfected with pCMV-1/3Nr-AS
proliferate slowly, they maintained their spindle shape
morphology.

7.2.3.   SOFT AGAR COLONY FORMATION OF Nr-CAM
ANTISENSE EXPRESSING CELLS

Results presented in Figure 12 demonstrate that
expression of Nr-CAM in the anti-sense direction (GM-Anti-Nr-
CAM) inhibits the number of soft agar colonies compared to
results observed with non-transfected GB cells (GB) and GB
cells transfected with control plasmid only (GB-PFS). As
illustrated, overexpression of antisense hNr-CAM caused 81%
inhibition in number of soft agar colonies formed. Colonies
formed by untransfected 5GB cells and control transfected 5GB
cells with pCMV-neo were larger than those expressing hNr-CAM
antisense.

7.2.4.   EXPRESSION OF Nr-CAM IN hNr-CAM-ANTISENSE EXPRESSING CELLS

The expression of *hNr-CAM* in cells expressing *hNr-CAM* antisense was evaluated using the Northern blot analysis technique described in Section 7.1.6.

As shown in Figure 13, over-expression of the *hNr-CAM* anti-sense caused approximately 60% reduction in the native *hNr-CAM* expression. A logical explanation of this could be that in antisense *hNr-CAM* transfected cells, the natural transcript is made constitutively and a percentage of it is detected by Northern blot analysis regardless of RNase mediated degradation of antisense *hNr-CAM* bound to natural transcript (See Figure 14).

7.2.5.   CELL CYCLE ANALYSIS OF CELLS EXPRESSING hNr-CAM ANTISENSE

To study cell cycle status of 2CMV-neo and pCMV-1/3Nr-AS transfected cells, flow cytometry was performed on 5GB cell cycle status. Approximately 6×10^6 5GB cells transfected with pCMVneo, or pCMV-Nr1/3AS cells were harvested by trypsinization followed by fixation in 80% ethanol (vol/vol) fixative (Sigma, St. Louis MO) and incubated for 24 hours at -20°C. The cells were then stained with Propidium Iodide for 30 minutes at room temperature in the dark. After filtering to remove debris, the cells were read on a FACS Calibur cell sorter (Becton Dickinson). Twenty thousand gated events were counted and the results analyzed using ModFit Lt 2.0 software (Becton Dickinson). Results are presented in Table 3.

TABLE 3

EFFECT OF HNR-CAM ANTISENSE OVER-EXPRESSION

CELL CYCLE PHASES	% OF CELLS
pCMV-neo Transfected Cells	
G0-G1	72.76
G2-M	10.00
S	17.24
G2-G1	1.83
pCMV-1/3Nr-AS Transfected Cells	
G0-G1	89.98
G2-M	3.66
S	6.36
G2-G1	1.83

As shown in Table 3, a 63% decrease in S phase, 20% increase in G0-G1 phase and a 63.4% decrease in G2-M was observed in pCMV-1/3Nr-AS transfected cells as compared to pCMV-neo transfected cells. This set of results clearly demonstrates that antisense *hNr-CAM* causes a lengthening of specific phases of 5GB glioblastoma cells, *i.e.*, causes lengthening of the cell cycle.

7.2.6. EFFECT OF ANTISENSE *hNR-CAM* ON MIGRATION AND INVASION

Glioblastoma cells are highly invasive and they penetrate into surrounding normal brain tissue during their genesis (Kleihues and Cavenee, Pathology and genetics of tumors of the nervous system. Lyon, France: International Agency for Research on Cancer, 1997). To determine if antisense *hNr-CAM* could alter the migration capacity of glioblastoma cells, a cell migration assay was performed on

5GB cells transfected with pCMV-neo or pCMV-1/3Nr-AS. Equal
number of cells (1x10^6) were plated on a 8μm pore size
polycarbonate membrane filter. Cells that migrated through
the membrane after 3 days were counted after fixation and
5 staining with hematoxylin. Results are shown in Figure 18.

As shown in Figure 18, anti-sense *hNr-CAM* over-
expression caused a 30% inhibition in the migration ability
of 5GB glioblastoma cells.

In addition, an invasion assay was performed (See,
Ridder and Calliauw, 1992, *Neurosurgery*, <u>31</u>:1043-1048) to
10 determine if antisense *hNr-CAM* could inhibit the invasion
properties of glioblastoma cells.

Briefly, 825ng of ECM gel was coated on to 8μm pore
size polycarbonate membrane filter. Equal number of 5GB
cells (1x10^4) were plated on to the ECM gel. Cells that
15 migrated through the ECM gel after 4 and 7 days were counted
after fixation and staining with hematoxylin. Results are
presented in Figure 19.

As shown in Figure 19, approximately 90% inhibition
of cell invasion was observed in pCMV-1/3Nr-AS transfected
20 5GB cells as compared to pCMV-neo transfected cells.

7.2.7.   EFFECT OF RADIATION OF CELLS
ANTISENSE hNr-CAM

Tumor cells are in general more resistant to
radiation. To determine if treatment antisense hNr-CAM
25 transfected glioblastoma cells are UV radiation sensitive,
the following experiment was conducted. Approximately 1x10^4
glioblastoma cell (transfected either with pCMV-neo or pCMV-
1/3Nr-AS) were plated in triplicates in 60mm diameter
petridishes. Cells were then exposed to 100 units of UV
30 radiation. As observed 65% of antisense *hNr-CAM* transfected
cells died as compared to 27% death in pCMV-neo transfected
cells.

The percentage of surviving cells undergoing apoptosis was also determined. To do so, the UV radiation experiment was repeated and cells undergoing apoptosis were identified using Apoptosis kit from BMB (Indianapolis, IA).
5 Results are presented in Figure 20.

As shown in Figure 20, a 17 fold increase in the number of cells undergoing apoptosis was observed. These results clearly suggest that antisense hNr-CAM over-expression caused 5GB glioblastoma cells to become more sensitive to UV radiation.
10

7.2.8.   ANTISENSE hNr-CAM INHIBITS GB TUMOR GROWTH IN VIVO

In one experiment, designated "Experiment 1", 5 nude mice were subcutaneously with $3.0x10^6$ 5GB glioblastoma
15 cells (pCMV-neo or pCMV-1/3Nr-AS transfected). Results are presented in Table 4.

As shown in Table 4, tumor growth was observed in three of the five mice that were injected with 5GB (pCMV-neo transfected) cells. No tumor growth was observed in mice injected with pCMV-1/3Nr-AS transfected 5GB cells.
20

To increase the efficiency of this type of experiment, in another experiment, designated "Experiment 2", $1x10^7$ 5GB cells (transfected with pCMV-neo or pCMV1/3Nr-AS) were injected into seven nude mice. Tumor size was measured 38 days post-injection. Results are presented in Table 4.
25

In Experiment 2, six of seven mice injected with cells transfected with pCMV-neo developed tumor. No tumor growth was observed in mice that were injected with cells transfected with pCMV-1/3Nr-AS vector.

Photographic illustration of three examples (each)
30 of mice injected with pCMV-neo or pCMV-1/3Nr-AS vectors is presented in Figure 21.

Results from this experiment clearly demonstrated
that anti-sense *hNr-CAM* inhibits tumorigenic properties of
5GB glioblastoma cells *in vivo*. Injection of antisense hNr-
CAM expressing glioblastoma cells caused inhibition of tumor
5 formation.

TABLE 4

THE EFFECT OF ANTISENSE *NNR-CAM* EXPRESSION ON TUMOR FORMATION *IN VIVO*

Experiment 1		Experiment 2	
Mice injected (5GB glioblastoma cells)		Mice injected (5GB glioblastoma cells)	
pCMV-neo	pCMV1/3Nr-AS	pCMV-neo	pCMV1/3Nr-AS
Tumor Volume (mm³)		Tumor Volume (mm³)	
400	NT	75	NT
726	NT	650	NT
936	NT	63	NT
NT	NT	196	NT
NT	NT	365	NT
		196	NT
		NT	NT

NT = No Tumor
1 = In experiment 1, tumor size was measured 70 days post injection.
2 = In experiment 2, tumor size was measured 38 days post injection.

7.2.9. INTRATUMORAL INOCULATION OF PLASMID-EXPRESSING ANTISENSE *hNR-CAM* CAUSED REDUCTION IN GLIOBLASTOMA TUMOR GROWTH

To determine if antisense *hNr-CAM* could cause
30 reduction in tumor growth *in vivo*, the effect of direct
intra-tumoral injection of an antisense hNr-CAM expressing
plasmid mixed with liposomes was analyzed.

In a first set of experiments, three athymic nude
mice were injected with 1 x 10⁵ 5GB (glioblastoma) cells
subcutaneously. 72 days post-implantation, 50 μg of either
pCMVneo (control) (one animal) or pCMV1/3Nr-AS (two animals)
plasmids were mixed with DMRIE (liopsomes) reagent
(Gibco/BRL) and injected twice a week for four weeks around
the tumor site. Tumor size was measured twice a week with a
caliper and tumor volume was determined. Results are shown
in Figure 22A.

As demonstrated in Figure 22A, animals which
received anti-sense hNr-CAM injected directly into tmor
showed not only slower tumor growth but also tumor regression
compared to the control animal.

In another set of experiments, 20 athymic nude mice
were implanted with 3x3mm pieces of glioblastoma tumor. 28
days post implantation, 300μg of either pCMVneo or pCMV1/3Nr-
AS plasmids were mixed with DMRIE reagent (Gibco/BRL) to a
final of 300μl volume and injected twice a week for four
weeks around the tumor. Control mice were injected with the
same volume of 1xPBS or no treatment. Tumor size was
measured twice a week with a caliper and tumor volume was
determined. Results are shown in Figure 22B.

As shown in Figure 22B, direct intra-tumoral
injection of plasmid expressing the antisense *hNr-CAM* caused
slower tumor growth. Results from this set of experiments
demonstrate that targeting of the *hNr-CAM* gene is an
advantageous strategy for treating human glioblastoma tumors.

7.2.10. ROLE OF *hNr-CAM* IS NOT CONFINED TO ONE CELL LINE

As a model to study the role of *hNr-CAM* in
malignant gliomas, we used the 5GB glioblastoma cell line.
Glioblastoma tumor cells are heterogeneous and tumors
isolated from different patients show different genetic

characteristics. Thus, in order to demonstrate the fact that
hNr-CAM is a good genetic target for gene therapy of human
glioblastomas we demonstrated the anti-tumorigenic properties
of antisense *hNr-CAM* in different glioblastoma cell lines.

5   Using the antisense *hNr-CAM* expressing vector
(pCMV-1/3Nr-AS), we have not been able to block nHr-CAM
expression in GB 1690 glioblastoma cells. This could
possibly be due to cellular factors interfering with the
binding of the antisense molecule to the appropriate site on
hNr-CAM message.
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        To overcome this problem, we decided to target a
different region of the *hNr-CAM* gene.

        We have PCR amplified 1360 bases of the *hNr-CAM*
gene (position 1410-2746) and cloned it in the antisense
direction into precut pCMV-neo vector. This clone was termed
15 as pCMV-2/3Nr-AS (Spanning the 2/3rd region of *hNr-CAM* gene).
Preliminary results are shown in Figures 23 (A-C).

        The results obtained have demonstrated that pCMV-
2/3Nr-AS transfected GB1690 cells changed in cell morphology
and formed fewer numbers of soft agar colonies as compared to
20 pCMV-neo transfected cells (Figure 17).

        On the basis of this result, it is concluded that
hNr-CAM expression requirement for glioblastoma cell
proliferation and tumorigenic properties is not confined to
just one cell line.

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7.2.11.   ADDITIONAL STUDIES

        It is envisaged that anti-sense *hNr-CAM* can be
over-expressed in other human malignant glioma cell lines,
such as 1690-CRL, 16-HTB, 138-HTB and other species cells
such as, *e.g.*, two rat glioma cell lines, C6 glioma and 9L
30
gliosarcoma. To demonstrate the fact that antisense human
Nr-CAM can bind to rat *Nr-CAM* and inhibit its function, we
performed a nucleotide sequence comparison between the rat

and human *Nr-CAM* sequence. As shown in Figure 24, 87%
sequence similarity was observed between the human and rat
Nr-CAM nucleotide sequence. On the basis of this result, we
conclude that human *Nr-CAM* antisense molecule is capable of
5 binding to rat *Nr-CAM*.

It is further envisaged that antisense
phosphorothioate oligonucleotides can be used to inhibit the
expression of *hNr CAM* in glioblastoma cells.

Antisense phosphorothioate oligonucleotides can be
delivered effectively to several different regions of the
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brain using high-flow microinfusion technology. Targeting of
the *hNrCAM* gene using antisense phosphorothioate
oligonucleotides will be an effective way of treating human
glioblastoma tumors in a clinical setting.

As a non-limiting illustrative example, the
15 following is presented. Briefly, we have designed three
phosphorothioate oligonucleotides (ODNs) against the
translational initiation site of *hNr-CAM* (see Table 5 below).

Table 5 schematicly illustrates phosphorothioate
oligonucleotides (ODAs) for *hNr-CAM* gene. ODNs H-1, H-2 and
20 H-3 are designed against *hNr-CAM*; OL-4,OL-5 and OL-6 are
random ODNs that can be used as controls. ATG sequence is
indicated in bold. Three random ODNs that can serve as
controls (OL-4, OL-5 and OL-6) are available commercially
from Oliogos Etc. Inc. (Wilsonville, OR).

The effect of ODNs on inhibition of *hNr-CAM*
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expression can be evaluated using the methodology described
previously (Anfossi, *et al.*, 1989 *Proc Natl Acad Sci USA*,
<u>86</u>:3379-3383). Briefly, 5GB, HTB-16 and GB1690 cells are
plated per well in 96-well plates in media without ODNs.
Twenty-four hours later, the culture is media changed to
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contain a final concentration of 1mmole/L, 3mmol/L, or
10mmol/L ODNs. Control cultures receive fresh culture media
without ODNs. After 4-5 days post-transfection, cell

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proliferation is analyzed using a cell proliferation assay
kit from Promega (Madison, WI). Expression is analyzed using
immunocytochemistry methods described previously (Sehgal, et
al., 1998, *Int. J. Cancer*, 76(4):451-458). These
oligonucleotides are be tagged with fluorescent tags to
ensure their entry into the cells.

TABLE 5

ILLUSTRATIVE ODN'S FOR hNr-CAM

		SEQ. ID. No.
5'AGGAGTTAAGATGCTAATGCAGCTTAAAATA**ATG**CCGAAAAAGAAGCGCTTATCTGCGGGC3'	*hNr-CAM*	22
3'TCCTCAATTCTACGATTAC5' H-1		23
3'ACGTCGAATTTTATTACGGCT5' H-2		24
3'TTCTTCGCGAATAGACG5' H-3		25
5'ACTAGAGATACAGATCATAT3' OL-4		26
5'CATATACGATCGATCGATGC3' OL-5		27
5'GATAGTGCTGATCGATGCTA3' OL-6		28

It is further envisaged that the expression of *hNr-CAM* gene can be blocked using a replication defective retroviral system to deliver antisense *hNr-CAM*. Replication defective retroviral systems have been used in the past to deliver genes to a variety of tumor cell types (see, Kondo, et al., 1998, Cancer Res. 68:962-967; Boviaisis, et al., 1994, Human Gene Ther. 5:183-191).

