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(54) Title: HUMAN MELANOMA H11 GENE POLYNUCLEOTIDES, POLYPEPTIDES, AND METHODS OF USE

(57) Abstract: The present invention relates to the novel H11 gene isolated from melanoma cells, and to diagnostic and therapeutic methods based on use of polynucleotides or polypeptides derived from the H11 gene. Specifically, the invention provides isolated nucleic acid molecules encoding the H11 gene, as well as isolated nucleic acid fragments or derivatives thereof. The invention also provides H11 polypeptides encoded by such isolated nucleic acid molecules, antibodies binding to such polypeptides, recombinant vectors comprising such nucleic acid molecules, prokaryotic or eukaryotic host cells comprising such recombinant vectors, methods and compositions for use in the diagnosis and/or treatment of melanoma and other cancers, and methods for the treatment of conditions or diseases relating to epithelial cell growth, such as wound healing and psoriasis.

Human Melanoma H11 Gene Polynucleotides, Polypeptides, and Methods of Use

Background of the Invention

Statement as to Rights to Inventions Made Under Federally-Sponsored Research and Development

Part of the work performed during development of this invention was supported by grant R01CA75453 from the National Cancer Institute, National Institutes of Health. The U.S. Government has certain rights in this invention.

Field of the Invention

The present invention relates to the novel H11 gene isolated from melanoma cells, and to diagnostic and therapeutic methods based on use of polynucleotides or polypeptides derived from the H11 gene. Specifically, the invention provides isolated nucleic acid molecules encoding the H11 gene, as well as isolated nucleic acid fragments or derivatives thereof. The invention also provides H11 polypeptides encoded by such isolated nucleic acid molecules, antibodies binding to such polypeptides, recombinant vectors comprising such nucleic acid molecules, prokaryotic or eukaryotic host cells comprising such recombinant vectors, methods and compositions for use in the diagnosis and/or treatment of melanoma and other cancers, and methods for the treatment of conditions or diseases relating to epithelial cell growth, such as wound healing and psoriasis.

Related Art

Melanomas are aggressive, frequently metastatic tumors derived from either melanocytes or melanocyte related nevus cells ("Cellular and Molecular Immunology," Abbas A. K., Lechtman, A. H., Pober, J. S. (eds); W. B. Saunders Company, Philadelphia: pages 340-341 (1991)). Melanomas make up

approximately three percent of all skin cancers; the worldwide increase in melanoma is unsurpassed by any other neoplasm with the exception of lung cancer in women ("Cellular and Molecular Immunology," Abbas, A. K., Lechtiman, A. H., Pober, J. S. (eds); W. B. Saunders Company Philadelphia pages: 340-342 (1991); Kirkwood and Agarwala, *Principles and Practice of Oncoloqy* 7:1-16 (1993)). Even when melanoma is apparently localized to the skin, up to 30% of the patients will develop systemic metastasis and the majority will die (Kirkwood and Agarwala, *Principles and Practice of Oncoloqy* 7:1-16 (1993)). Classic modalities of treating melanoma include surgery, radiation and chemotherapy. In the past decade immunotherapy and gene therapy have emerged as new and promising methods for treating melanoma.

A number of melanoma-associated antigens have been discovered, making it possible to develop a number of experimental strategies for the treatment of melanoma, such as vaccination. See, for example, Bandman *et al.*, U. S. Patent No. 5,912,143 ("Polynucleotides Encoding a Human MAGE Protein Homolog"); Kawakami *et al.*, U.S. Patent No. 5,844,075 ("Melanoma Antigens and Their Use in Diagnostic and Therapeutic Methods"); and Robbins *et al.*, U. S. Patent No. 5,843,648 ("P15 and Tyrosinase Melanoma Antigens and Their Use in Diagnostic and Therapeutic Methods").

The lethal nature of melanoma has made the early detection of the disease critical. Thus a number of diagnostic methods for the detection of the disease have been suggested.

Melanoma-specific overexpression of individual proteins has been suggested as a prognostic marker for selecting patents at high risk for development of this cancer. For example, Väisänen *et al.* (*J. Pathol. 186*:51-58 (1998)) have discussed the possible value of MMP-2 immunoreactive protein (72 kDa Type IV collagenase) as a prognostic indicator of primary skin melanoma. Weiss *et al.* (*Arch. Dermatol. Res. 289*:573-577 (1997)) have reported that over-expression of Rap1-GAP, a protein which has been shown to inactivate Rap1 (Rap1 is a putative endogenous antagonist of Ras proteins), may be a useful

marker for identifying thin high-risk melanomas. Dunne, B.M., *et al.* (*Hum. Pathol. 29*:594-598 (1998)) have reported that MDR1 expression is an independent prognostic indicator of poor survival for individuals afflicted with uveal melanoma. Vogt *et al.* (*Clin. Cancer Res. 4*:791-797 (1998)) have suggested that over expression of Lerk-5/Eplg5 mRNA may be a useful marker for increased tumorigenicity and metastatic potential in human malignant melanomas as well. Over expression in melanoma tissues of BCL-2 (and underexpression of p53) have also been investigated as potential markers for melanoma (Saenz-Santamaría, M.C., *et al.*, *J. Cutan. Pathol. 21*:393-397 (1994); and Albino, A.P., *et al.*, *Melanoma Research 4*:35-45 (1994)).

All of these methods are still in the experimental stage. Given the current absence of any curative therapy for patients with metastatic melanoma, a need continues to exist for the development of new methods for the early diagnosis and effective treatment of this cancer.

Summary of the Invention

The present invention provides an isolated nucleic acid molecule comprising a polynucleotide that comprises a nucleotide sequence at least 90% identical to the nucleotide sequence shown in SEQ ID NO:1 (FIG. 1), to the H11 coding region of the SEQ ID NO:1 sequence, to fragments of the SEQ ID NO:1 sequence that are at least 10 contiguous nucleotides, or to their complements. The present invention also provides an isolated nucleic acid molecule comprising a polynucleotide which hybridizes under stringent hybridization conditions to a polynucleotide that comprises the sequence shown in SEQ ID NO:1, to the H11 coding region of the SEQ ID NO:1 sequence, to fragments of the SEQ ID NO:1 sequence that are at least 10 contiguous nucleotides, or to their complements.

The invention also provides an isolated nucleic acid molecule comprising a polynucleotide which encodes the amino acid sequence of an epitope-bearing portion of an H11 polypeptide, said H11 polypeptide comprising an amino acid

sequence at least 90% identical to the amino acid sequence shown in SEQ ID NO:2.

Further, the invention provides an isolated polypeptide comprising an amino acid sequence at least 95% identical to the amino acid sequence shown in SEQ ID NO:2, to an amino acid sequence encoded by the nucleic acid sequence shown in SEQ ID NO:1, or to an amino acid sequence of an epitope-bearing portion of either the SEQ ID NO:2 sequence or to a portion of the amino acid sequence encoded by the nucleic acid sequence shown in SEQ ID NO:1. The invention also provides a polypeptide comprising an epitope-bearing portion of an H11 polypeptide, which is produced or contained in a recombinant host cell.

In another embodiment, the invention provides a recombinant vector comprising an isolated nucleic acid molecule that comprises a polynucleotide comprising a nucleotide sequence at least 90% identical to the nucleotide sequence shown in SEQ ID NO:1, or to its complement. The invention also provides a method for making such a recombinant vector, particularly an expression vector, comprising inserting such a nucleic acid molecule into a vector.

Further, the invention provides a recombinant host cell, comprising an isolated nucleic acid molecule that comprises a polynucleotide comprising a nucleotide sequence at least 90% identical to the nucleotide sequence shown in SEQ ID NO:1, or to its complement. The invention also provides a method for making such a recombinant host cell, comprising introducing such a nucleic acid molecule into a host cell.

The invention also provides a method for producing an isolated H11 polypeptide, comprising culturing the above recombinant host cell under conditions such that said polypeptide is expressed, and isolating said polypeptide. Further, the invention provides an isolated H11 polypeptide produced according to this method. In particular, it provides an isolated polypeptide comprising an epitope-bearing portion of the H11 protein, as well as hybridoma cells producing this isolated polypeptide.

Moreover, the invention provides a method of producing an isolated H11-specific antibody comprising immunizing an animal with an isolated H11 polypeptide, and isolating an H11-specific antibody from said animal. The invention provides also an isolated H11-specific antibody produced according to this method, particularly one that is detectably labeled.

In yet another embodiment, the invention provides a method of detecting an H11 polypeptide in cells or tissue, comprising contacting a sample of said cells or said tissue with at least one antibody that specifically binds to an H11 polypeptide; and detecting said antibody which is bound in said sample.

The invention also provides a nucleic acid probe for the detection of the expression of an H11 polypeptide in a sample from an animal, comprising a nucleic acid molecule sufficient to specifically detect under stringent hybridization conditions the presence of a polynucleotide encoding said H11 polypeptide in said sample. The invention provides further a method of detecting the presence of a polynucleotide encoding an H11 polypeptide in a sample, comprising contacting said sample with this probe under conditions of hybridization, and detecting the formation of a hybrid of said probe and said polynucleotide.

The invention also provides a method of diagnosing or detecting cancer, particularly melanoma, in an animal, comprising contacting a sample of cells or tissue from said animal, with at least one antibody that specifically binds to an H11 polypeptide, and detecting said antibody which is bound in said sample, wherein detection of elevated levels of H11 polypeptide, compared to control cells or control tissue, indicates that said cells or said tissue are cancerous.

Further, the invention provides a method of treating a cancer in an animal, comprising introducing into cells of the animal in need thereof an antisense oligonucleotide that is complementary to a polynucleotide encoding an H11 protein, whereby said antisense oligonucleotide binds to said polynucleotide encoding an H11 protein and inhibits the growth of said cells and said cancer is

treated. In particular, the invention provides for the treatment of melanoma, ovarian cancer, or estrogen-dependent breast cancer using this method.

The invention also provides a method of preventing or inhibiting melanoma in an animal, comprising administering to the animal in need thereof an antigenic H11 polypeptide, whereby said H11 polypeptide causes the production within said animal of antibodies that specifically bind to said H11 polypeptide and prevents or inhibits the growth of melanoma tumors.

Moreover, the invention provides a method of enhancing wound healing in an animal, comprising introducing into cells of the wound in need thereof a polynucleotide construct comprising a gene expressing an H11 polypeptide, or fragment or analog thereof, whereby said H11 polypeptide or fragment or analog thereof is produced and the healing of the wound is enhanced.

In a further embodiment, the invention provides a method of treating a hyperproliferative skin disorder in an animal, comprising introducing into skin cells of the animal in need thereof an antisense oligonucleotide that is complementary to a polynucleotide encoding an H11 protein, whereby said antisense oligonucleotide binds to said polynucleotide encoding an H11 protein and inhibits the growth of said skin cells and treats said disorder. In particular, the invention provides for the treatment of the hyperproliferative skin disorder psoriasis, using this method.

The invention also provides an antisense oligonucleotide comprising a nucleotide sequence which is complementary to a polynucleotide encoding an H11 protein.

Brief Description of the Figures

FIG. 1 shows the nucleotide sequence of the H11 cDNA (SEQ ID NO:1) cloned from the HeLa cDNA expression library. Nucleotide numbering is on the left and the predicted open reading from ("ORF") has been translated. The polyA tract is represented by the terminal stretch of 16 A nucleotides (extending from

nucleotides 1828 to 1843). FIG. 1 also shows the predicted amino acid sequence of the H11 protein (SEQ ID NO:2). The sequence derived from the H11 open reading frame ("ORF") is shown with amino acid numbers given in the left column.

FIGs. 2A-2C show the results of Northern blot analysis of H11 RNA in human tissues. Northern blot analysis of poly(A)+ mRNA from the indicated human adult (FIGs. 2A and 2B) and fetal (FIG. 2C) tissues was done by sequentially probing blots with [^{32}P]-labeled H11 and actin cDNA probes as described in Example 1. Numbers on the left are molecular size markers. Arrow indicates the H11 RNA band.

FIGs. 3A and 3B show expression of H11 in human cell lines and tissues. FIG. 3A: RT-PCR was done with total cellular RNA from the following cell lines: G361, SK-MEL-31, SK-MEL-2 (melanoma, lanes 2-4), HeLa [cervical carcinoma (lane 5)], CATES [testicular cancer (lane 6)] and 293 [immortalized human embryonic kidney (lane 7)]. PCR with cDNA in the human cancer tissue cDNA panel (Clontech) included: pancreas (lane 8), lung [tissues 61-1178 (lane 9) and 2X-19 (lane 10)], breast (lane 11), colon (lane 12) and prostate (lane 13). Lane 14 represents RT-PCR of G361 RNA in the absence of reverse transcriptase. FIG. 3B: PCR with cDNA in human tissue cDNA panels (Clontech) including ovary (lane 2), ovarian cancer (lane 3), testis (lane 4), testicular cancer (lane 5). RT-PCR was done with total cellular RNA from primary melanoma [tissues 1 and 2 (lanes 6,9)], a benign nevus (lane 7), and normal melanocytes (lane 8). H11 and actin sense and antisense primers (0.5 μM) were used as described in Materials and Methods.

FIGs. 4A-4D show the expression of H11 phosphoprotein in melanoma. FIG. 4A: Extracts from [^{35}S]methionine labeled G361 (lanes 1-3) and 293 (lanes 4-6) cells were immunoprecipitated with H11 antibodies H11-10 (N; lanes 1,4),

H11-181 (C; lanes 2,5), or with preimmune serum (P; lanes 3,6). FIG. 4B: Extracts from [^{32}P]-orthophosphate-labeled G361 (lanes 1-3) and 293 (lanes 4-6) cells were immunoprecipitated with H11 antibodies H11-10 (N; lanes 1,4) and H11-181 (C; lanes 2,5) or with preimmune serum (P; lanes 3,6). FIG. 4C: Precipitates in FIG. 4B were immunoblotted with H11-181 antibody. FIG. 4D: The blot in FIG. 4C was stripped and immunoblotted with antibody to hsp27 (hsp).

FIG. 5 shows immunoperoxidase staining of G361 cells with anti-H11 antibody AB-10 (Panel 1) or preimmune serum (Panel 2). Staining in Panel 1 is cytoplasmic (arrow) and at the cell periphery.

FIGs. 6A-6C show indirect immunofluorescent (IFA) staining of melanoma cells. FIG. 6A: G361 cells were stained with antibody H11-10 and analyzed by confocal epifluorescence microscopy using optical sections at 0.5 μm intervals through the cell. Shown are 0 μm (Panel 1), 2 μm (Panel 2), 4 μm (Panel 3), 6 μm (Panel 4), 10 μm (Panel 5), and 17 μm (Panel 6). FIG. 6B: Higher (3x) magnification of one SK-MEL-31 cell examined at the 0 μm interval. FIG. 6C: Unfixed G361 cells resuspended by trypsinization and stained with antibody H11-10 (Panel 1) or preimmune serum (Panel 2).