The current state of retroviral gene transfer technology stems from the coordinated design of retroviral vectors and packaging cell lines. The development of packaging cell lines that package retroviral RNAs into infectious particles without the concomitant production of replication-competent virus created a new level of safety and control. To do this, the structural genes necessary for particle formation and replication, gag, pol, and env, were integrated into cell lines without the RNA packaging signal, psi+. Subsequent introduction of a retroviral vector

containing psi+, transcription and processing elements, and
the gene of interest produces high-titer, replication-
incompetent infectious virus. In other words, these
retroviral particles can infect target cells and transmit the
5 gene of interest, but cannot replicate within these cells
since they lack the viral structural genes. The separate
introduction and integration of the structural genes into the
packaging cell line minimizes the chances of producing
replication-competent virus due to recombination events
during cell proliferation.
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        As a non-limiting, illustrative exmmple, the
following is presented. A Retro-X™ system is used to deliver
and over-express antisense *hNr-CAM* gene in glioblastoma
cells. Retro-X™ system is a complete retroviral gene
expression system that can transduce up to 100% of cells.
15 Together with the RetroPackTM, PT67 cell line, the Retro-X
Vectors produce infectious, replication-incompetent
retrovirus that can be used to introduce a gene of interest
into a wide variety of mammalian cell types *in vitro* or *in
vivoy*. The highly efficient transduction machinery of
20 retroviruses can stably integrate the cloned gene into the
host genome of nearly all mitotically dividing cells. A
retroviral vector containing the gene of interest (*hNr-CAM*)
is first transfected into the packaging cell line.
Antibiotic selection can then be used to obtain a population
25 of cells that stably expresses the integrated vector and, if
desired, high-titer clones can be isolated from this
population. Virus produced by either stably transfected
cells can be used to infect target cells.

        The *hNr-CAM* gene (first 1.3Kb) is cloned into the
EcoRI and BamHI site of the pLXSN retroviral vector in
30 antisense direction. Human *hNr-CAM* specific primers (BT306,
5' CATAC<u>GAATTC</u>TAGATA CAACTAGTCTAATGCAGCTTAAAATAATGCC 3' SEQ.
ID. No.: 29; and BT307, 5' AGATAGATCCGCGGATATCCATATT

CATTAGAGGCATTGGGATCCCATAC 3' SEQ. ID. No.: 30) are used to
PCR amplify 1/3rd portion of the *hNr-CAM* gene. PCR is carried
out using Gene Amp PCR kit from Perkin Elmer (Branchburg, NJ)
under the following conditions: 4µl of dNTP mix, 2µl
(100mg/µl) each of *hNr-CAM* specific primers, 4µl of 25µM
$MgCl_2$, 125ng of cDNA template and 5 units of Amplitaq DNA
polymerase. PCR amplification is carried out for 1 cycle at
94°C 3 min, 61°C 1 min, 72°C 4 min, then for 30 cycles at 94°C
1 min 61°C 1 min, 72°C 4 min followed by 1 cycle at 94°C 1
min, 61°C 1 min, 72°C 10 min.

The PCR product is run on a 1% agarose gel. The
band is then cut out and DNA was purified using gene clean
system. DNA fragment is then digested with 5units/µl EcoRI
and BamHI for 2 hours at 37°C. Digested DNA is then run on a
2% agarose gel. The 1.3Kb fragment is then cut out, purified
and cloned in the antisense direction into EcoRI and BamHI
sites of the pLXSN retroviral vector. Orientation of the
cloned *hNr-CAM* gene is confirmed by sequencing. These
particular plasmids containing *hNr-CAM* anti-sense direction
are termed "pLXSN-1/3Nr-AS". PLXSN and pLXSN-1/3Nr vectors
are transfected into Retropack™ PT67 cell line using the
lipofectamine reagent. Cell lines are plated at a density of
3 x 10⁴ cells/60mm. Twenty four hours after plating the
cells are washed with serum-free media and transfected with
lipofectamine reagent plus plasmid DNA (5µg) diluted in 1 ml
of serum-free media. Cells are incubated at 37°C for 5 hours
after which the reagent is replaced with media containing 10%
FBS and cultures were incubated at 37°C for 72 hours. Media
is then changed to an identical medium but containing adding
1000 µg/ml G418 and incubated at 37°C for 96 hours. At this
point, the medium is changed to one containing 200 µg/ml
G418. Cultures are subsequently maintained in a medium
containing 200 µg/ml G418, changing the medium every 72-96
hours. Retroviral particles are then harvested by aspirating

the cell culture media into a sterile tube (approximately 10^5-10^6 recombinant virus particles/ml).

Viral titer is then determined using a protocol recommended by Clontech. Human glioblastoma cell lines (5GB, 1690-CRL, 1620-CRL) and two rat glioma cell lines C6 and 9L gliosarcoma cells are plated at a density of 3×10^5 in two 100mm plates. Viral particles harvested from PT67 cell line culture are filtered through a 0.45μm filter and added on to human and rat glioma cell lines. Polybrene is added to a final concentration of 4μg/ml and incubated for 48 hours at 37°C. Cells are harvested and analyzed for *hNr-CAM* expression using Northern blot analysis. Cells that are expressing low levels of *hNr-CAM* are expanded in culture.

Northern blot analysis for the expression of *hNr-CAM*. Cell clones that are expressing low level of *hNr-CAM* are expanded in culture. Approximately 1×10^7 glioblastoma cells 1690-CRL, 1620-CRL, HTB-16, C6, 9L gliosarcoma) will be injected subcutaneously into the flanks of ten female athymic nude mice (two sites each). Mice are then kept in a germ free environment. Tumor growth vill be analyzed every week for at least fourteen weeks and compared between anti-sense *hNr-CAM* and mock infected glioma cell lines.

It is appreciated that the physiological conditions under which a tumor develops in brain can be quite different from when it develops subcutaneously. As a non-limiting, illustrative example, the following is presented. The effect of *hNr-CAM* (antisense in 1690-CRL, 1620-CRL, HTB-16, C6, 9L cells) on tumor formation in brain can be assessed as described previously for a mutant EGFR (Nishikawa, et al., 1994, Proc. Natl. Acad. Sci. USA 91:7727-7731). Briefly, 5×10^5 cells transfected or infected with the antisense *hNr-CAM* in 50-100μl of 1xPBS are inoculated into the cerebral hemisphere (using stereotectic instrument) of eight nude mice for each cell line. As a positive control for tumor growth,

U87MGΔEGFR cells that are known to form tumors in nude mice brains, can be used (Nishikawa, *supra*). 5x10⁵ U87MGΔEGFR cells are inoculated into the cerebral hemisphere of nude mice. Another group of nude nice are injected with pCMV-neo

5 transfected or mock infected 1690-CRL, 1620-CRL, HTB-16, C6, 9L cells. Brains from all of these mice are removed at two week intervals, embedded in OCT compound, frozen in liquid nitrogen and stored at -80°C. 6μm sections are cut on a cryostat and immunocytochemistry are performed, for example,

10 using the three markers described below.

GFAP (Glial Fibrillary Acidic Protein): Malignant glioma cells are known to cause gliosis of the surrounding tissue upon invasion (Mikkelsen, et al., 1998, Brain Tumor, Invasion, Bilogical, Clinical and Therapeutic Considerations,

15 Wiley Liss, N.Y.). Thus, the tissue area at the edge of originally transplanted glioblastoma cells and the mice brain tissue, can be stained by performing immunocytochemistry for the GFAP protein. After recovery of the brain tissue it is embedded in OCT blocks. Several 6μm sections are cut on a

20 cryostat. After washing sections in 1xPBS buffer, 200 μl of diluted (1:80) rabbit anti-human GFAP are applied to slides. Slides are incubated for 18 hours at 4°C in a humid chamber. After washing in 1xPBS, FITC conjugated anti-rabbit immunoglobulins (1:50) (DAKO, A/S, Denmark) are applied and

25 the slides are incubated at 24°C for 30 minutes in a humid chamber. Cells are washed with 1xPBS and then stained with Hematoxylin (Richard Allen Scientific, Richland MI) for 30 seconds. Slides are then treated with a clarifying agent (Richard Allen Scientific, Richland MI) for 2 seconds and then in bluing agent (Richard Allen Scientific, Richland MI).

30 After washing in water, slides are coverslipped with 2% DABCO (Sigma, St. Louis MO) in 50% glycerol/1xPBS, and visualized with a Zeiss Axioskop UV microscope.

Cathepsin B (CB): CB is a cysteine protease that is expressed in gliomas directly in relation to grade of malignancy. CB is capable of degrading proteoglycans, a major component of the brain extracellular matrix, and could

5 be involved in the process of glial tumor cell invasion into peritumoral normal brain (Mikkelsen, *supra*). Each of the inoculated glioma cells (GB1690, C6, 9L cells transfected with pCMV-neo or pCMV-1/3Nr-AS) can examined for CB expression by performing immunocytochemistry for CB protein.

10 It has been shown that the relative degree of granular stain for glioma cell lines immunocytochemically *in vivo* correlates directly to the agree of invasiveness of the tumor displayed. To study the CB expression by immunocytochemistry, the same protocol as described above for the GEAP protein is followed. Human glioblastoma cell line U251MGn can be used as a

15 positive control for CB staining.

Ki67: Ki67 is a protein that is expressed at high levels in actively dividing cells (Mikkelsen, *supra*).
Immunocytochemistry can be performed on mouse brain sections

20 against the Ki67 antigen using a rabbit anti-human antibody. The protocol for immunocytochemistry is the same as described above for the GFAP protein. U251MGn glioblastoma injected mice brain sections can be used as positive control for Ki67 specific staining. Direct comparison of the staining of

25 these three markers provides the information about the extent of invasion of inoculated tumor cells.

    8.   **EXAMPLE: IDENTIFICATION OF GENES ALTERED BY hNr-CAM**

        In order to identify genes that are altered by the *hNr-CAM* gene product in 5GB gliioblastoma cells, we compared

30 the expression of 5000 genes in pCMV-neo or pCMV-1/3Nr-AS transfected 5GB glioblastoma cells using the Array technique. Two identical Human GeneFilters™ were differentially

hybridized with cDNA prepared from pCMV-neo or pCMV-1/3Nr-AS
transfected 5GB glioblastoma cells. Two identical array
membranes containing 5000 genes were purchase dfrom REsearch
Genetics. The membranes were prehyridized in a pre-

5 hybridization solution for 12 hours. Hybridization was done
with a 1x105 cpm/ml cDNA probe. Ths probe was prepared by
carrying out 1st strand synthesis from pCMV-neo or pCMV-
1/3Nr-AS transfected 5GB glioblastoma cells 1μg polyA+mRNA.
First strand cDNA synthesis was carried out using the
Advantage cDNA synthesis kit from Clontech. The membranes

10 were washed in a wash solution (0.1%SDS/1XSSC) for 30 minutes
at room temperature and then at 50°. Membranes were then
exposed to X-ray film. Results are presented in Figures 26
(A and B).

         As shown in Figures 26 (A and B), two genes were

15 identified that were differentially expressed. Selectin
(endothelial adhesion molecule 2) was detected in pCMV-neo
transfected cells (Figure 26B) and not n pCMV-1/3Nr-AS
(Figure 26A) transfected 5GB cells. A novel gene (accession
H7785) was detected in pCMV-1/3Nr-AS (Figure 26A)

20 transfected and not in pCMV-neo transfected (Figure 26B) 5GB
cells. We are not only interested in exploring the role of
these genes in glioblastoma cells in the context of *hNr-CAM*
over-expression but also inunderstanding the mechanism by
which *hNr-CAM* modulates the expression of these genes.

25   The present invention is not to be limited in scope
by the microorganism deposited or the specific embodiments
described herein. Indeed, various modifications of the
invention in addition to those described herein will become
apparent to those skilled in the art from the foregoing
description and accompanying figures. Such modifications are

30 intended to fall within the scope of the appended claims.

Various publications are cited herein, the
disclosures of which are incorporated by reference in their
entireties.

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WHAT IS CLAIMED IS:

1.   A pharmaceutical composition for the inhibition or
treatment of tumorigenesis comprising an antisense nucleic
acid complementary to at least a portion of an RNA transcript
of a *Nr-CAM* gene in an amount effective to inhibit
hyperproliferation of a tumor cell.

2.   A pharmaceutical composition for the inhibition
or treatment of tumorigenesis comprising an antibody to Nr-
CAM in an amount effective to inhibit hyperproliferation of a
tumor cell.

3.   A method of treating, inhibiting or preventing
a disease or disorder involving cell overproliferation in a
subject comprising administering to a subject in which such
treatment or prevention is desired an effective amount of a
molecule that inhibits Nr-CAM function.

4.   The method according to claim 3 in which the
disease or disorder is a malignancy.

5.   The method according to claim 3 in which the
disease or disorder is selected from the group consisting of
brain cancer, leukemia and B cell lymphoma.

6.   The method according to claim 3 in which the
subject is a human.

7.   The method according to claim 5 in which the
brain cancer is selected from the group consisting of
glioblastoma, glioma, meningioma, astrocytoma,
medulloblastoma, neuroectodermal cancer and neuroblastoma.

8.   The method according to claim 7 in which the glioblastoma is glioblastoma multiforme.

9.   The method according to claim 3 in which the disease or disorder is selected from the group consisting of premalignant conditions, benign tumors, hyperproliferative disorders, and benign dysproliferative disorders.

10.   The method according to claim 3 in which the molecule that inhibits Nr-CAM function is selected from the group consisting of an anti-Nr-CAM antibody or a fragment thereof, a Nr-CAM derivative or analog that is capable of being bound by an anti-Nr-CAM antibody, a Nr-CAM antisense nucleic acid, and a nucleic acid comprising at least a portion of a *Nr-CAM* gene into which a heterologous nucleotide sequence has been inserted such that said heterologous sequence inactivates the biological activity of at least a portion of the *Nr-CAM* gene, in which the *Nr-CAM* gene portion flanks the heterologous sequence so as to promote homologous recombination with a genomic *Nr-CAM* gene.

11.   The method according to claim 3 in which the molecule that inhibits Nr-CAM function is an oligonucleotide which (a) consists of at least six nucleotides; (b) comprises a sequence complementary to at least a portion of an RNA transcript of a *Nr-CAM* gene; and (c) is hybridizable to the RNA transcript under moderately stringent conditions.

12.   A method of inhibiting, treating or preventing a disease or disorder involving cell proliferation in a subject comprising administering to a subject in need of such treatment an effective amount of a molecule that promotes Nr-CAM function.

13. The method according to claim 12, in which the
disease or disorder is selected from the group consisting of
degenerative disorders, growth deficiencies,
hypoproliferative disorders, physical trauma, lesions, and
5 wounds.