FIGs. 7A and 7B show growth inhibition of melanoma cells after treatment with ODNs complementary to the H11 translation initiation site. FIG. 7A: G361 cells were either untreated (shaded), or treated for 24hrs with 30 μM scrambled ODN (scODN; diagonal slashes), aODN-1 (solid) or aODN-2 (open) at 10 μM or 30 μM. Cells were labeled with [^{3}H]-TdR during the last 4hrs of treatment and data are presented as [^{3}H]-TdR incorporation (CPM x 1000 $\pm$ SEM). FIG. 7B: G361 (solid), HeLa (open) and 293 (diagonal slashes) cells were treated with aODN-1, aODN-2 or sODN for 24hrs and labeled during the last 4 hrs of treatment with [^{3}H]-TdR. Data are expressed as % growth inhibition

calculated relative to that of scODN (whose inhibition does not exceed 15% of the proliferation rate shown by untreated cells) using the formula [1-([^{3}H)-TdR incorporation of treated cells/[^{3}H]-TdR incorporation of control cells)] x 100.

FIG. 8 shows inhibition of H11 expression by H11 antisense ODNs. Extracts from G361 cells treated for 24hrs with 30 μM aODN-2 (lanes 1,4), 30 μM sODN (lanes 2,5) or untreated (lanes 3,6) and labeled for the last 4 hrs of treatment with [^{35}S]-methionine were directly electrophoresed on an 8.5% SDS-acrylamide gel (lanes 1-3) or immunoprecipitated with H11-10 antibody (lanes 4-6). Molecular weight markers are shown on the right. H11 and actin are identified.

FIGs. 9A-9C show H11 protein expression and kinase activity. The results of Western blot and immunocomplex PK assay experiments of bacterially-expressed GST-H11 fusion proteins are presented. Antibodies directed either to the N-terminal (AB-10) or the C-terminal (AB-181) portions of H11 were used to probe for the presence of fusion protein in extracts of either induced ("I") or uninduced control ("U") bacterial cultures. FIG. 9A shows the results of a Western blot of extracts from either induced or uninduced bacterial cultures. Both H11 antibodies were used to probe for the presence of the GST-H11 fusion protein. FIG. 9B shows the results of immunocomplex protein kinase (PK) assays of the GST-H11 fusion protein precipitated from induced or uninduced bacterial culture lysates using AB-10 or AB-181 antibody. FIG. 9C represents the Western blot of the gel shown in FIG. 6B. "N" = N-terminal AB-10 H11 antibody; "C" = C-terminal AB-181 antibody; "GST" = antibody specific to GST; and "PRE" = Preimmune serum control.

FIGs. 10A-10G show immunoperoxidase staining of melanoma tissue and benign or dysplastic nevi with H11 antibody. FIG.10A: Melanoma tissue stained with H11 antibody. FIG. 10B: H&E staining of same tissue as in Fig. 10A. FIG.

10C: Melanoma in hair follicle stained with H11 antibody. Note outlined, hatched areas in Figs. 10A and 10C, indicating brown staining representing H11 presence. FIG. 10D: Benign nevus stained by H&E. FIG. 10E: Same tissue as in Fig. 10D, stained with H11 antibody. Note absence of outlined, hatched areas, indicating absence of brown staining and thus absence of H11. FIG. 10F: Dysplastic nevus stained by H&E. FIG. 10G: Same tissue as in Fig. 10F, stained with H11 antibody, is positive for H11. Areas of brown staining, indicating presence of H11, are outlined and hatched, as in FIGs. 10A and 10C.

FIG. 11 shows H11 expression in breast and prostate cancer. The figure shows the results of immunoblotting of extracts from breast cancer cells (MCF7) or prostate cancer cells (DU145) with the anti-H11 antibody AB-181 or preimmune serum. The results indicate specific expression of H11 in these cancer cells. Compare with Figures 4C and 4D, lanes 4-6, which show that 293 cells are negative for H11 expression.

Detailed Description of the Preferred Embodiments

The present invention relates to the novel H11 melanoma gene and to diagnostic and therapeutic methods based on the use of polynucleotides or polypeptides, and fragments or derivatives thereof, derived from the H11 gene.

The H11 gene was discovered through observation that one of the genes of the herpes simplex virus, large subunit of ribonucleotide reductase (RR1), has an epitope that cross-reacts with tumor genes. The HSV RR1 gene differs from its counterparts in eukaryotic and prokaryotic cells and in other viruses in that it possesses a unique one-third 5′-terminal domain, the protein product of which has serine/threonine (ser/thr)-specific protein kinase ("PK") activity (Chung, T.D., *et al., J. Virol. 63*:3389-3398 (1989); Peng, T., *et al., Virology 216*:184-196 (1996); Nelson, J.W., *et al., J. Biol. Chem. 271*:17021-17027 (1996); Cooper, J.,

et al., J. Virol. 69:4979-4985 (1995); Aurelian, L., *Frontiers in Bioscience 3*:D237-249 (1998); Smith, C.C., *et al., Virology 200*:598-612 (1994); Smith, C.C. & Aurelian, L., *Virology 234*:235-242 (1997); Smith, C.C., *et al., J. Gen. Virol. 73*:1417-1428 (1992); Smith, C.C., *et al., Virology 217*:425-434 (1996)). The HSV-2 RR1 PK minigene causes neoplastic transformation of immortalized human cells, a function that requires an intact protein kinase activity (Hunter, J.C.R., *et al., Virology 210*:345-360 (1995); Hunter, J.C.R., *et al., Int. J. Oncol. 7*:515-522 (1995); Smith, C.C., *et al., Virology 200*:598-612 (1994); Smith, C.C., *et al., J. Gen. Virol. 73*:1417-1428 (1992); Aurelian, L., *Frontiers in Bioscience 3*:D237-249 (1998)). Computer assisted phylogenetic analyses indicated that RR1 PK belongs to the family of growth factor receptors serine-/threonine PKs that are involved in cellular gene regulation (at the level of cell proliferation and its inhibition) as well as playing central roles in cellular differentiation and apoptosis. However, the HSV-1 and HSV-2 RR1 PK genes are on their own branch of the phylogenetic tree, suggesting that they belong to a hitherto unrecognized subfamily (Hunter, J.C.R., *et al., Int. J. Oncol. 7*:515-522 (1995)). The only other recognized subfamily member is FAST, which has a relatively low level of homology to RR1 PK. FAST was isolated from lymphocytes but it is widely distributed in human tissues, including brain, placenta, lung, liver and pancreas, and it is activated during Fas-mediated apoptosis (Tian, Q., *et al., J. Exp. Med. 182*:865-874 (1995)).

Immunohistochemistry and immunoblotting studies with antibody to a peptide within the HSV-2 RR1 PK oncoprotein, indicated that certain tumor, but not normal cells, contain a cross-reacting protein. Specifically, antibody to an epitope (SPESER) (SEQ ID NO:14) identified a 20-25 kDa protein in HeLa and melanoma cells that was not expressed in normal cervical cells or melanocytes (unpublished). The corresponding full-length cDNA clone was isolated from a HeLa cDNA library and sequenced. The DNA sequence of the clone showed no homology to any sequence registered in the GenBank or European Molecular

biology Laboratory databases, indicating that the cloned gene (dubbed H11) is novel.

The isolated H11 gene consists of a long cDNA with small open reading frame, indicating that it is highly regulated and that it could be involved in development. The corresponding H11 protein is present and expressed in normal skin at low levels, but is highly over-expressed in melanoma. The protein is also expressed in normal keratinocytes (basal epidermal cells), but not in normal melanocytes or nevi. In addition, it is found in certain normal and cancerous human cell lines, primarily those with significant epithelial components (for example, normal prostate and estrogen dependent breast cancer). Experiments indicate that its expression may be upregulated during carcinogenesis. Moreover, treatment of melanoma cells with H11 specific antisense oligonucleotides has been shown to inhibit H11 expression and markedly decrease cell proliferation.

These results demonstrate that H11 expression is required for the growth of melanoma cells. The results also suggest that H11 may be involved in the development and/or progression of human melanoma. Thus H11 expression may be used diagnostically, as a method for detecting melanoma. It may also be used therapeutically in methods for the prevention and/or treatment of melanoma, such as antisense therapy and use of vaccines to suppress H11 expression in melanoma cells. Because the H11 overexpression has been detected in other cancers such as estrogen-dependent breast cancer and some prostate cancers (FIG. 11), as well as ovarian cancer (FIG. 3B), H11 expression may be used both diagnostically and therapeutically for these cancers as well.

The results of these studies and others indicate that H11 is likely to be required for basal cell replication (*i.e.*, cells do not replicate when this protein is inhibited). Stimulation of the expression of this gene therefore results in cell replication and growth. Thus, gene therapy methods may be used to introduce the H11 gene into cells to enhance cell growth (*e.g.*, to enhance wound healing). Alternatively, gene therapy methods (*e.g.*, antisense therapy) may be used to suppress normal H11 expression in skin cells (keratinocytes) and thus reduce the

abnormally high level of cell growth accompanying skin conditions such as psoriasis.

I. Definitions

Nucleic Acid Sequence. "Nucleic acid sequence" as used herein refers to an oligonucleotide, nucleotide, or polynucleotide, and fragments or portions thereof, and to DNA or RNA of genomic or synthetic origin which may be single- or double-stranded, and represent the sense or antisense strand.

Amino Acid Sequence. "Amino acid sequence" as used herein refers to an oligopeptide, peptide, polypeptide, or protein sequence, and fragments or portions thereof, and to naturally occurring or synthetic molecules.

Isolated Polynucleotide Molecule. The term "isolated polynucleotide molecule" is intended to refer to a nucleic acid molecule, DNA or RNA, which has been removed from its native environment For example, recombinant DNA molecules contained in a vector are considered isolated for the purposes of the present invention. Further examples of isolated DNA molecules include recombinant DNA molecules maintained in heterologous host cells or purified (partially or substantially) DNA molecules in solution. Isolated RNA molecules include *in vivo* or *in vitro* RNA transcripts of the DNA molecules of the present invention. Isolated nucleic acid molecules according to the present invention further include such molecules produced synthetically.

Variant. A "variant" of H11, as used herein, refers to an amino acid sequence that is altered by one or more amino acids. The variant may have "conservative" changes, wherein a substituted amino acid has similar structural or chemical properties, e.g., replacement of leucine with isoleucine. More rarely, a variant may have "nonconservative" changes, e.g., replacement of a glycine with a tryptophan. Similar minor variations may also include amino acid deletions or insertions, or both. Guidance in determining which amino acid residues may be substituted, inserted, or deleted without abolishing biological or

immunological activity may be found using computer programs well known in the art, for example, DNASTAR software.

Deletion. A "deletion," as used herein, refers to a change in either amino acid or nucleotide sequence in which one or more amino acid or nucleotide residues, respectively, are absent.

Insertion. An "insertion" or "addition," as used herein, refers to a change in an amino acid or nucleotide sequence resulting in the addition of one or more amino acid or nucleotide residues, respectively, as compared to the naturally occurring molecule.

Substitution. A "substitution," as used herein, refers to the replacement of one or more amino acids or nucleotides by different amino acids or nucleotides, respectively.

Biologically active. The term "biologically active," as used herein, refers to a protein having structural, regulatory, or biochemical functions of a naturally occurring molecule. Likewise, "immunologically active" refers to the capability of the natural, recombinant, or synthetic H11, or any oligopeptide thereof, to induce a specific immune response in appropriate animals or cells and to bind with specific antibodies.

Substantially pure. As used herein means that the desired purified protein is essentially free from contaminating cellular components, said components being associated with the desired protein in nature, as evidenced by a single band following polyacrylamide-sodium dodecyl sulfate gel electrophoresis. Contaminating cellular components may include, but are not limited to, proteinaceous, carbohydrate, or lipid impurities.

The term "substantially pure" is further meant to describe a molecule which is homogeneous by one or more purity or homogeneity characteristics used by those of skill in the art. For example, a substantially pure H11 will show constant and reproducible characteristics within standard experimental deviations for parameters such as the following: molecular weight, chromatographic migration, amino acid composition, amino acid sequence, blocked or unblocked

N-terminus, HPLC elution profile, biological activity, and other such parameters. The term, however, is not meant to exclude artificial or synthetic mixtures of the factor with other compounds. In addition, the term is not meant to exclude H11 fusion proteins isolated from a recombinant host.

Amplification. "Amplification" as used herein refers to the production of additional copies of a nucleic acid sequence and is generally carried out using polymerase chain reaction (PCR) technologies well known in the art (Dieffenbach, C. W. and G. S. Dveksler (1995) PCR Primer, a Laboratory Manual, Cold Spring Harbor Press, Plainview, N.Y.).

Hybridization. The term "hybridization", as used herein, refers to any process by which a strand of nucleic acid binds with a complementary strand through base pairing.

Homology. The term "homology", as used herein, refers to a degree of complementarity. There may be partial homology or complete homology (i.e., identity). A partially complementary sequence is one that at least partially inhibits an identical sequence from hybridizing to a target nucleic acid; it is referred to using the functional term "substantially homologous." The inhibition of hybridization of the completely complementary sequence to the target sequence may be examined using a hybridization assay (Southern or northern blot, solution hybridization and the like) under conditions of low stringency. A substantially homologous sequence or probe will compete for and inhibit the binding (i.e., the hybridization) of a completely homologous sequence or probe to the target sequence under conditions of low stringency. This is not to say that conditions of low stringency are such that non-specific binding is permitted; low stringency conditions require that the binding of two sequences to one another be a specific (i.e., selective) interaction. The absence of non-specific binding may be tested by the use of a second target sequence which lacks even a partial degree of complementarity (e.g., less than about 30% identity); in the absence of non-specific binding, the probe will not hybridize to the second non-complementary target sequence.

Stringent conditions. As known in the art, numerous equivalent conditions may be employed to comprise either low or high stringency conditions. Factors such as the length and nature (DNA, RNA, base composition) of the sequence, nature of the target (DNA, RNA, base composition, presence in solution or immobilization, etc.), and the concentration of the salts and other components (e.g., the presence or absence of formamide, dextran sulfate and/or polyethylene glycol) are considered and the hybridization solution may be varied to generate conditions of either low or high stringency different from, but equivalent to, the above listed conditions.

By "stringent conditions" is intended overnight incubation at 42 °C in a solution comprising: 50% formamide, 5 x SSC (750 mM NaCl, 15 mM trisodium citrate), 50 mM sodium phosphate (pH 7.6), 5 x Denhardt's solution, 10% dextran sulfate, and 20 μg/ml denatured, sheared salmon sperm DNA (ssDNA), followed by washing the filters in 0.1 x SSC at about 65 °C. As will be understood by those of skill in the art, the stringency of hybridization may be altered in order to identify or detect identical or related polynucleotide sequences.

Portion. The term "portion," as used herein, with regard to a protein (as in "a portion of a given protein") refers to fragments of that protein. The fragments may range in size from four amino acid residues to the entire amino acid sequence minus one amino acid. Thus, a protein "comprising at least a portion of the amino acid sequence shown in SEQ ID NO:2" encompasses the full-length human H11 protein (SEQ ID NO:2) and fragments thereof. Similarly, the term "portion," as used with regard to a polynucleotide or a nucleotide sequence (as in "a portion of a polynucleotide" or a "portion of a nucleotide sequence") refers to fragments of that polynucleotide or sequence. The fragments may range in size from ten bases or base pairs to the entire polynucleotide sequence minus one base or base pair.