14. A method of diagnosing a disease or disorder
characterized by an aberrant level of Nr-CAM RNA or protein
in a subject, comprising measuring the level of Nr-CAM RNA or
protein in a sample derived from the subject, in which an
10 increase or decrease in the level of Nr-CAM RNA or protein,
relative to the level of Nr-CAM RNA or protein found in an
analogous sample from another subject not having the disease
or disorder, indicates the presence of the disease or
disorder in the subject.
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15. A method of diagnosing or screening for the
presence of or a predisposition for developing a disease or
disorder involving cell overproliferation in a subject
comprising detecting Nr-CAM DNA, RNA or protein derived from
20 the subject in which the presence of said Nr-CAM DNA, RNA or
protein indicates the presence of the disease or disorder or
a predisposition for developing the disease or disorder.

16. A kit comprising in one or more containers a
molecule selected from the group consisting of an anti-Nr-CAM
25 antibody, a nucleic acid probe capable of hybridizing to a
Nr-CAM RNA, a pair of nucleic acid primers capable of priming
amplification of at least a portion of a Nr-CAM nucleic acid,
an anti-Nr-CAM ligand antibody, a nucleic acid probe capable
of hybridizing to an Nr-CAM ligand encoding RNA and a pair of
30 nucleic acid primers capable of priming amplification of at
least a portion of an Nr-CAM ligand encoding nucleic acid.

17.   A pharmaceutical composition for the
inhibition or treatment of tumorigenesis comprising an
antisense nucleic acid complementary to at least a portion of
an RNA transcript of an Nr-CAM ligand encoding gene in an
5 amount effective to inhibit hyperproliferation of a tumor
cell.

18.   A pharmaceutical composition for the
inhibition or treatment of tumorigenesis comprising an
antibody to an Nr-CAM ligand encoding gene in an amount
10 effective to inhibit hyperproliferation of a tumor cell.

19.   A method of inhibiting, treating or preventing
a disease or disorder involving cell overproliferation in a
subject comprising administering to a subject in which such
15 treatment or prevention is desired an effective amount of a
molecule that inhibits an Nr-CAM ligand encoding gene
function.

20.   A method of diagnosing a disease or disorder
20 characterized by an aberrant level of an Nr-CAM ligand
encoding RNA or protein in a subject, comprising measuring
the level of an Nr-CAM ligand encoding RNA or protein in a
sample derived from the subject, in which an increase or
decrease in the level of said RNA or protein, relative to the
level of said RNA or protein found in an analogous sample
25 from another subject not having the disease or disorder,
indicates the presence of the disease or disorder in the
subject.

21.   A method of diagnosing or screening for the
30 presence of or a predisposition for developing a disease or
disorder involving cell overproliferation in a subject
comprising detecting an Nr-CAM ligand encoding DNA, RNA or

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protein derived from the subject in which the presence of
said DNA, RNA or protein indicates the presence of the
disease or disorder or a predisposition for developing the
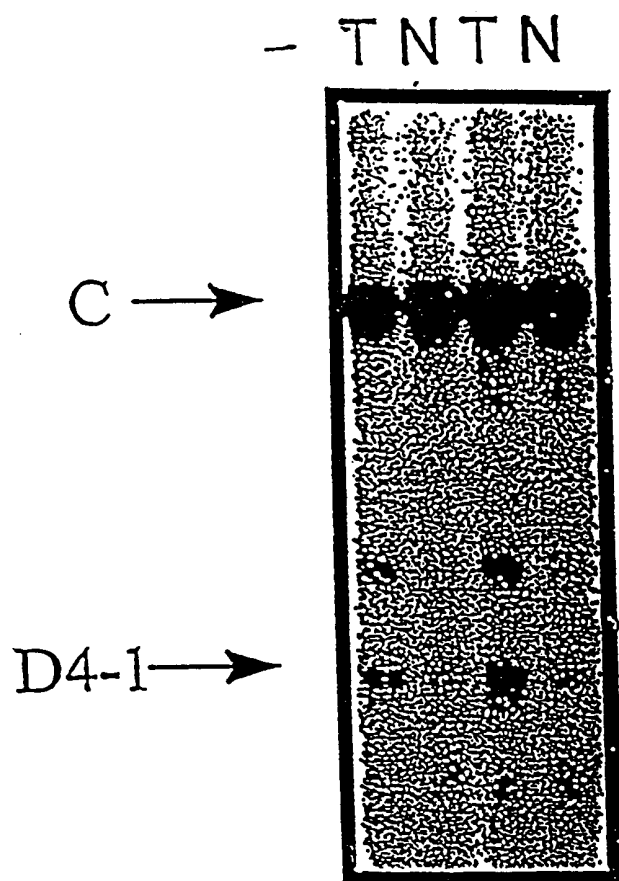disease or disorder.

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FIGURE 1

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   1 cttcaaagtt ccccgcatga aaattactta aacgttgcac acaacgtttc cgaaaatctt
  61 ttgtgaaaga agaaaaggaa attcagtgtg tgagtctcag caggagttaa gctaatgcag
 121 cttaaattga tgccgaaaaa gaagcgctta tctgcgggca gagtgccct gattctcttc
 181 ctgtgccaga tgattagtgc actggaagta cctcttgatc caaaacttct tgaagacttg
 241 gtacagcctc caaccatcac ccaacagtct ccaaaagatt acattattga ccctcgggag
 301 aatattgtaa tccagtgtga agccaaaggg aaaccgcccc caagcttttc ctggacccgt
 361 aatgggactc attttgacat cgataaagac cctctggtca ccatgaagcc tggcacagga
 421 acgctcataa ttaacatcat gagcgaaggg aaagctgaga cctatgaagg agtctatcag
 481 tgtacagcaa ggaacgaacg cggagctgca gtttctaata acattgttgt ccgcccatcc
 541 agatcaccat tgtggaccaa agaaaaactt gaaccaatca cacttcaaag tggtcagtct
 601 ttagtacttc cctgcagacc cccaattgga ttaccaccac ctataatatt ttggatggat
 661 aattcctttc aaagacttcc acaaagtgag agagtttctc aaggtttgaa tggggacctt
 721 tattttcca atgtcctccc agaggacacc cgcgaagact atatctgtta tgctagattt
 781 aatcatactc aaaccataca gcagaagcaa cctatttctg tgaaggtgat ttcagtggat
 841 gaattgaatg acactataga tgctaatttg agtgacactg agttttatgg tgctaaatca
 901 agtagagaga ggccaccaac attttttaact ccagaaggca atgcaagtaa caaagaggaa
 961 ttaagaggaa atgtgctttc actggagtgc attgcagaag gactgcctac cccaattatt
1021 tactgggcaa aggaagatgg aatgctaccc aaaaacagga cagtttataa gaactttgag
1081 aaaaccttgc agatcattca tgtttcagaa gcagactctg gaaattacca atgtatagca
1141 aaaaatgcat taggagccat ccaccatacc atttctgtta gagttaaagc ggctccatac
1201 tggatcacag ccctcaaaa tcttgtgctg tccccaggag aggatgggac cttgatctgc
1261 agagctaatg gcaaccccaa acccagaatt agctggttaa caaatggagt cccaatagaa
1321 attgccctg atgacccag cagaaaaata gatggcgata ccattgtttt ttcaaatgtt
1381 caagaaagat caagtgcagt atatcagtc taatgcctctt atgactatgg atattactg
1441 gcaaacgcat ttgtaaatgt gctggctgag ccaccacgaa tcctcacacc tgcaaacaca
1501 ctctaccagg tcattgcaaa caggcctgct ttactagact gtgccttctt tgggtctcct
1561 ctcccaacca tcgagtggtt taaaggagct aaaggaagtg ctcttcatga agatatttat
1621 gttttacatg aaaatggaac tttggaaatc aagatgctca catggatcgt taaagaaatt
1681 cctgtggccc aaaaggacag tacaggaact tatacgtgtg ttgcaaggaa taaattaggg
1741 atggcaaaga atgaagttca cttacagccc gaatatgcag ttgtgcaaag agggagcatg
1801 gtgtcctttg aatgcaaagt gaaacatgat cacaccttat ccctcactgt cctgtggctg
1861 aaggacaaca gggaactgcc cagtgatgaa aggttcactg ttgacaagga tcatctagtg
1921 gtagctgatg tcagtgacga tgacagcggg acctacacgt gtgtggccaa caccactctg
1981 gacagcgtct ccgccagcgc tgtgcttagc gttgttgctc ctactccaac tccagctccc
2041 gtttacgatg tcccaaatcc tccctttgac ttagaactga cagatcaact tgacaaaagt
2101 gttcagctgt catggaccc aggcgatgac aacaatagcc ccattacaaa attcatcatc
2161 gaatatgaag atgcaatgca caagccaggg ctgtggcacc accaaactga agttttctgga
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2281 gcagtgaaca gcattgggaa gagcttgccc agcgaggcgt ctgagcagta tttgacgaaa
2341 gcctcagaac cagataaaaa ccccacagct gtggaaggac tgggatcaga gcctgataat
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2461 aaagttagct ggcgccagaa agatggtgat gatgaatgga catctgtggt tgtggcaaat
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2581 gccctgaatg acatgggggtt tgcccccgag ccagctgtag tcatgggaca ttctggagaa
2641 gacctcccaa tggtggctcc tgggaacgtg cgtgtgaatg tggtgaacag tacttagcc
2701 gaggtgcact gggacccagt acctctgaaa agcatccgag gacacctaca aggctaggg
2761 atttactatt ggaagaccca gagttcatct aaaagaaaca gacgtcacat tgagaaaaag
2821 atcctcacct tccaaggcag caagactcat ggcatgttgc cggggctaga gccctttagc
2881 cactacacac tgaatgtccg agtggtcaat gggaaagggg agggcccagc cagccctgac
2941 agagtcttta tactccaga aggagtcccc agtgctcct cgtctttgaa gattgtgaat
3001 ccaacactgg actctctcac tttggaatgg gatccaccga gccacccgaa tggcattttg
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3181 cgataaagt tttatttcta tgcacaaaca tcagcaggat caggaagtca aattacagag
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3481 ttaaagggtc taatgccagg aacagcatac aaagtttgag ttggtgctgt gggggactct
3541 ggttttgtga gttcaggga. tgtgtttgag acaggcccag cgatggcaag ccggcaggtg
3601 gatattgcaa ctcagggctg gttcattggt ctgatgtgtg ctgttgctct ccttatctta
3661 attttgctga ttgtttgctt catcagaaga aacaagggtg gtaaatatcc agttaaagaa
3721 aaggaagatg cccatgctga ccctgaaatc cagcctatga ggaagatga tgggacattt
3781 ggagaataca gtgatgcaga agaccacaag cctttgaaa aggaagtcg aactccttca
3841 gacaggactg tgaaaaaaga agatagtgac gacagcctag ttgactatgg agaaggggtt
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3961 gagccggctg aaggaaacga aagctcagag gcacctttctc ctgtcaatgc catgaattcc
4021 tttgtttaat ttttaagctc aaagccaata ttcgatttct ctagaatgtt tatcctaagc
4081 tcttgtttgt cagccctctc atactatgaa aatatgggta gagagtatat tttc
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FIGURE 2A

MPKKKRLSAGRVPLILFLCQMISALEVPLDPKLLEDLVQPPTIT
QQSPKDYIIDPRENIVIQCEAKGKPPPSFSWTRNGTHFDIDKDPLVTMKPGTGTLIIN
IMSEGKAETYEGVYQCTARNERGAAVSNNIVVRPSRSPLWTKEKLEPITLQSGQSLVL
PCRPPIGLPPPIIFWMDNSFQRLPQSERVSQGLNGDLYFSNVLPEDTREDYICYARFN
HTQTIQQKQPISVKVISVDELNDTIAANLSDTEFYGAKSSRERPPTFLTPEGNASNKE
ELRGNVLSLECIAEGLPTPIIYWAKEDGMLPKNRTVYKNFEKTLQIIHVSEADSGNYQ
CIAKNALGAIHHTISVRVKAAPYWITAPQNLVLSPGEDGTLICRANGNPKPRISWLTN
GVPIEIAPDDPSRKIDGDTIIFSNVQERSSAVYQCNASNEYGYLLANAFVNVLAEPPR
ILTPANTLYQVIANRPALLDCAFFGSPLPTIEWFKGAKGSALHEDIYVLHENGTLEIK
DATWIVKEIPVAQKDSTGTYTCVARNKLGMAKNEVHLQPEYAVVQRGSMVSFECKVKH
DHTLSLTVLWLKDNRELPSDERFTVDKDHLVVADVSDDDSGTYTCVANTTLDSVSASA
VLSVVAPTPTPAPVYDVPNPPFDLELTDQLDKSVQLSWTPGDDNNSPITKFIIEYEDA
MHKPGLWHHQTEVSGTQTTAQLKLSPYVNYSFRVMAVNSIGKSLPSEASEQYLTKASE
PDKNPTAVEGLGSEPDNLEITWKPLNGFESNGPGLQYKVSWRQKDGDDEWTSVVVANV
SKYIVSGTPTFVPYLIKVQALNDMGFAPEPAVVMGHSGEDLPMVAPGNVRVNVVNSTL

AEVHWDPVPLKSIRGHLQGYRIYYWKTQSSSKRNRRHIEKKILTFQGSKTHGMLPGLE
PFSHYTLNVRVVNGKGEGPASPDRVFNTPEGVPSAPSSLKIVNPTLDSLTLEWDPPSH
PNGILTEYTLKYQPINSTHELGPLVDLKIPANKTRWTLKNLNFSTRYKFYFYAQTSAG
SGSQITEEAVTTVDEAGILPPDVGAGKVQAVNTRISNLTAAAAETYANISWEYEGPEH
VNFYVEYGVAGSKEEWRKEIVNGSRSFFGLKGLMPGTAYKVRVGAVGDSGFVSSEDVF
ETGPAMASRQVDIATQGWFIGLMCAVALLILILLIVCFIRRNKGGKYPVKEKEDAHAD
PEIQPMKEDDGTFGEYSDAEDHKPLKKGSRTPSDRTVKKEDSDDSLVDYGEGVNGQFN
EDGSFIGQYSGKKEKEPAEGNESSEAPSPVNAMNSFV