Transformation. "Transformation," as defined herein, describes a process by which exogenous DNA enters and changes a recipient cell. It may occur under natural or artificial conditions using various methods well known in

the art. Transformation may rely on any known method for the insertion of foreign nucleic acid sequences into a prokaryotic or eukaryotic host cell. The method is selected based on the host cell being transformed and may include, but is not limited to, viral infection, electroporation, lipofection, "naked" DNA transfection, and particle bombardment. Such "transformed" cells include stably transformed cells in which the inserted DNA is capable of replication either as an autonomously replicating plasmid or as part of the host chromosome. They also include cells which transiently express the inserted DNA or RNA for limited periods of time.

Epitope. The term "epitope" or "antigenic determinant," as used herein, refers to that portion of a molecule that makes contact with a particular antibody. When a protein or fragment of a protein is used to immunize a host animal, numerous regions of the protein may induce the production of antibodies which bind specifically to a given region or three-dimensional structure on the protein; these regions or structures are referred to as antigenic determinants. An antigenic determinant may compete with the intact antigen (i.e., the immunogen used to elicit the immune response) for binding to an antibody.

Specific binding. The terms "specific binding" or "specifically binding," as used herein, in reference to the interaction of an antibody and a protein or peptide, mean that the interaction is dependent upon the presence of a particular structure (i.e., the antigenic determinant or epitope) on the protein; in other words, the antibody is recognizing and binding to a specific protein structure rather than to proteins in general. For example, if an antibody is specific for epitope "A", the presence of a protein containing epitope A (or free, unlabeled A) in a reaction containing labeled "A" and the antibody will reduce the amount of labeled A bound to the antibody.

Sample. The term "sample," as used herein, is used in its broadest sense. A biological sample suspected of containing nucleic acid encoding H11 or fragments thereof may comprise a cell, chromosomes isolated from a cell (e.g., a spread of metaphase chromosomes), genomic DNA (in solution or bound to a

solid support such as for Southern analysis), RNA (in solution or bound to a solid support such as for northern analysis), cDNA (in solution or bound to a solid support), an extract from cells or a tissue, and the like.

Antibody. As used herein, the term "antibody" refers to intact molecules as well as fragments thereof, such as Fa, $F(ab')_2$, and Fv, which are capable of binding the epitopic determinant. The term "antibody" refers both to monoclonal antibodies which are a substantially homogeneous population and to polyclonal antibodies which are heterogeneous populations. Polyclonal antibodies are derived from the sera of animals immunized with an antigen. Monoclonal antibodies (mAbs) to specific antigens may be obtained by methods known to those skilled in the art. *See*, for example, Kohler and Milstein, *Nature 256*:495-497 (1975) and U.S. Patent No. 4,376,110. Such antibodies may be of any immunoglobulin class including IgG, IgM, IgE, IgA, IgD and any subclass thereof. Antibodies that bind H11 polypeptides can be prepared using intact polypeptides or fragments containing small peptides of interest as the immunizing antigen. The polypeptide or peptide used to immunize an animal can be derived from the transition of RNA or synthesized chemically, and can be conjugated to a carrier protein, if desired. Commonly used carriers that are chemically coupled to peptides include bovine serum albumin and thyroglobulin. The coupled peptide is then used to immunize the animal (e.g., a mouse, a rat, or a rabbit).

Cloning vector. A plasmid or phage DNA or other DNA sequence which is able to replicate autonomously in a host cell, and which is characterized by one or a small number of restriction endonuclease recognition sites at which such DNA sequences may be cut in a determinable fashion without loss of an essential biological function of the vector, and into which a DNA fragment may be spliced in order to bring about its replication and cloning. The cloning vector may further contain a marker suitable for use in the identification of cells transformed with the cloning vector. Markers, for example, provide tetracycline resistance or ampicillin resistance.

Expression vector. A vector similar to a cloning vector but which is capable of enhancing the expression of a gene which has been cloned into it, after transformation into a host. The cloned gene is usually placed under the control of (i.e., operably linked to) certain control sequences such as promoter sequences. Promoter sequences may be either constitutive or inducible.

Recombinant host. According to the invention, a recombinant host may be any prokaryotic or eukaryotic host cell which contains the desired cloned genes on an expression vector or cloning vector. This term is also meant to include those prokaryotic or eukaryotic cells that have been genetically engineered to contain the desired gene(s) in the chromosome or genome of that organism. For examples of such hosts, *see* Sambrook *et al., Molecular Cloning: A Laboratory Manual*, Second Edition, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York (1989).

Recombinant vector. Any cloning vector or expression vector which contains the desired cloned gene(s).

Gene. A DNA sequence that contains information needed for expressing a polypeptide or protein. For example, the term "gene," as used herein with reference to H11, is intended to refer to a DNA sequence that encodes the H11 protein. Thus, "H11 gene" or "gene expressing H11" may refer to either a segment of genomic DNA encoding the H11 protein or to a cDNA sequence encoding the H11 protein.

Expression. Expression is the process by which a polypeptide is produced from a structural gene. The process involves transcription of the gene into mRNA and the translation of such mRNA into polypeptide(s).

Antisense RNA gene/Antisense RNA. In eukaryotes, mRNA is transcribed by RNA polymerase II. However, it is also known that one may construct a gene containing a RNA polymerase II template wherein a RNA sequence is transcribed which has a sequence complementary to that of a specific mRNA but is not normally translated. Such a gene construct is herein termed an "antisense RNA gene" and such a RNA transcript is termed an "antisense RNA."

Antisense RNAs are not normally translatable due to the presence of translation stop codons in the antisense RNA sequence.

Antisense oligonucleotide. A DNA or RNA molecule or a derivative of a DNA or RNA molecule containing a nucleotide sequence which is complementary to that of a specific mRNA. An antisense oligonucleotide binds to the complementary sequence in a specific mRNA and inhibits translation of the mRNA. There are many known derivatives of such DNA and RNA molecules. See, for example, U.S. Patent Nos. 5,602,240, 5,596,091, 5,506,212, 5,521,302, 5,541,307, 5,510,476, 5,514,787, 5,543,507, 5,512,438, 5,510,239, 5,514,577, 5,519,134, 5,554,746, 5,276,019, 5,286,717, 5,264,423, as well as WO96/35706, WO96/32474, WO96/29337 (thiono triester modified antisense oligodeoxynucleotide phosphorothioates), WO94/17093 (oligonucleotide alkylphosphonates and alkylphosphothioates), WO94/08004 (oligonucleotide phosphothioates, methyl phosphates, phosphoramidates, dithioates, bridged phosphorothioates, bridge phosphoramidates, sulfones, sulfates, ketos, phosphate esters and phosphorobutylamines (van der Krol *et al., Biotech. 6*:958-976 (1988); Uhlmann *et al., Chem. Rev. 90*:542-585 (1990)), WO94/02499 (oligonucleotide alkylphosphonothioates and arylphosphonothioates), and WO92/20697 (3'-end capped oligonucleotides). Particular H11 antisense oligonucleotides of the present invention include derivatives such as S-oligonucleotides (phosphorothioate derivatives or S-oligos, *see*, Jack Cohen, *Oligodeoxynucleotides, Antisense Inhibitors of Gene Expression*, CRC Press (1989)). S-oligos (nucleoside phosphorothioates) are isoelectronic analogs of an oligonucleotide (O-oligo) in which a nonbridging oxygen atom of the phosphate group is replaced by a sulfur atom. The S-oligos of the present invention may be prepared by treatment of the corresponding O-oligos with 3*H*-1,2-benzodithiol-3-one-1,1-dioxide which is a sulfur transfer reagent. *See* Iyer *et al., J. Org. Chem. 55*:4693-4698 (1990); and Iyer *et al., J. Am. Chem. Soc. 112*:1253-1254 (1990).

Antisense therapy. A method of treatment wherein antisense oligonucleotides are administered to a patient in order to inhibit the expression of the corresponding protein.