FIGURE 2B

```
HUMAN Nr-CAM    4097    TCTCATACTATGAACATATGGGTAGAGAGTATATTTTC                4134
                        *************************************
D4-1            1       TCTCATACTATGAACATATGGGTAGAGAGTATATTTTCTGCTGT    44
                        ************* ************** ** ** ******** **
RAT Nr-CAM      3713    TCTCATACTATGGACATATGGGTAGAAAGAATGTTTTCTGCGGT    3757


D4-1            45      ATGTTAGTATTATGAGAATAGTTACAGCAAAAACATAACTCAGT    89
                        ** * ******** **** **      ** *'* * * * *****
RAT Nr-CAM      3758    ATATGAGTATTATAAGAACAGAGCAAGAACATAACTCAGTCAGT    3802


D4-1            90      CAAAGTATATGTTAATATGAACTGGAATGCAAAAGTGCATACTT    134
                        ** * *** *************** ** *** *
RAT Nr-CAM      3802    CAGATGATACGTTAATATGAACTGGGGTGAAAAGG           3831


D4-1            135     TTTCATTCAAAATGGGTATTCTTGATTTCCTAAAAAAAAAAAAA  179
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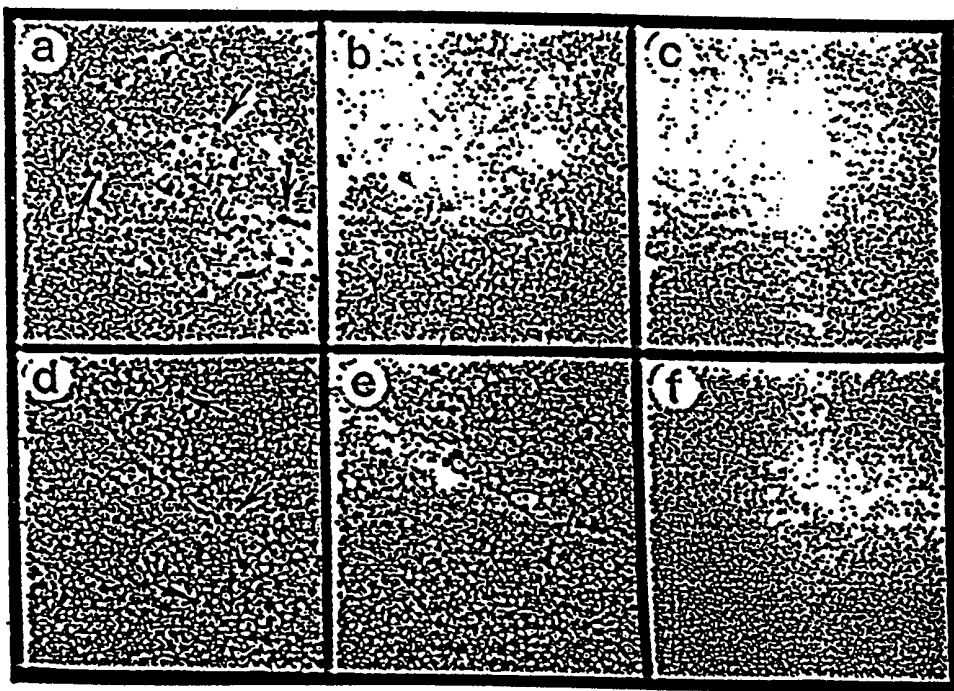
FIGURE 2C

hNr-CAM

hNr-CAM1/3AS

CMV PROMOTER
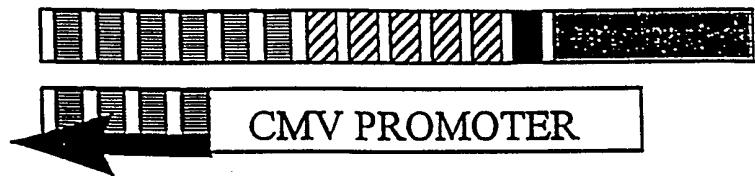
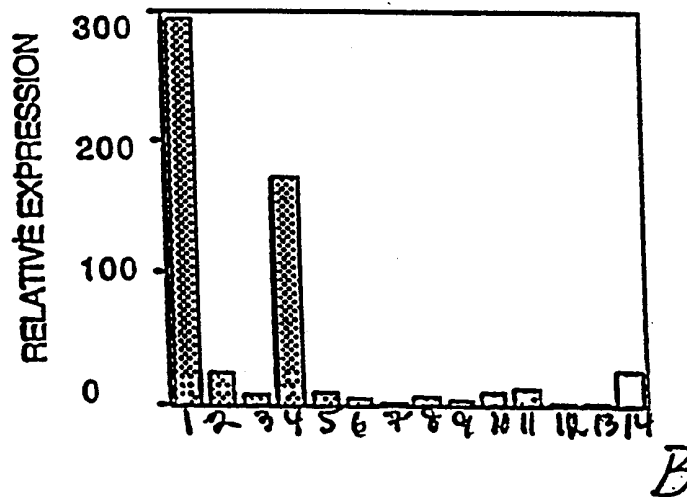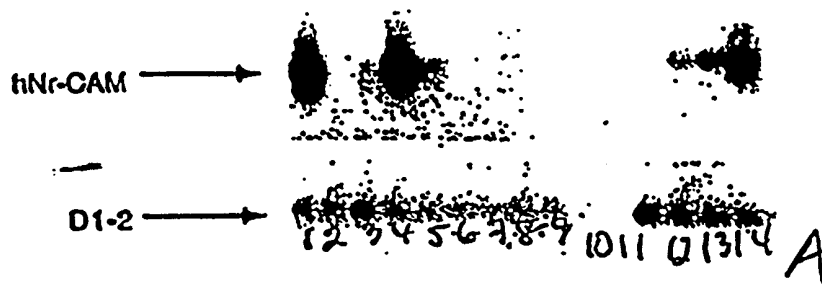▤ Ig domain   ▨ Fibronectin   ■ Transmembrane
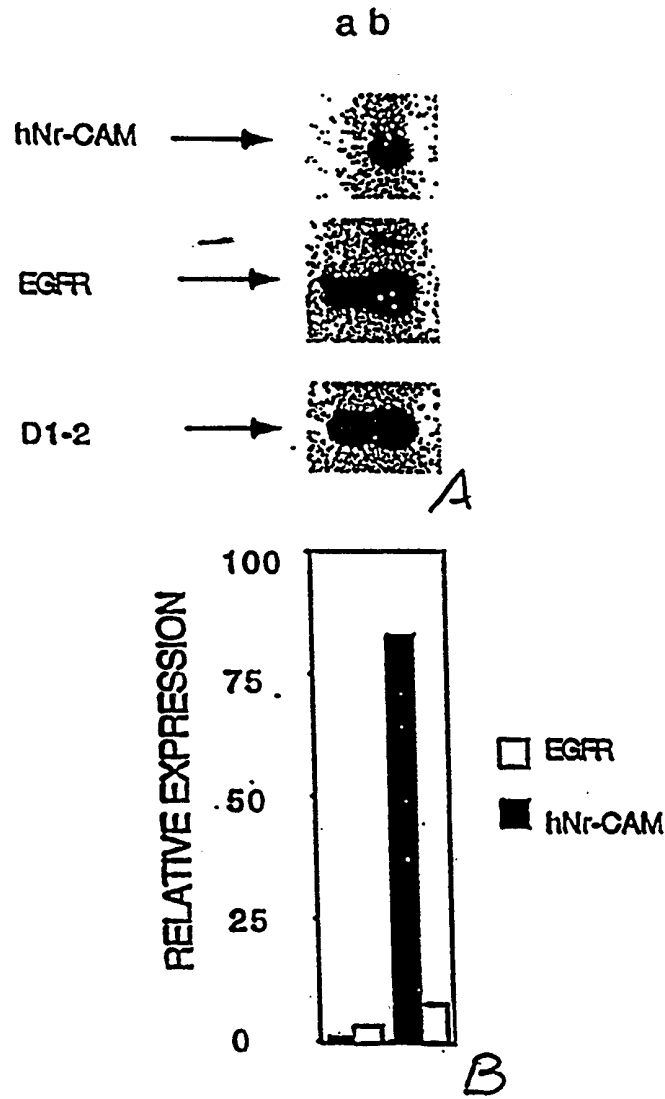   domain   domain

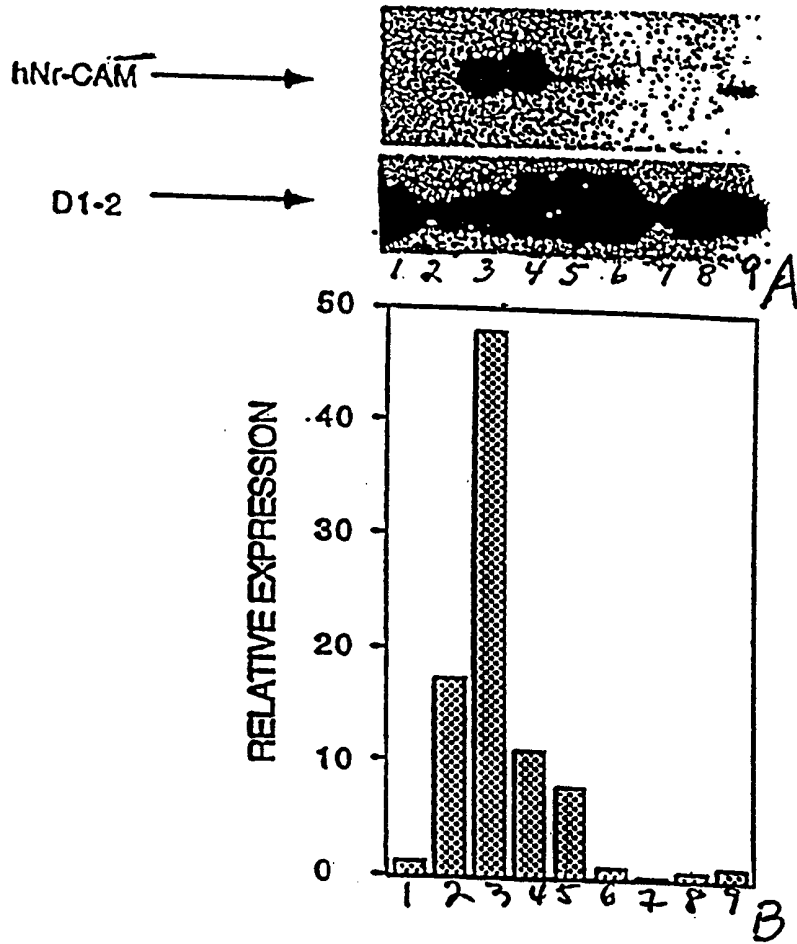▦ Cytoplasmic tail

FIGURE 2D

FIGURES 3A-F

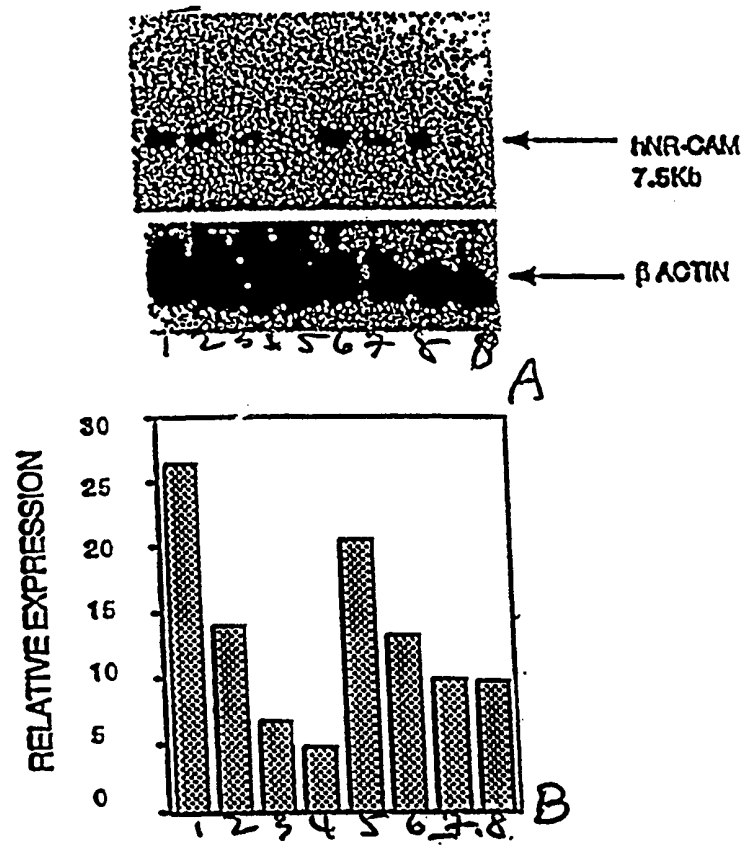**FIGURES 4A-B**

FIGURES 5A-B

FIGURES 6A-B

FIGURES 7A-B

FIGURES 8A-B

hNr-CAM

1 2 3 4
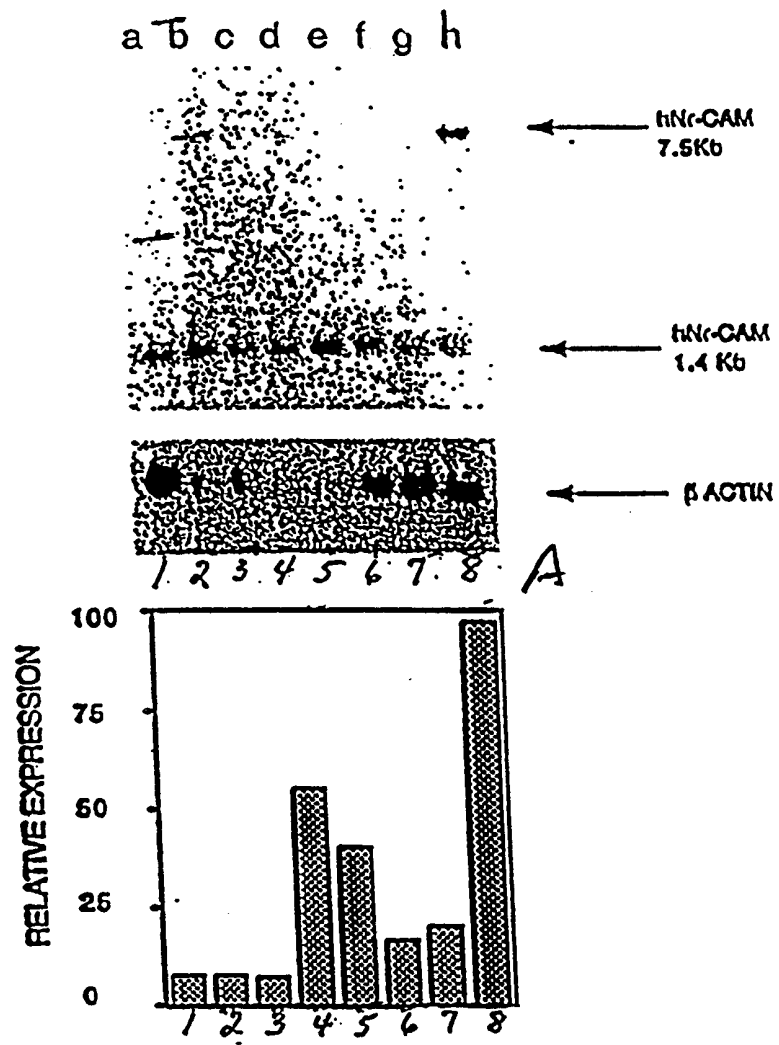
FIGURE 9

FIGURES 10A-B

FIGURE 11

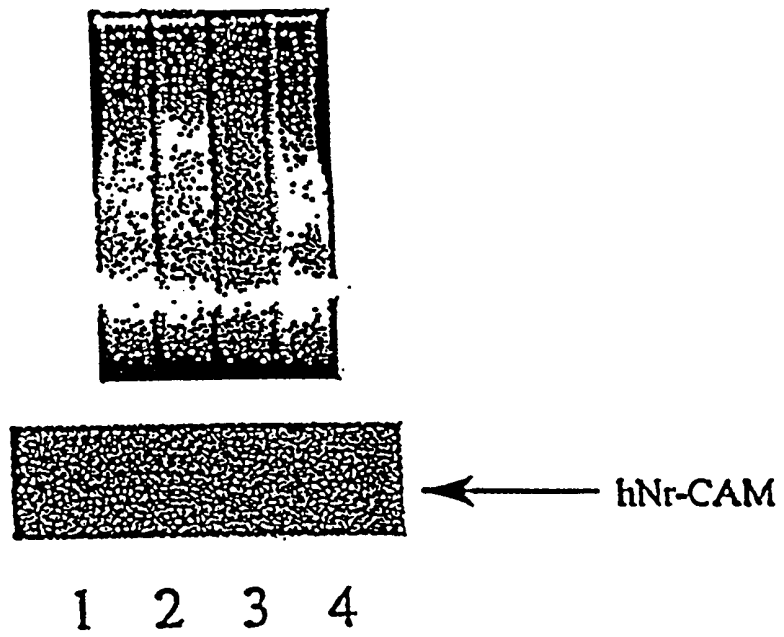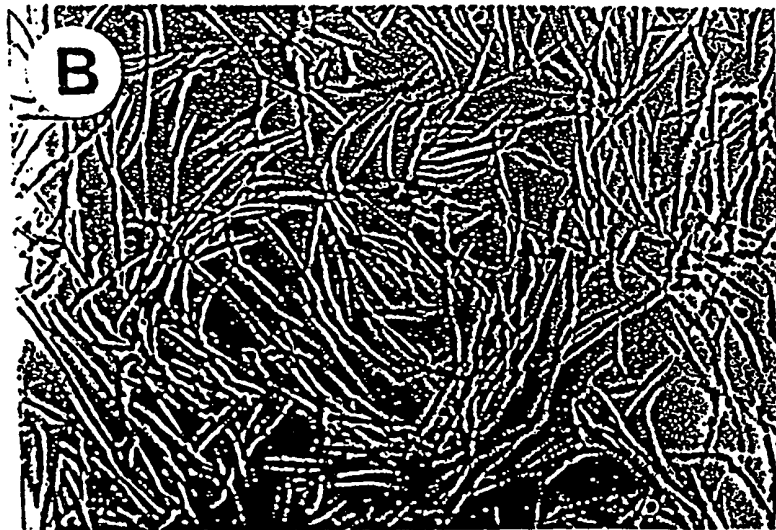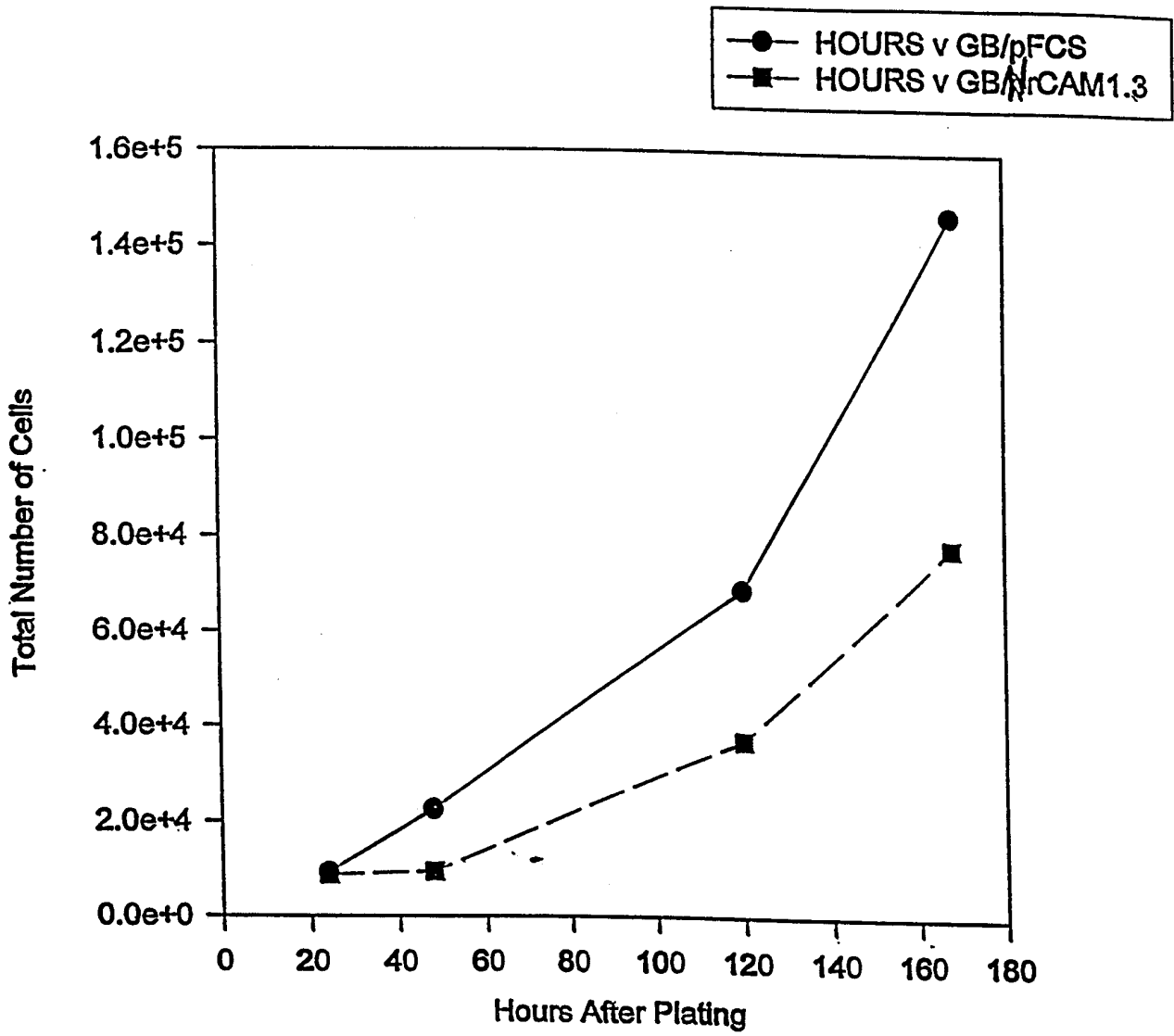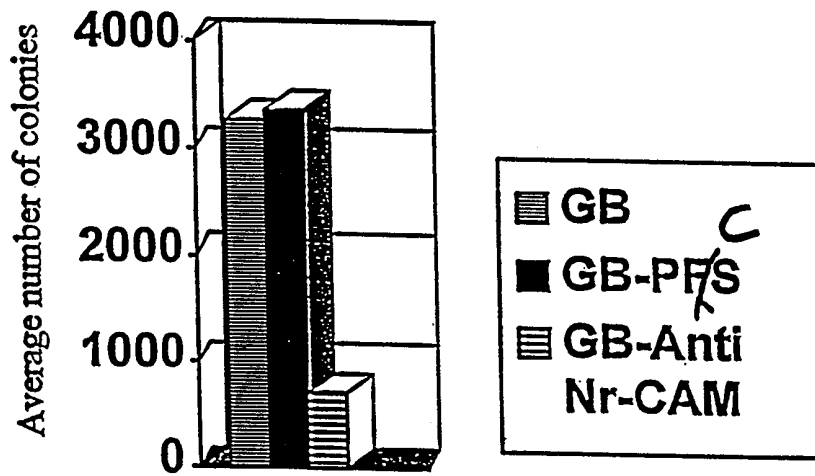**FIGURE 12**

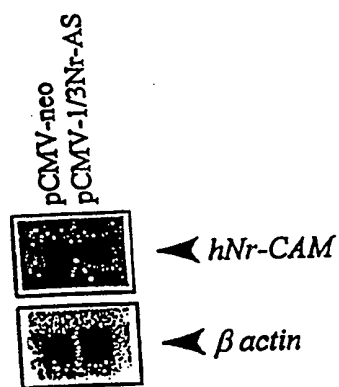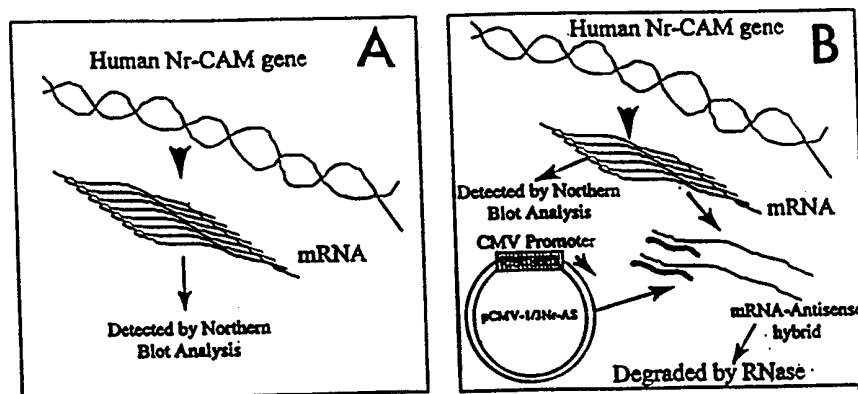FIGURE 13

FIGURE 14A-B
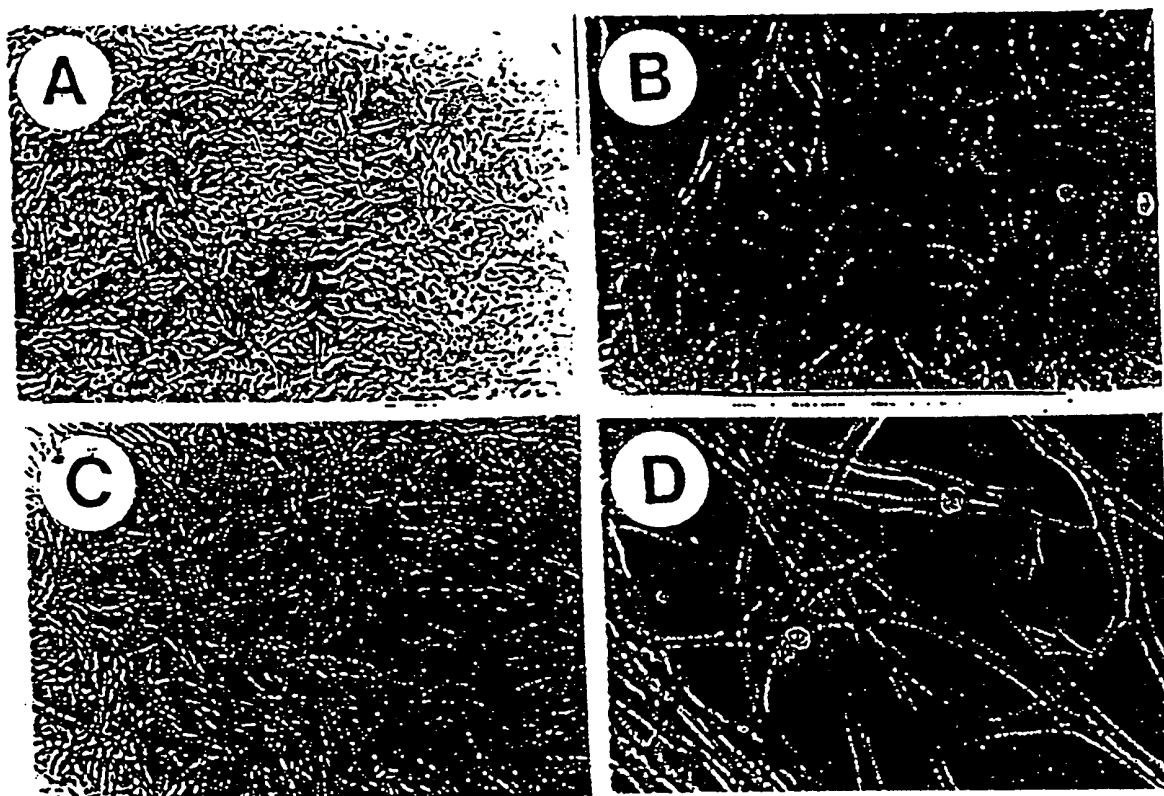
FIGURE 15A-D
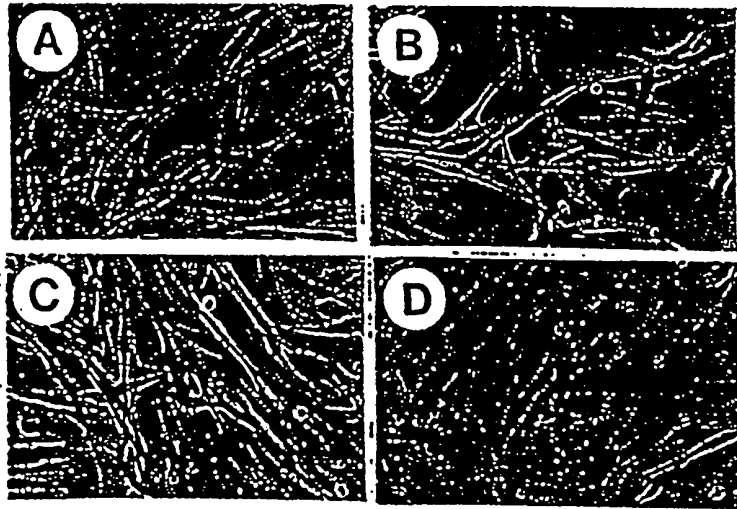
FIGURE 16A-D
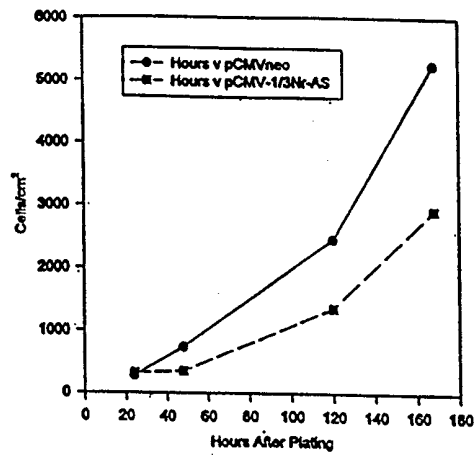
FIGURE 17
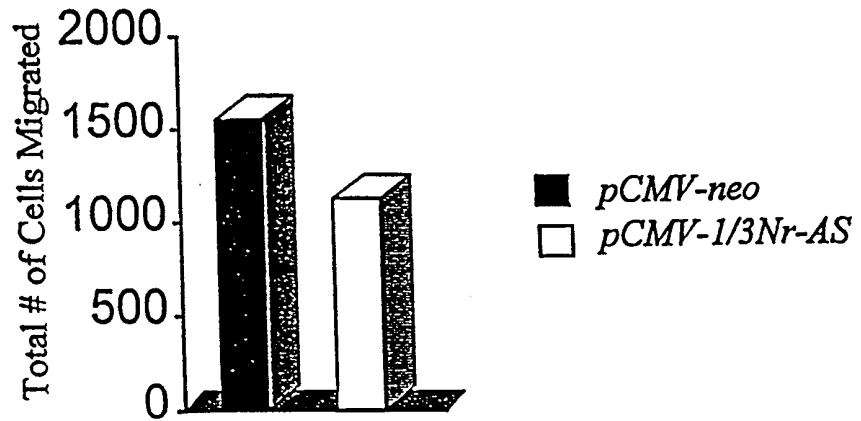
FIGURE 18
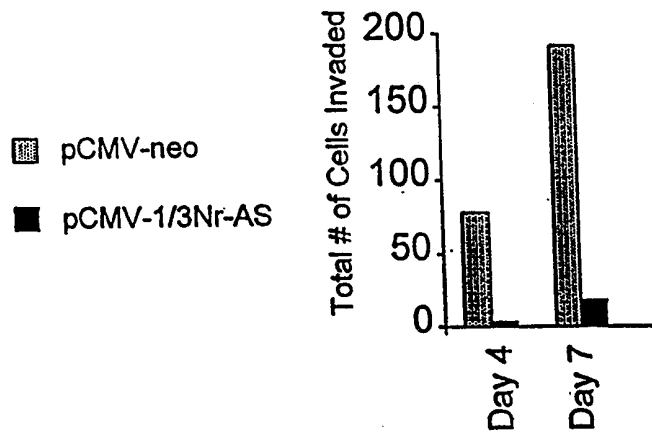
FIGURE 19
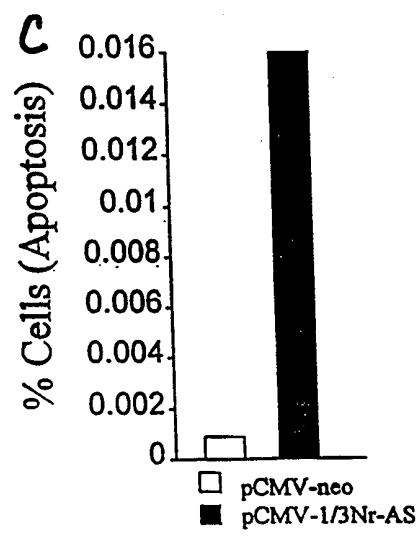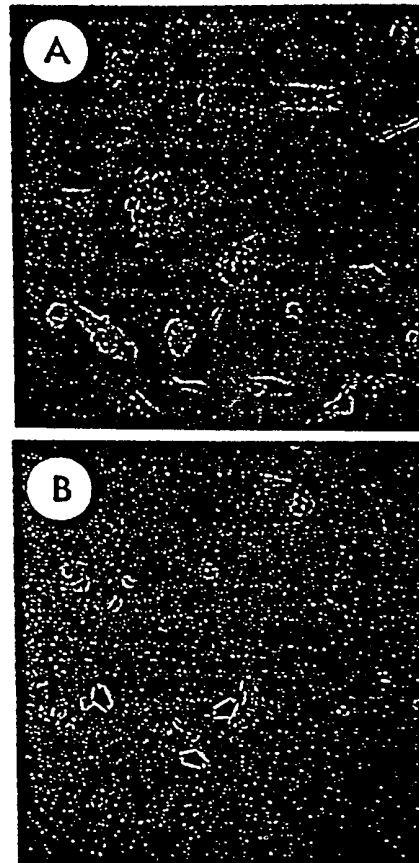
FIGURE 20A-C

FIGURE 21

FIGURE 22A

FIGURE 22B

FIGURE 23A-C

FIGURE 24

FIGURE 25A

FIGURE 25B

pCMV-1/3Nr-AS
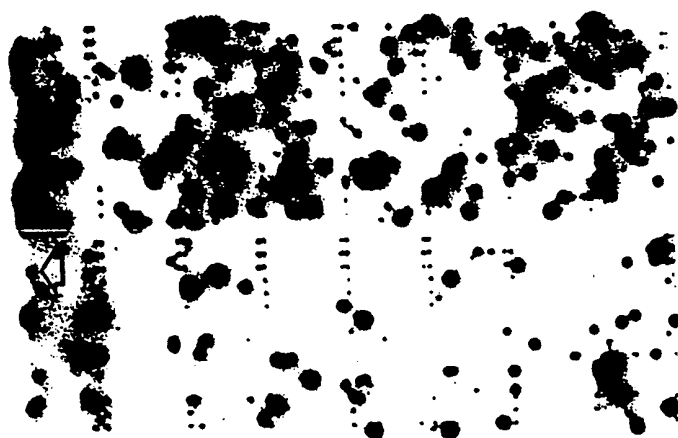
FIGURE 26A

pCMV-neo
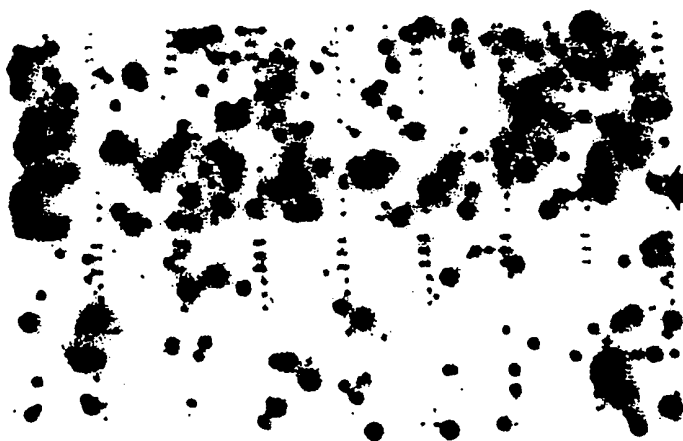
FIGURE 26B

SEQUENCE LISTING

<110> PACIFIC NORTHWEST CANCER FOUNDATION

<120> Nr-CAM GENE, NUCLEIC ACIDS AND NUCLEIC ACID PRODUCTS
      FOR THERAPEUTIC AND DIAGNOSTIC USES FOR TUMORS

<130> 8511-021-228

<140> PCT/US99/
<141> 1999-04-27

<150> 60/112,098
<151> 1998-12-14

<150> 60/083,152
<151> 1998-04-27

<160> 32

<170> PatentIn Ver. 2.0

<210> 1
<211> 4134
<212> DNA
<213> Homo sapiens

<220>
<221> CDS
<222> (130)..(4029)

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ttgtgaaaga agaaaaggaa attcagtgtg tgagtctcag caggagttaa gctaatgcag 120

cttaaaata atg ccg aaa aag aag cgc tta tct gcg ggc aga gtg ccc ctg 171
         Met Pro Lys Lys Lys Arg Leu Ser Ala Gly Arg Val Pro Leu
          1   5   10

att ctc ttc ctg tgc cag atg att agt gca ctg gaa gta cct ctt gat   219
Ile Leu Phe Leu Cys Gln Met Ile Ser Ala Leu Glu Val Pro Leu Asp
 15   20   25   30

cca aaa ctt ctt gaa gac ttg gta cag cct cca acc atc acc caa cag   267
Pro Lys Leu Leu Glu Asp Leu Val Gln Pro Pro Thr Ile Thr Gln Gln
                 35   40   45

tct cca aaa gat tac att att gac cct cgg gag aat att gta atc cag   315
Ser Pro Lys Asp Tyr Ile Ile Asp Pro Arg Glu Asn Ile Val Ile Gln
             50   55   60

tgt gaa gcc aaa ggg aaa ccg ccc cca agc ttt tcc tgg acc cgt aat   363
Cys Glu Ala Lys Gly Lys Pro Pro Pro Ser Phe Ser Trp Thr Arg Asn
             65   70   75

1

SUBSTITUTE SHEET (RULE 26)

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ggg act cat ttt gac atc gat aaa gac cct ctg gtc acc atg aag cct    411
Gly Thr His Phe Asp Ile Asp Lys Asp Pro Leu Val Thr Met Lys Pro
    80                  85                  90

ggc aca gga acg ctc ata att aac atc atg agc gaa ggg aaa gct gag    459
Gly Thr Gly Thr Leu Ile Ile Asn Ile Met Ser Glu Gly Lys Ala Glu
95                  100                 105                 110

acc tat gaa gga gtc tat cag tgt aca gca agg aac gaa cgc gga gct    507
Thr Tyr Glu Gly Val Tyr Gln Cys Thr Ala Arg Asn Glu Arg Gly Ala
                115                 120                 125

gca gtt tct aat aac att gtt gtc cgc cca tcc aga tca cca ttg tgg    555
Ala Val Ser Asn Asn Ile Val Val Arg Pro Ser Arg Ser Pro Leu Trp
            130                 135                 140

acc aaa gaa aaa ctt gaa cca atc aca ctt caa agt ggt cag tct tta    603
Thr Lys Glu Lys Leu Glu Pro Ile Thr Leu Gln Ser Gly Gln Ser Leu
            145                 150                 155

gta ctt ccc tgc aga ccc cca att gga tta cca cca cct ata ata ttt    651
Val Leu Pro Cys Arg Pro Pro Ile Gly Leu Pro Pro Pro Ile Ile Phe
    160                 165                 170

tgg atg gat aat tcc ttt caa aga ctt cca caa agt gag aga gtt tct    699
Trp Met Asp Asn Ser Phe Gln Arg Leu Pro Gln Ser Glu Arg Val Ser
175                 180                 185                 190

caa ggt ttg aat ggg gac ctt tat ttt tcc aat gtc ctc cca gag gac    747
Gln Gly Leu Asn Gly Asp Leu Tyr Phe Ser Asn Val Leu Pro Glu Asp
                195                 200                 205

acc cgc gaa gac tat atc tgt tat gct aga ttt aat cat act caa acc    795
Thr Arg Glu Asp Tyr Ile Cys Tyr Ala Arg Phe Asn His Thr Gln Thr
            210                 215                 220

ata cag cag aag caa cct att tct gtg aag gtg att tca gtg gat gaa    843
Ile Gln Gln Lys Gln Pro Ile Ser Val Lys Val Ile Ser Val Asp Glu
            225                 230                 235

ttg aat gac act ata gct gct aat ttg agt gac act gag ttt tat ggt    891
Leu Asn Asp Thr Ile Ala Ala Asn Leu Ser Asp Thr Glu Phe Tyr Gly
    240                 245                 250

gct aaa tca agt aga gag agg cca cca aca ttt tta act cca gaa ggc    939
Ala Lys Ser Ser Arg Glu Arg Pro Pro Thr Phe Leu Thr Pro Glu Gly
255                 260                 265                 270

aat gca agt aac aaa gag gaa tta aga gga aat gtg ctt tca ctg gag    987
Asn Ala Ser Asn Lys Glu Glu Leu Arg Gly Asn Val Leu Ser Leu Glu
                275                 280                 285

tgc att gca gaa gga ctg cct acc cca att att tac tgg gca aag gaa   1035
Cys Ile Ala Glu Gly Leu Pro Thr Pro Ile Ile Tyr Trp Ala Lys Glu
                290                 295                 300
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2

SUBSTITUTE SHEET (RULE 26)

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Asp Gly Met Leu Pro Lys Asn Arg Thr Val Tyr Lys Asn Phe Glu Lys
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acc ttg cag atc att cat gtt tca gaa gca gac tct gga aat tac caa     1131
Thr Leu Gln Ile Ile His Val Ser Glu Ala Asp Ser Gly Asn Tyr Gln
    320             325             330

tgt ata gca aaa aat gca tta gga gcc atc cac cat acc att tct gtt     1179
Cys Ile Ala Lys Asn Ala Leu Gly Ala Ile His His Thr Ile Ser Val
335             340             345             350

aga gtt aaa gcg gct cca tac tgg atc aca gcc cct caa aat ctt gtg     1227
Arg Val Lys Ala Ala Pro Tyr Trp Ile Thr Ala Pro Gln Asn Leu Val
            355             360             365

ctg tcc cca gga gag gat ggg acc ttg atc tgc aga gct aat ggc aac     1275
Leu Ser Pro Gly Glu Asp Gly Thr Leu Ile Cys Arg Ala Asn Gly Asn
            370             375             380

ccc aaa ccc aga att agc tgg tta aca aat gga gtc cca ata gaa att     1323
Pro Lys Pro Arg Ile Ser Trp Leu Thr Asn Gly Val Pro Ile Glu Ile
            385             390             395

gcc cct gat gac ccc agc aga aaa ata gat ggc gat acc att att ttt     1371
Ala Pro Asp Asp Pro Ser Arg Lys Ile Asp Gly Asp Thr Ile Ile Phe
    400             405             410

tca aat gtt caa gaa aga tca agt gca gta tat cag tgc aat gcc tct     1419