Complementary DNA (cDNA). A "complementary DNA," or "cDNA" gene includes recombinant genes synthesized by reverse transcription of mRNA and from which intervening sequences (introns) have been removed.

Fragment. A "fragment" of a molecule such as H11 is meant to refer to any polypeptide subset of that molecule.

Derivative. The term "derivative" or "functional derivative" is intended to include the "variants," "analogues," or "chemical derivatives" of the molecule. A "variant" of a molecule such as H11 is meant to refer to a naturally occurring molecule substantially similar to either the entire molecule, or a fragment thereof. An "analogue" of a molecule such as H11 is meant to refer to a non-natural molecule substantially similar to either the entire molecule or a fragment thereof.

A molecule is said to be "substantially similar" to another molecule if the sequence of amino acids in both molecules is substantially the same, and if both molecules possess a similar biological activity. Thus, provided that two molecules possess a similar activity, they are considered variants as that term is used herein even if one of the molecules contains additional amino acid residues not found in the other, or if the sequence of amino acid residues is not identical.

As used herein, a molecule is said to be a "chemical derivative" of another molecule when it contains additional chemical moieties not normally a part of the molecule. Such moieties may improve the molecule's solubility, absorption, biological half-life, etc. The moieties may alternatively decrease the toxicity of the molecule, eliminate or attenuate any undesirable side effect of the molecule, etc. Examples of moieties capable of mediating such effects are disclosed in *Remington's Pharmaceutical Sciences* (1980) and will be apparent to those of ordinary skill in the art.

Melanoma. The term "melanoma" includes, but is not limited to, melanomas, metastatic melanomas, melanomas derived from either melanocytes

or melanocytes related nevus cells, melanocarcinomas, melanoepitheliomas, melanosarcomas, melanoma in situ, superficial spreading melanoma, nodular melanoma, lentigo maligna melanoma, acral lentiginous melanoma, invasive melanoma or familial atypical mole and melanoma (FAM-M) syndrome. Such melanomas in mammals may be caused by chromosomal abnormalities, degenerative growth and developmental disorders, mitogenic agents, ultraviolet radiation (UV), viral infections, inappropriate tissue expression of a gene, alterations in expression of a gene and presentation on a cell, or carcinogenic agents. The aforementioned melanomas can be diagnosed, assessed or treated by methods described in the present application.

II. H11 Polynucleotides, Polypeptides, and Antibodies

Unless otherwise indicated, all nucleotide sequences determined by sequencing a DNA molecule herein were determined using an automated DNA sequencer (UMAB Biopolymer Laboratory), and all amino acid sequences of polypeptides encoded by DNA molecules determined herein were predicted by translation of a DNA sequence determined as above. Therefore, as is known in the art for any DNA sequencing method, any nucleotide sequence determined herein may contain some errors. Nucleotide sequences determined by automation are typically at least about 90% identical, more typically at least about 95% to at least about 99.9% identical to the actual nucleotide sequence of the sequenced DNA molecule. As is also known in the art, a single insertion or deletion in a determined nucleotide sequence compared to the actual sequence will cause a frame shift in translation of the nucleotide sequence such that the predicted amino acid sequence encoded by a determined nucleotide sequence will be completely different from the amino acid sequence actually encoded by the sequenced DNA molecule, beginning at the point of such an insertion or deletion.

Unless otherwise indicated, each "nucleotide sequence" set forth herein is presented as a sequence of deoxyribonucleotides (abbreviated A, G , C and T).

However, by "nucleotide sequence" of a nucleic acid molecule or polynucleotide is intended, for a DNA molecule or polynucleotide, a sequence of deoxyribonucleotides, and for an RNA molecule or polynucleotide, the corresponding sequence of ribonucleotides (A, G, C and U), where each thymidine deoxyribonucleotide (T) in the specified deoxyribonucleotide sequence is replaced by the ribonucleotide uridine (U). For instance, reference to an RNA molecule having the sequence of SEQ ID NO:1 set forth using deoxyribonucleotide abbreviations is intended to indicate an RNA molecule having a sequence in which each deoxyribonucleotide A, G or C of SEQ ID NO:1 has been replaced by the corresponding ribonucleotide A, G or C, and each deoxyribonucleotide T has been replaced by a ribonucleotide U.

Using the information provided herein, such as the nucleotide sequence of SEQ ID NO:1, a nucleic acid molecule of the present invention encoding an H11 polypeptide may be obtained using standard cloning and screening procedures, such as those for cloning cDNAs using any one of a number of commercially available cDNA libraries. Alternative, a cDNA library can be constructed using methods known to those skilled in the art. See, for example, *Molecular Cloning, A Laboratory Manual*, 2nd. edition, Sambrook, J., Fritsch, E. F. and Maniatis, T., eds., Cold Spring Harbor Laboratory Press , Cold Spring Harbor, N.Y. (1989). Illustrative of the invention, the nucleic acid molecule described in Fig. 1 was discovered by screening a HeLa cDNA. The determined nucleotide sequence of the H11 cDNA (SEQ ID NO:1) contains an open reading frame encoding a protein of 196 amino acid residues and a deduced molecular weight of about 21.5 kDa. The amino acid sequence of the predicted H11 protein (SEQ ID NO:2) is shown in Fig. 1.

As indicated, nucleic acid molecules of the present invention may be in the form of RNA, such as mRNA, or in the form of DNA, including, for instance, cDNA and genomic DNA obtained by cloning or produced synthetically. The DNA may be double-stranded or single-stranded. Single-stranded DNA or RNA

may be the coding strand, also known as the sense strand, or it may be the non-coding strand, also referred to as the anti-sense strand.

Isolated nucleic acid molecules of the present invention include DNA molecules comprising an open reading frame (ORF) and having the nucleotide sequence shown in SEQ ID NO:1 (Fig. 1); DNA molecules comprising a sequence encoding an H11 protein having the amino acid sequence shown in SEQ ID NO:2 (Fig. 1); and DNA molecules which comprise a sequence substantially different from those described above but which, due to the degeneracy of the genetic code, still encode the H11 protein. Of course, the genetic code is well known in the art. Thus, it is routine for one skilled in the art to generate the degenerate variants described above.

In another aspect, the invention provides isolated nucleic acid molecules having the nucleic acid sequence shown in SEQ ID NO:1; or isolated nucleic acid molecules encoding an H11 polypeptide having the amino acid sequence shown in SEQ ID NO:2; and variants of these.

The invention further provides an isolated nucleic acid molecule having a sequence complementary to the nucleotide sequence shown in SEQ ID NO:1; or complementary to a nucleotide sequence encoding an H11 protein having the amino acid sequence shown in SEQ ID NO:2. Such isolated molecules, particularly DNA molecules, are useful as probes for gene mapping, by *in situ* hybridization with chromosomes, and for detecting expression of the H11 gene in human tissue, for instance, by Northern blot analysis.

The present invention is further directed to fragments of the isolated nucleic acid molecules described herein. By a fragment of an isolated nucleic acid molecule having the nucleotide sequence shown in SEQ ID NO:1 is intended fragments at least about 15 nt, and more preferably at least about 20 nt, still more preferably at least about 30 nt, and even more preferably, at least about 40 nt in length which are useful as diagnostic probes and primers as discussed herein. Of course, larger fragments 50, 100, 150, 200, 250, 300, 350, 400, 500, or 600 nt in length are also useful according to the present invention as are fragments

corresponding to most, if not all, of the nucleotide sequence of SEQ ID NO:1. By a fragment at least 20 nt in length, for example, is intended fragments which include 20 or more contiguous bases from the nucleotide sequence of SEQ ID NO:1. Since the SEQ ID NO:1 nucleotide sequence has been provided, generating such DNA fragments would be routine to the skilled artisan. For example, restriction endonuclease cleavage or shearing by sonication could easily be used to generate fragments of various sizes. Alternatively, such fragments could be generated synthetically.