Ser Asn Val Gln Glu Arg Ser Ser Ala Val Tyr Gln Cys Asn Ala Ser
415             420             425             430

aat gaa tat gga tat tta ctg gca aac gca ttt gta aat gtg ctg gct     1467
Asn Glu Tyr Gly Tyr Leu Leu Ala Asn Ala Phe Val Asn Val Leu Ala
            435             440             445

gag cca cca cga atc ctc aca cct gca aac aca ctc tac cag gtc att     1515
Glu Pro Pro Arg Ile Leu Thr Pro Ala Asn Thr Leu Tyr Gln Val Ile
            450             455             460

gca aac agg cct gct tta cta gac tgt gcc ttc ttt ggg tct cct ctc     1563
Ala Asn Arg Pro Ala Leu Leu Asp Cys Ala Phe Phe Gly Ser Pro Leu
            465             470             475

cca acc atc gag tgg ttt aaa gga gct aaa gga agt gct ctt cat gaa     1611
Pro Thr Ile Glu Trp Phe Lys Gly Ala Lys Gly Ser Ala Leu His Glu
    480             485             490

gat att tat gtt tta cat gaa aat gga act ttg gaa atc aaa gat gct     1659
Asp Ile Tyr Val Leu His Glu Asn Gly Thr Leu Glu Ile Lys Asp Ala
495             500             505             510

aca tgg atc gtt aaa gaa att cct gtg gcc caa aag gac agt aca gga     1707
Thr Trp Ile Val Lys Glu Ile Pro Val Ala Gln Lys Asp Ser Thr Gly
            515             520             525
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3

SUBSTITUTE SHEET (RULE 26)

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act tat acg tgt gtt gca agg aat aaa tta ggg atg gca aag aat gaa        1755
Thr Tyr Thr Cys Val Ala Arg Asn Lys Leu Gly Met Ala Lys Asn Glu
            530             535             540

gtt cac tta cag ccc gaa tat gca gtt gtg caa aga ggg agc atg gtg        1803
Val His Leu Gln Pro Glu Tyr Ala Val Val Gln Arg Gly Ser Met Val
            545             550             555

tcc ttt gaa tgc aaa gtg aaa cat gat cac acc tta tcc ctc act gtc        1851
Ser Phe Glu Cys Lys Val Lys His Asp His Thr Leu Ser Leu Thr Val
        560             565             570

ctg tgg ctg aag gac aac agg gaa ctg ccc agt gat gaa agg ttc act        1899
Leu Trp Leu Lys Asp Asn Arg Glu Leu Pro Ser Asp Glu Arg Phe Thr
575             580             585             590

gtt gac aag gat cat cta gtg gta gct gat gtc agt gac gat gac agc        1947
Val Asp Lys Asp His Leu Val Val Ala Asp Val Ser Asp Asp Asp Ser
            595             600             605

ggg acc tac acg tgt gtg gcc aac acc act ctg gac agc gtc tcc gcc        1995
Gly Thr Tyr Thr Cys Val Ala Asn Thr Thr Leu Asp Ser Val Ser Ala
            610             615             620

agc gct gtg ctt agc gtt gtt gct cct act cca act cca gct ccc gtt        2043
Ser Ala Val Leu Ser Val Val Ala Pro Thr Pro Thr Pro Ala Pro Val
            625             630             635

tac gat gtc cca aat cct ccc ttt gac tta gaa ctg aca gat caa ctt        2091
Tyr Asp Val Pro Asn Pro Pro Phe Asp Leu Glu Leu Thr Asp Gln Leu
        640             645             650

gac aaa agt gtt cag ctg tca tgg acc cca ggc gat gac aac aat agc        2139
Asp Lys Ser Val Gln Leu Ser Trp Thr Pro Gly Asp Asp Asn Asn Ser
655             660             665             670

ccc att aca aaa ttc atc atc gaa tat gaa gat gca atg cac aag cca        2187
Pro Ile Thr Lys Phe Ile Ile Glu Tyr Glu Asp Ala Met His Lys Pro
            675             680             685

ggg ctg tgg cac cac caa act gaa gtt tct gga aca cag acc aca gcc        2235
Gly Leu Trp His His Gln Thr Glu Val Ser Gly Thr Gln Thr Thr Ala
            690             695             700

cag ctg aag ctg tct cct tac gtg aac tac tcc ttc cgc gtg atg gca        2283
Gln Leu Lys Leu Ser Pro Tyr Val Asn Tyr Ser Phe Arg Val Met Ala
            705             710             715

gtg aac agc att ggg aag agc ttg ccc agc gag gcg tct gag cag tat        2331
Val Asn Ser Ile Gly Lys Ser Leu Pro Ser Glu Ala Ser Glu Gln Tyr
            720             725             730

ttg acg aaa gcc tca gaa cca gat aaa aac ccc aca gct gtg aaa gga        2379
Leu Thr Lys Ala Ser Glu Pro Asp Lys Asn Pro Thr Ala Val Glu Gly
735             740             745             750
```

4

SUBSTITUTE SHEET (RULE 26)

```
ctg gga tca gag cct gat aat ttg gag att acg tgg aag ccc ttg aat    2427
Leu Gly Ser Glu Pro Asp Asn Leu Glu Ile Thr Trp Lys Pro Leu Asn
            755                 760                 765

ggt ttc gaa tct aat ggg cca ggc ctt cag tac aaa gtt agc tgg cgc    2475
Gly Phe Glu Ser Asn Gly Pro Gly Leu Gln Tyr Lys Val Ser Trp Arg
            770                 775                 780

cag aaa gat ggt gat gat gaa tgg aca tct gtg gtt gtg gca aat gta    2523
Gln Lys Asp Gly Asp Asp Glu Trp Thr Ser Val Val Val Ala Asn Val
            785                 790                 795

tcc aaa tat att gtc tca ggc acg cca acc ttt gtt cca tac ctg atc    2571
Ser Lys Tyr Ile Val Ser Gly Thr Pro Thr Phe Val Pro Tyr Leu Ile
            800                 805                 810

aaa gtt cag gcc ctg aat gac atg ggg ttt gcc ccc gag cca gct gta    2619
Lys Val Gln Ala Leu Asn Asp Met Gly Phe Ala Pro Glu Pro Ala Val
815                 820                 825                 830

gtc atg gga cat tct gga gaa gac ctc cca atg gtg gct cct ggg aac    2667
Val Met Gly His Ser Gly Glu Asp Leu Pro Met Val Ala Pro Gly Asn
            835                 840                 845

gtg cgt gtg aat gtg gtg aac agt acc tta gcc gag gtg cac tgg gac    2715
Val Arg Val Asn Val Val Asn Ser Thr Leu Ala Glu Val His Trp Asp
            850                 855                 860

cca gta cct ctg aaa agc atc cga gga cac cta caa ggc tat cgg att    2763
Pro Val Pro Leu Lys Ser Ile Arg Gly His Leu Gln Gly Tyr Arg Ile
            865                 870                 875

tac tat tgg aag acc cag agt tca tct aaa aga aac aga cgt cac att    2811
Tyr Tyr Trp Lys Thr Gln Ser Ser Ser Lys Arg Asn Arg Arg His Ile
            880                 885                 890

gag aaa aag atc ctc acc ttc caa ggc agc aag act cat ggc atg ttg    2859
Glu Lys Lys Ile Leu Thr Phe Gln Gly Ser Lys Thr His Gly Met Leu
895                 900                 905                 910

ccg ggg cta gag ccc ttt agc cac tac aca ctg aat gtc cga gtg gtc    2907
Pro Gly Leu Glu Pro Phe Ser His Tyr Thr Leu Asn Val Arg Val Val
            915                 920                 925

aat ggg aaa ggg gag ggc cca gcc agc cct gac aga gtc ttt aat act    2955
Asn Gly Lys Gly Glu Gly Pro Ala Ser Pro Asp Arg Val Phe Asn Thr
            930                 935                 940

cca gaa gga gtc ccc agt gct ccc tcg tct ttg aag att gtg aat cca    3003
Pro Glu Gly Val Pro Ser Ala Pro Ser Ser Leu Lys Ile Val Asn Pro
            945                 950                 955

aca ctg gac tct ctc act ttg gaa tgg gat cca ccg agc cac ccg aat    3051
Thr Leu Asp Ser Leu Thr Leu Glu Trp Asp Pro Pro Ser His Pro Asn
            960                 965                 970
```

5

SUBSTITUTE SHEET (RULE 26)

```
ggc att ttg aca gag tac acc tta aag tat cag cca att aac agc aca    3099
Gly Ile Leu Thr Glu Tyr Thr Leu Lys Tyr Gln Pro Ile Asn Ser Thr
975                 980                 985                 990


cat gaa tta ggc cct ctg gta gat ttg aaa att cct gcc aac aag aca    3147
His Glu Leu Gly Pro Leu Val Asp Leu Lys Ile Pro Ala Asn Lys Thr
                995                 1000                1005


cgg tgg act tta aaa aat tta aat ttc agc act cga tat aag ttt tat    3195
Arg Trp Thr Leu Lys Asn Leu Asn Phe Ser Thr Arg Tyr Lys Phe Tyr
            1010                1015                1020


ttc tat gca caa aca tca gca gga tca gga agt caa att aca gag gaa    3243
Phe Tyr Ala Gln Thr Ser Ala Gly Ser Gly Ser Gln Ile Thr Glu Glu
        1025                1030                1035


gca gta aca act gtg gat gaa gct ggt att ctt cca cct gat gta ggt    3291
Ala Val Thr Thr Val Asp Glu Ala Gly Ile Leu Pro Pro Asp Val Gly
        1040                1045                1050


gca ggc aaa gtt caa gct gta aat acc agg atc agc aat ctt act gct    3339
Ala Gly Lys Val Gln Ala Val Asn Thr Arg Ile Ser Asn Leu Thr Ala
1055                1060                1065                1070


gca gct gct gag acc tat gcc aat atc agt tgg gaa tat gag gga cca    3387
Ala Ala Ala Glu Thr Tyr Ala Asn Ile Ser Trp Glu Tyr Glu Gly Pro
                1075                1080                1085


gag cat gtg aac ttt tat gtt gaa tat ggt gta gca ggc agc aaa gaa    3435
Glu His Val Asn Phe Tyr Val Glu Tyr Gly Val Ala Gly Ser Lys Glu
                1090                1095                1100


gaa tgg aga aaa gaa att gta aat ggt tct cgg agc ttc ttt ggg tta    3483
Glu Trp Arg Lys Glu Ile Val Asn Gly Ser Arg Ser Phe Phe Gly Leu
            1105                1110                1115


aag ggt cta atg cca gga aca gca tac aaa gtt cga gtt ggt gct gtg    3531
Lys Gly Leu Met Pro Gly Thr Ala Tyr Lys Val Arg Val Gly Ala Val
        1120                1125                1130


ggg gac tct ggt ttt gtg agt tca gag gat gtg ttt gag aca ggc cca    3579
Gly Asp Ser Gly Phe Val Ser Ser Glu Asp Val Phe Glu Thr Gly Pro
1135                1140                1145                1150


gcg atg gca agc cgg cag gtg gat att gca act cag ggc tgg ttc att    3627
Ala Met Ala Ser Arg Gln Val Asp Ile Ala Thr Gln Gly Trp Phe Ile
                1155                1160                1165


ggt ctg atg tgt gct gtt gct ctc ctt atc tta att ttg ctg att gtt    3675
Gly Leu Met Cys Ala Val Ala Leu Leu Ile Leu Ile Leu Leu Ile Val
            1170                1175                1180


tgc ttc atc aga aga aac aag ggt ggt aaa tat cca gtt aaa gaa aag    3723
Cys Phe Ile Arg Arg Asn Lys Gly Gly Lys Tyr Pro Val Lys Glu Lys
            1185                1190                1195
```

6

SUBSTITUTE SHEET (RULE 26)

```
gaa gat gcc cat gct gac cct gaa atc cag cct atg aag gaa gat gat      3771
Glu Asp Ala His Ala Asp Pro Glu Ile Gln Pro Met Lys Glu Asp Asp
    1200              1205              1210

ggg aca ttt gga gaa tac agt gat gca gaa gac cac aag cct ttg aaa      3819
Gly Thr Phe Gly Glu Tyr Ser Asp Ala Glu Asp His Lys Pro Leu Lys
1215              1220              1225              1230

aaa gga agt cga act cct tca gac agg act gtg aaa aaa gaa gat agt      3867
Lys Gly Ser Arg Thr Pro Ser Asp Arg Thr Val Lys Lys Glu Asp Ser
              1235              1240              1245

gac gac agc cta gtt gac tat gga gaa ggg gtt aat ggc cag ttc aat      3915
Asp Asp Ser Leu Val Asp Tyr Gly Glu Gly Val Asn Gly Gln Phe Asn
              1250              1255              1260

gag gat ggc tcc ttt att gga caa tac agt ggt aag aaa gag aaa gag      3963
Glu Asp Gly Ser Phe Ile Gly Gln Tyr Ser Gly Lys Lys Glu Lys Glu
         1265              1270              1275

ccg gct gaa gga aac gaa agc tca gag gca cct tct cct gtc aac gcc      4011
Pro Ala Glu Gly Asn Glu Ser Ser Glu Ala Pro Ser Pro Val Asn Ala
    1280              1285              1290

atg aat tcc ttt gtt taa tttttaagct caaagccaat attccatttc            4059
Met Asn Ser Phe Val
1295                1300

tctagaatgt ttatcctaag ctcttgtttg tcagccctct catactatga acatatgggt   4119

agagagtata ttttc                                                    4134
```

```
<210> 2
<211> 1299
<212> PRT
<213> Homo sapiens

<400> 2
Met Pro Lys Lys Lys Arg Leu Ser Ala Gly Arg Val Pro Leu Ile Leu
  1               5                  10                  15

Phe Leu Cys Gln Met Ile Ser Ala Leu Glu Val Pro Leu Asp Pro Lys
             20                  25                  30

Leu Leu Glu Asp Leu Val Gln Pro Pro Thr Ile Thr Gln Gln Ser Pro
           35                  40                  45

Lys Asp Tyr Ile Ile Asp Pro Arg Glu Asn Ile Val Ile Gln Cys Glu
         50                  55                  60

Ala Lys Gly Lys Pro Pro Pro Ser Phe Ser Trp Thr Arg Asn Gly Thr
65                  70                  75                  80

His Phe Asp Ile Asp Lys Asp Pro Leu Val Thr Met Lys Pro Gly Thr
                 85                  90                  95
```

7

Gly Thr Leu Ile Ile Asn Ile Met Ser Glu Gly Lys Ala Glu Thr Tyr
              100   105   110

Glu Gly Val Tyr Gln Cys Thr Ala Arg Asn Glu Arg Gly Ala Ala Val
          115   120   125

Ser Asn Asn Ile Val Val Arg Pro Ser Arg Ser Pro Leu Trp Thr Lys
      130   135   140

Glu Lys Leu Glu Pro Ile Thr Leu Gln Ser Gly Gln Ser Leu Val Leu
145   150   155   160

Pro Cys Arg Pro Pro Ile Gly Leu Pro Pro Pro Ile Ile Phe Trp Met
              165   170   175

Asp Asn Ser Phe Gln Arg Leu Pro Gln Ser Glu Arg Val Ser Gln Gly
              180   185   190

Leu Asn Gly Asp Leu Tyr Phe Ser Asn Val Leu Pro Glu Asp Thr Arg
          195   200   205

Glu Asp Tyr Ile Cys Tyr Ala Arg Phe Asn His Thr Gln Thr Ile Gln
      210   215   220

Gln Lys Gln Pro Ile Ser Val Lys Val Ile Ser Val Asp Glu Leu Asn
225   230   235   240

Asp Thr Ile Ala Ala Asn Leu Ser Asp Thr Glu Phe Tyr Gly Ala Lys
              245   250   255

Ser Ser Arg Glu Arg Pro Pro Thr Phe Leu Thr Pro Glu Gly Asn Ala
          260   265   270

Ser Asn Lys Glu Glu Leu Arg Gly Asn Val Leu Ser Leu Glu Cys Ile
          275   280   285

Ala Glu Gly Leu Pro Thr Pro Ile Ile Tyr Trp Ala Lys Glu Asp Gly
      290   295   300

Met Leu Pro Lys Asn Arg Thr Val Tyr Lys Asn Phe Glu Lys Thr Leu
305   310   315   320

Gln Ile Ile His Val Ser Glu Ala Asp Ser Gly Asn Tyr Gln Cys Ile
          325   330   335

Ala Lys Asn Ala Leu Gly Ala Ile His His Thr Ile Ser Val Arg Val
          340   345   350

Lys Ala Ala Pro Tyr Trp Ile Thr Ala Pro Gln Asn Leu Val Leu Ser
          355   360   365

Pro Gly Glu Asp Gly Thr Leu Ile Cys Arg Ala Asn Gly Asn Pro Lys
      370   375   380

Pro Arg Ile Ser Trp Leu Thr Asn Gly Val Pro Ile Glu Ile Ala Pro
385   390   395   400

8

SUBSTITUTE SHEET (RULE 26)

Asp Asp Pro Ser Arg Lys Ile Asp Gly Asp Thr Ile Ile Phe Ser Asn
            405   410   415

Val Gln Glu Arg Ser Ser Ala Val Tyr Gln Cys Asn Ala Ser Asn Glu
            420   425   430

Tyr Gly Tyr Leu Leu Ala Asn Ala Phe Val Asn Val Leu Ala Glu Pro
            435   440   445

Pro Arg Ile Leu Thr Pro Ala Asn Thr Leu Tyr Gln Val Ile Ala Asn
    450   455   460

Arg Pro Ala Leu Leu Asp Cys Ala Phe Phe Gly Ser Pro Leu Pro Thr
465   470   475   480

Ile Glu Trp Phe Lys Gly Ala Lys Gly Ser Ala Leu His Glu Asp Ile
            485   490   495

Tyr Val Leu His Glu Asn Gly Thr Leu Glu Ile Lys Asp Ala Thr Trp
            500   505   510