Preferred nucleic acid fragments of the present invention include nucleic acid molecules encoding epitope-bearing portions of the H11 protein. In particular, such nucleic acid fragments of the present invention include nucleic acid molecules encoding peptides that represent amino acids 10-29 or 181-193 of the sequence shown in SEQ ID NO:2. Methods for determining other such epitope-bearing portions of the H11 protein are described in detail below.

In another aspect, the invention provides an isolated nucleic acid molecule comprising a polynucleotide which hybridizes under stringent hybridization conditions to a portion of the polynucleotide in a nucleic acid molecule of the invention described above, for instance, a polynucleotide having the sequence of SEQ ID NO:1. By "stringent hybridization conditions" is intended overnight incubation at 42°C in a solution comprising: 50% formamide, 5x SSC (150 mM NaCl, 15mM trisodium citrate), 50 mM sodium phosphate (pH 7.6), 5x Denhardt's solution, 10% dextran sulfate, and 20 g/ml denatured, sheared salmon sperm DNA, followed by washing the filters in 0.1x SSC at about 65°C.

By a polynucleotide which hybridizes to a "portion" of a polynucleotide is intended a polynucleotide (either DNA or RNA) hybridizing to at least about 15 nucleotides (nt), and more preferably at least about 20 nt, still more preferably at least about 30 nt, and even more preferably about 30-70 nt of the reference polynucleotide. These are useful as diagnostic probes and primers as discussed above and in more detail below.

Of course, polynucleotides hybridizing to a larger portion of the reference polynucleotide (e.g., a polynucleotide having the sequence shown in SEQ ID NO:1), for instance, a portion 50, 100, 150, 200, 250, 300, 350, 400, 500, or 600 nt in length, or even to the entire length of the reference polynucleotide, are also useful as probes according to the present invention, as are polynucleotides corresponding to most, if not all, of the nucleotide sequence shown in SEQ ID NO:1. By a portion of a polynucleotide of "at least 20 nt in length," for example, is intended 20 or more contiguous nucleotides from the nucleotide sequence of the reference polynucleotide (e.g., the nucleotide sequence as shown in SEQ ID NO:1). As indicated, such portions are useful diagnostically either as a probe according to conventional DNA hybridization techniques or as primers for amplification of a target sequence by the polymerase chain reaction (PCR), as described, for instance, in *Molecular Cloning, A Laboratory Manual*, 2nd. edition, Sambrook, J., Fritsch, E. F. and Maniatis, T., eds., Cold Spring Harbor Laboratory Press , Cold Spring Harbor, N.Y. (1989), the entire disclosure of which is hereby incorporated herein by reference.

Generating polynucleotides which hybridize to a portion of an H11 polynucleotide molecule as described herein is routine to the skilled artisan. For example, restriction endonuclease cleavage or shearing by sonication of polynucleotides having the sequence shown in SEQ ID NO:1 is easily used to generate DNA polynucleotide fragments of various sizes that hybridize to a portion of H11 protein molecules. Alternatively, the hybridizing polynucleotides of the present invention may be generated synthetically according to known techniques. Of course, a polynucleotide which hybridizes only to a poly A sequence (such as the 3′ terminal poly(A) tract of the H11 cDNA shown in SEQ ID NO: 1, or to a complementary stretch of T (or U) resides, is not included in a polynucleotide of the invention used to hybridize to a portion of a nucleic acid of the invention, since such a polynucleotide would hybridize to any nucleic acid molecule containing a poly (A) stretch or the complement thereof (e.g., practically any double-stranded cDNA clone).

As indicated, nucleic acid molecules of the present invention which encode an H11 polypeptide may include, but are not limited to, those encoding the amino acid sequence of the polypeptide shown in SEQ ID NO:2, with or without additional non-coding sequences, including, for example (but not limited to), introns and non-coding 5′ and 3′ sequences such as the transcribed, non-translated sequences that play a role in transcription and mRNA processing (*e.g.*, splicing and polyadenylation signals) and that play a role in ribosome binding and stability of mRNA; and coding sequences which code for additional amino acids, such as those which provide additional functionalities. Thus, the sequence encoding the polypeptide may be fused to a marker sequence, such as a sequence encoding a peptide which facilitates purification of the fused polypeptide. In certain preferred embodiments of this aspect of the invention, the marker amino acid sequence is a hexa-histidine peptide, such as the tag provided in a pQE vector (Qiagen, Inc.), among others, many of which are commercially available. As described in Gentz *et al., Proc. Natl. Acad. Sci. USA 86*:821-824 (1989), for instance, hexa-histidine provides for convenient purification of the fusion protein. The "HA" tag is another peptide useful for purification which corresponds to an epitope derived from the influenza hemagglutinin protein, which has been described by Wilson *et al., Cell 37*: 767 (1984). As discussed below, other such fusion proteins include an H11-GST fusion protein.

The present invention further relates to variants of the nucleic acid molecules of the present invention, which encode portions, analogs or derivatives of the H11 protein. Variants may occur naturally, such as a natural allelic variant. By an "allelic variant" is intended one of several alternate forms of a gene occupying a given locus on a chromosome of an organism. *Genes II*, Lewin, B., ed., John Wiley & Sons, New York (1985). Non-naturally occurring variants may be produced using art-known mutagenesis techniques.

Such variants include those produced by nucleotide substitutions, deletions or additions. The substitutions, deletions or additions may involve one or more nucleotides. The variants may be altered in coding regions, non-coding

regions, or both. Alterations in the coding regions may produce conservative or non-conservative amino acid substitutions, deletions or additions. Especially preferred among these are silent substitutions, additions and deletions, which do not alter the properties and activities of the H11 protein or portions thereof. Also especially preferred in this regard are conservative substitutions. Most highly preferred are nucleic acid molecules encoding an H11 protein having the amino acid sequence shown in SEQ ID NO:2.

Further embodiments of the invention include isolated nucleic acid molecules comprising a polynucleotide having a nucleotide sequence at least 95% identical, and more preferably at least 96%, 97%, 98% or 99% identical to (a) a nucleotide sequence encoding the polypeptide having the amino acid sequence of SEQ ID NO:2 or to portions of the SEQ ID NO:2 sequence; or (b) a nucleotide sequence complementary to the nucleotide sequence in (a).

By a polynucleotide having a nucleotide sequence at least, for example, 95% "identical" to a reference nucleotide sequence encoding an H11 polypeptide is intended that the nucleotide sequence of the polynucleotide is identical to the reference sequence except that the polynucleotide sequence may include up to five point mutations per each 100 nucleotides of the reference nucleotide sequence encoding the H11 polypeptide. In other words, to obtain a polynucleotide having a nucleotide sequence at least 95% identical to a reference nucleotide sequence, up to 5% of the nucleotides in the reference sequence may be deleted or substituted with another nucleotide, or a number of nucleotides up to 5% of the total nucleotides in the reference sequence may be inserted into the reference sequence. These mutations of the reference sequence may occur at the 5′ or 3′ terminal positions of the reference nucleotide sequence or anywhere between those terminal positions, interspersed either individually among nucleotides in the reference sequence or in one or more contiguous groups within the reference sequence.

As a practical matter, whether any particular nucleic acid molecule is at least 95%, 96%, 97%, 98% or 99% identical to, for instance, the nucleotide

sequence shown in SEQ ID NO:1 can be determined conventionally using known computer programs such as the Bestfit program (Wisconsin Sequence Analysis Package, Version 8 for Unix, Genetics Computer Group, University Research Park, 575 Science Drive, Madison, WI 53711). Bestfit uses the local homology algorithm of Smith and Waterman, *Advances in Applied Mathematics* 2:482-489 (1981), to find the best segment of homology between two sequences. When using Bestfit or any other sequence alignment program to determine whether a particular sequence is, for instance, 95% identical to a reference sequence according to the present invention, the parameters are set, of course, such that the percentage of identity is calculated over the full length of the reference nucleotide sequence and that gaps in homology of up to 5% of the total number of nucleotides in the reference sequence are allowed. Alternatively, the "% identity" between two nucleic acid sequences can be determined using the "fastA" computer algorithm (Pearson, W.R. & Lipman, D.J., *Proc. Natl. Acad. Sci. USA* 85:2444 (1988)) with the default parameters.