Ile Val Lys Glu Ile Pro Val Ala Gln Lys Asp Ser Thr Gly Thr Tyr
            515   520   525

Thr Cys Val Ala Arg Asn Lys Leu Gly Met Ala Lys Asn Glu Val His
    530   535   540

Leu Gln Pro Glu Tyr Ala Val Val Gln Arg Gly Ser Met Val Ser Phe
545   550   555   560

Glu Cys Lys Val Lys His Asp His Thr Leu Ser Leu Thr Val Leu Trp
            565   570   575

Leu Lys Asp Asn Arg Glu Leu Pro Ser Asp Glu Arg Phe Thr Val Asp
            580   585   590

Lys Asp His Leu Val Val Ala Asp Val Ser Asp Asp Asp Ser Gly Thr
            595   600   605

Tyr Thr Cys Val Ala Asn Thr Thr Leu Asp Ser Val Ser Ala Ser Ala
    610   615   620

Val Leu Ser Val Val Ala Pro Thr Pro Thr Pro Ala Pro Val Tyr Asp
625   630   635   640

Val Pro Asn Pro Pro Phe Asp Leu Glu Leu Thr Asp Gln Leu Asp Lys
            645   650   655

Ser Val Gln Leu Ser Trp Thr Pro Gly Asp Asp Asn Asn Ser Pro Ile
            660   665   670

Thr Lys Phe Ile Ile Glu Tyr Glu Asp Ala Met His Lys Pro Gly Leu
            675   680   685

Trp His His Gln Thr Glu Val Ser Gly Thr Gln Thr Thr Ala Gln Leu
690   695   700

9

SUBSTITUTE SHEET (RULE 26)

Lys Leu Ser Pro Tyr Val Asn Tyr Ser Phe Arg Val Met Ala Val Asn
705   710   715   720

Ser Ile Gly Lys Ser Leu Pro Ser Glu Ala Ser Glu Gln Tyr Leu Thr
                725   730   735

Lys Ala Ser Glu Pro Asp Lys Asn Pro Thr Ala Val Glu Gly Leu Gly
            740   745   750

Ser Glu Pro Asp Asn Leu Glu Ile Thr Trp Lys Pro Leu Asn Gly Phe
        755   760   765

Glu Ser Asn Gly Pro Gly Leu Gln Tyr Lys Val Ser Trp Arg Gln Lys
    770   775   780

Asp Gly Asp Asp Glu Trp Thr Ser Val Val Val Ala Asn Val Ser Lys
785   790   795   800

Tyr Ile Val Ser Gly Thr Pro Thr Phe Val Pro Tyr Leu Ile Lys Val
                805   810   815

Gln Ala Leu Asn Asp Met Gly Phe Ala Pro Glu Pro Ala Val Val Met
            820   825   830

Gly His Ser Gly Glu Asp Leu Pro Met Val Ala Pro Gly Asn Val Arg
    835   840   845

Val Asn Val Val Asn Ser Thr Leu Ala Glu Val His Trp Asp Pro Val
    850   855   860

Pro Leu Lys Ser Ile Arg Gly His Leu Gln Gly Tyr Arg Ile Tyr Tyr
865   870   875   880

Trp Lys Thr Gln Ser Ser Ser Lys Arg Asn Arg Arg His Ile Glu Lys
            885   890   895

Lys Ile Leu Thr Phe Gln Gly Ser Lys Thr His Gly Met Leu Pro Gly
            900   905   910

Leu Glu Pro Phe Ser His Tyr Thr Leu Asn Val Arg Val Val Asn Gly
        915   920   925

Lys Gly Glu Gly Pro Ala Ser Pro Asp Arg Val Phe Asn Thr Pro Glu
    930   935   940

Gly Val Pro Ser Ala Pro Ser Ser Leu Lys Ile Val Asn Pro Thr Leu
945   950   955   960

Asp Ser Leu Thr Leu Glu Trp Asp Pro Pro Ser His Pro Asn Gly Ile
            965   970   975

Leu Thr Glu Tyr Thr Leu Lys Tyr Gln Pro Ile Asn Ser Thr His Glu
        980   985   990

Leu Gly Pro Leu Val Asp Leu Lys Ile Pro Ala Asn Lys Thr Arg Trp
    995   1000   1005

10

SUBSTITUTE SHEET (RULE 26)

Thr Leu Lys Asn Leu Asn Phe Ser Thr Arg Tyr Lys Phe Tyr Phe Tyr
    1010   1015   1020

Ala Gln Thr Ser Ala Gly Ser Gly Ser Gln Ile Thr Glu Glu Ala Val
025   1030   1035   1040

Thr Thr Val Asp Glu Ala Gly Ile Leu Pro Pro Asp Val Gly Ala Gly
            1045   1050   1055

Lys Val Gln Ala Val Asn Thr Arg Ile Ser Asn Leu Thr Ala Ala Ala
            1060   1065   1070

Ala Glu Thr Tyr Ala Asn Ile Ser Trp Glu Tyr Glu Gly Pro Glu His
        1075   1080   1085

Val Asn Phe Tyr Val Glu Tyr Gly Val Ala Gly Ser Lys Glu Glu Trp
    1090   1095   1100

Arg Lys Glu Ile Val Asn Gly Ser Arg Ser Phe Phe Gly Leu Lys Gly
105   1110   1115   1120

Leu Met Pro Gly Thr Ala Tyr Lys Val Arg Val Gly Ala Val Gly Asp
            1125   1130   1135

Ser Gly Phe Val Ser Ser Glu Asp Val Phe Glu Thr Gly Pro Ala Met
            1140   1145   1150

Ala Ser Arg Gln Val Asp Ile Ala Thr Gln Gly Trp Phe Ile Gly Leu
        1155   1160   1165

Met Cys Ala Val Ala Leu Leu Ile Leu Ile Leu Leu Ile Val Cys Phe
        1170   1175   1180

Ile Arg Arg Asn Lys Gly Gly Lys Tyr Pro Val Lys Glu Lys Glu Asp
185   1190   1195   1200

Ala His Ala Asp Pro Glu Ile Gln Pro Met Lys Glu Asp Asp Gly Thr
            1205   1210   1215

Phe Gly Glu Tyr Ser Asp Ala Glu Asp His Lys Pro Leu Lys Lys Gly
            1220   1225   1230

Ser Arg Thr Pro Ser Asp Arg Thr Val Lys Lys Glu Asp Ser Asp Asp
        1235   1240   1245

Ser Leu Val Asp Tyr Gly Glu Gly Val Asn Gly Gln Phe Asn Glu Asp
    1250   1255   1260

Gly Ser Phe Ile Gly Gln Tyr Ser Gly Lys Lys Glu Lys Glu Pro Ala
265   1270   1275   1280

Glu Gly Asn Glu Ser Ser Glu Ala Pro Ser Pro Val Asn Ala Met Asn
            1285   1290   1295

Ser Phe Val

<210> 3

11

SUBSTITUTE SHEET (RULE 26)

```
<211> 38
<212> DNA
<213> Homo sapiens

<400> 3
tctcatacta tgaacatatg ggtagagagt atattttc                              38


<210> 4
<211> 123
<212> DNA
<213> Rattus norvegicus

<400> 4
tctcatacta tggacatatg ggtagaaaga atgttttctg cggtatatga gtattataag 60

aacagagcaa gaacataact cagtcagtca gatgatacgt taatatgaac tggggtgaaa 120

agg                                                                   123


<210> 5
<211> 176
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: clone D4-1

<400> 5
tctcatacta tgaacatatg ggtagagagt atattttctg ctgtatgtta gtattatgag 60

aatagttaca gcaaaaacat aactcagtca aagtatatgt taatatgaac tggaatgcaa 120

aagtgcatac tttttcattc aaaatgggta ttcttgattt cctaaaaaaa aaaaaa     176


<210> 6
<211> 38
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: primer

<400> 6
tagatacaac tagtcaatgc ctctaatgaa tatggata                              38


<210> 7
<211> 38
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: primer

<400> 7
```

12

SUBSTITUTE SHEET (RULE 26)

agatagatcc gcggaatagt aaatccgata gccttgta   38

<210> 8
<211> 15
<212> DNA
<213> Artificial Sequence

<220>
<221> modified_base
<222> (1)
<223> n=a, c, g, or t

<220>
<223> Description of Artificial Sequence: primer

<400> 8
ngctgctctc atact   15

<210> 9
<211> 24
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: primer

<400> 9
aacatatggg tagagagtat attt   24

<210> 10
<211> 23
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: primer

<400> 10
ctttgcattc cagttcatat taa   23

<210> 11
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: primer

<400> 11
tgtggtgaca gatcacggct   20

<210> 12
<211> 21

13

<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: primer

<400> 12
cagctcaaac ctgtgatttc c   21

<210> 13
<211> 60
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: primer

<400> 13
aataggtatt ggtgaattta aagactcact ctccataaat gctacgaata ttaaacactt 60

<210> 14
<211> 21
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: primer

<400> 14
cggagcaata tgaaatgatc t   21

<210> 15
<211> 19
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: primer

<400> 15
gcaaatacag ctcctattg   19

<210> 16
<211> 43
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: primer

<400> 16
gctgtatgtt agtattatga gaatagttac agcaaaaaca taa   43

SUBSTITUTE SHEET (RULE 26)

```
<210> 17
<211> 40
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: primer

<400> 17
taggcctgac tggcattgta ttagcaaact catcactaga                      40


<210> 18
<211> 37
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: primer

<400> 18
tagatacaac tagtctaatg cagcttaaaa taatgcc                         37


<210> 19
<211> 39
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: primer

<400> 19
agatagatcc gcggatatcc atattcatta gaggcattg                       39


<210> 20
<211> 38
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: primer

<400> 20
tagatacaac tagtcaatgc ctctaatgaa tatggata                        38


<210> 21
<211> 38
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: primer

<400> 21
agatagatcc gcggaatagt aaatccgata gccttgta                        38
```

15

```
<210> 22
<211> 61
<212> DNA
<213> Homo sapiens

<220>
<223> Description of Artificial Sequence: primer

<400> 22
aggagttaag atgctaatgc agcttaaaat aatgccgaaa aagaagcgct tatctgcggg    60

c                                                                    61


<210> 23
<211> 19
<212> DNA
<213> Homo sapiens

<400> 23
cattagcatc ttaactcct                                                 19


<210> 24
<211> 21
<212> DNA
<213> Homo sapiens

<400> 24
tcggcattat tttaagctgc a                                              21


<210> 25
<211> 17
<212> DNA
<213> Homo sapiens

<400> 25
gcagataagc gcttctt                                                   17


<210> 26
<211> 20
<212> DNA
<213> Homo sapiens

<400> 26
actagagata cagatcatat                                                20


<210> 27
<211> 20
<212> DNA
<213> Homo sapiens

<400> 27
catatacgat cgatcgatgc                                                20
```

16

SUBSTITUTE SHEET (RULE 26)

```
<210> 28
<211> 20
<212> DNA
<213> Homo sapiens

<400> 28
gatagtgctg atcgatgcta                                              20


<210> 29
<211> 48
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: primer

<400> 29
catacgaatt ctagatacaa ctagtctaat gcagcttaaa ataatgcc            48


<210> 30
<211> 50
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: primer

<400> 30
agatagatcc gcggatatcc atattcatta gaggcattgg gatcccatac          50


<210> 31
<211> 1371
<212> DNA
<213> Homo sapiens

<400> 31
atgccgaaaa agaagcgctt atctgcgggc agagtgcccc tgattctctt cctgtgccag 60

atgattagtg cactggaagt acctcttgat ccaaaacttc ttgaagactt ggtacagcct 120

ccaaccatca cccaacagtc tccaaaagat tacattattg accctcggga gaatattgta 180

atccagtgtg aagccaaagg gaaaccgccc ccaagctttt cctggacccg taatgggact 240

cattttgaca tcgataaaga ccctctggtc accatgaagc ctggcacagg aacgctcata 300

attaacatca tgagcgaagg gaaagctgag acctatgaag gagtctatca gtgtacagca 360

aggaacgaac gcggagctgc agtttctaat aacattgttg tccgcccatc cagatcacca 420

ttgtggacca agaaaaaact tgaaccaatc acacttcaaa gtggtcagtc tttagtactt 480

ccctgcagac ccccaattgg attaccacca cctataatat tttggatgga taattccttt 540
```

SUBSTITUTE SHEET (RULE 26)

```
caaagacttc cacaaagtga gagagtttct caaggtttga atggggacct ttatttttcc 600

aatgtcctcc cagaggacac ccgcgaagac tatatctgtt atgctagatt taatcatact 660

caaaccatac agcagaagca acctatttct gtgaaggtga tttcagtgga tgaattgaat 720

gacactatag ctgctaattt gagtgacact gagttttatg gtgctaaatc aagtagagag 780

aggccaccaa cattttttaac tccagaaggc aatgcaagta caaagagga attaagagga 840

aatgtgcttt cactggagtg cattgcagaa ggactgccta ccccaattat ttactgggca 900

aaggaagatg gaatgctacc caaaaacagg acagtttata agaactttga gaaaaccttg 960

cagatcattc atgtttcaga agcagactct ggaaattacc aatgtatagc aaaaaatgca 1020

ttaggagcca tccaccatac catttctgtt agagttaaag cggctccata ctggatcaca 1080

gcccctcaaa atcttgtgct gtccccagga gaggatggga ccttgatctg cagagctaat 1140

ggcaacccca acccagaat tagctggtta acaaatggag tcccaataga aattgcccct 1200

gatgacccca gcagaaaaat agatggcgat accattattt tttcaaatgt tcaagaaaga 1260

tcaagtgcag tatatcagtg caatgcctct aatgaatatg gatatttact ggcaaacgca 1320

tttgtaaatg tgctggctga gccaccacga atcctcacac ctgcaaacac a          1371
```

```
<210> 32
<211> 1371
<212> DNA
<213> Rattus norvegicus

<400> 32
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SUBSTITUTE SHEET (RULE 26)

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SUBSTITUTE SHEET (RULE 26)

INTERNATIONAL SEARCH REPORT	International application No.
	PCT/US99/09039

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :A61K 48/00; CO7H 21/04
US CL :514/44; 536/24.5

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 514/44; 536/24.5

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, STN CAPLUS
search terms: nr-cam, neural-glial-related or associated-cellular adhesion molecule, antisense

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y, P	US 5,766,888 A (SOBOL et al) 16 June 1998, col. 45-46.	15-16 20-21
Y,P	US 5,872,225 A (LEMMON) 16 February 1999, col. 1-2.	1-21
A,P	US 5,789,573 A (BAKER et al) 04 August 1998, col 1, lines 41-64.	1-21
A	STANLEY CROOKE, Basic Principles of Antisense Therapeutics, Antisense Research and Application, Springer-Verlag Berlin Heidelberg, 07 July 1998, p. 2-3.	1-21

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance		
"E"	earlier document published on or after the international filing date	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed	"&"	document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
06 JULY 1999	25 AUG 1999
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231	Authorized officer JANET LEE EPPS
Facsimile No. (703) 305-3230	Telephone No. (703) 308-0196

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