The present invention also relates to vectors which include the isolated DNA molecules of the present invention, host cells which are genetically engineered with the recombinant vectors, and the production of H11 polypeptides or fragments thereof by recombinant techniques.

Recombinant constructs may be introduced into host cells using well known techniques such as infection, transduction, transfection, transvection, electroporation and transformation. The vector may be, for example, a phage, plasmid, viral or retroviral vector. Retroviral vectors may be replication competent or replication defective. In the latter case, viral propagation generally will occur only in complementing host cells.

The polynucleotides may be joined to a vector containing a selectable marker for propagation in a host. Generally, a plasmid vector is introduced in a precipitate, such as a calcium phosphate precipitate, or in a complex with a charged lipid. If the vector is a virus, it may be packaged *in vitro* using an appropriate packaging cell line and then transduced into host cells.

The vectors employed may comprise cis-acting control regions to the polynucleotide of interest. Appropriate trans-acting factors may be supplied by the host, supplied by a complementing vector or supplied by the vector itself upon introduction into the host.

The vectors employed may provide for specific expression, which may be inducible and/or cell type-specific. Inducible vectors may be those inducible by environmental factors that are easy to manipulate, such as temperature and nutrient additives.

Expression vectors useful in the present invention include chromosomal-, episomal- and virus-derived vectors, e.g., vectors derived from bacterial plasmids, bacteriophage, yeast episomes, yeast chromosomal elements, viruses such as baculoviruses, papova viruses, vaccinia viruses, adenoviruses, fowl pox viruses, pseudorabies viruses and retroviruses, and vectors derived from combinations thereof, such as cosmids and phagemids.

The DNA insert should be operatively linked to an appropriate promoter, such as the phage lambda PL promoter, the *E. coli lac, trp* and *tac* promoters, the SV40 early and late promoters and promoters of retroviral LTRs, to name a few. Other suitable promoters will be known to the skilled artisan. The expression constructs will further contain sites for transcription initiation, termination and, in the transcribed region, a ribosome binding site for translation. The coding portion of the mature transcripts expressed by the constructs will preferably include a translation initiating at the beginning and a termination codon (UAA, UGA or UAG) appropriately positioned at the end of the polypeptide to be translated.

As indicated, the expression vectors will preferably include at least one selectable marker. Such markers may include dihydrofolate reductase or neomycin resistance for eukaryotic cell culture and tetracycline or ampicillin resistance genes for culturing in *E. coli* and other bacteria. Representative examples of appropriate hosts include, but are not limited to, bacterial cells, such as *E. coli*, *Streptomyces* and *Salmonella typhimurium* cells; fungal cells, such as

yeast cells; insect cells such as *Drosophila* S2 and *Spodoptera* Sf9 cells; animal cells such as CHO, COS and Bowes melanoma cells; and plant cells. Appropriate culture mediums and conditions for the above-described host cells are known in the art.

Among vectors preferred for use in bacteria include pQE70, pQE60 and pQE-9, available from Qiagen; pBS vectors, Phagescript vectors, Bluescript vectors, pNH8A, pNH16a, pNH18A, pNH46A, available from Stratagene; and ptrc99a, pKK223-3, pKK233-3, pDR540, pRIT5 available from Pharmacia. Among preferred eukaryotic vectors are pWLNEO, pSV2CAT, pOG44, pXT1 and pSG available from Stratagene; and pSVK3, pBPV, pMSG and pSVL available from Pharmacia. Other suitable vectors will be readily apparent to the skilled artisan.

Among known bacterial promoters suitable for use in the present invention include the *E. coli lac*I and *lac*Z promoters, the T3 and T7 promoters, the *gpt* promoter, the lambda PR and PL promoters and the *trp* promoter. Suitable eukaryotic promoters include the CMV immediate early promoter, the HSV thymidine kinase promoter, the early and late SV40 promoters, the promoters of retroviral LTRs, such as those of the Rous sarcoma virus (RSV), and metallothionein promoters, such as the mouse metallothionein-I promoter.

Introduction of the construct into the host cell can be effected by calcium phosphate transfection, DEAE-dextran mediated transfection, cationic lipid-mediated transfection, electroporation, transduction, infection or other methods. Such methods are described in many standard laboratory manuals, such as Davis *et al., Basic Methods In Molecular Biology* (1986).

Transcription of the DNA encoding the polypeptides of the present invention by higher eukaryotes may be increased by inserting an enhancer sequence into the vector. Enhancers are cis-acting elements of DNA, usually about from 10 to 300 bp that act to increase transcriptional activity of a promoter in a given host cell-type. Examples of enhancers include the SV40 enhancer, which is located on the late side of the replication origin at bp 100 to 270, the

cytomegalovirus early promoter enhancer, the polyoma enhancer on the late side of the replication origin, and adenovirus enhancers.

The polypeptide may be expressed in a modified form, such as a fusion protein, and may include additional heterologous functional regions. For instance, peptide moieties may be added to the polypeptide to facilitate purification. Such regions may be removed prior to final preparation of the polypeptide. The addition of peptide moieties to polypeptides to engender secretion or excretion, to improve stability and to facilitate purification, among others, are familiar and routine techniques in the art.

The H11 protein can be recovered and purified from recombinant cell cultures by well-known methods including ammonium sulfate or ethanol precipitation, acid extraction, anion or cation exchange chromatography, phosphocellulose chromatography, hydrophobic interaction chromatography, affinity chromatography, hydroxylapatite chromatography and lectin chromatography. Polypeptides of the present invention include naturally purified products, products of chemical synthetic procedures, and products produced by recombinant techniques from a prokaryotic or eukaryotic host, including, for example, bacterial, yeast, higher plant, insect and mammalian cells. Depending upon the host employed in a recombinant production procedure, the polypeptides of the present invention may be glycosylated or may be non-glycosylated. In addition, polypeptides of the invention may also include an initial modified methionine residue, in some cases as a result of host-mediated processes.

The invention further provides an isolated H11 polypeptide having the amino acid sequence shown in SEQ ID NO:2, or a peptide or polypeptide comprising a portion of the above polypeptide. The terms "peptide" and "oligopeptide" are considered synonymous (as is commonly recognized) and each term can be used interchangeably as the context requires to indicate a chain of at least to amino acids coupled by peptidyl linkages. The word "polypeptide" is used herein for chains containing more than ten amino acid residues. All

oligopeptide and polypeptide formulas or sequences herein are written from left to right and in the direction from amino terminus to carboxy terminus.

It will be recognized in the art that some amino acid sequences of the H11 polypeptide can be varied without significant effect of the structure or function of the protein. If such differences in sequence are contemplated, it should be remembered that there will be critical areas on the protein which determine activity. In general, it is possible to replace residues which form the tertiary structure, provided that residues performing a similar function are used. In other instances, the type of residue may be completely unimportant if the alteration occurs at a non-critical region of the protein.

Thus, the invention further includes variations of H11 polypeptides which include regions of the H11 protein such as the protein portions discussed below. Such mutants include deletions, insertions, inversions, repeats, and type substitutions (for example, substituting one hydrophilic residue for another, but not strongly hydrophilic for strongly hydrophobic as a rule). Small changes or such "neutral" amino acid substitutions will generally have little effect on H11 function.

Typically seen as conservative substitutions are the replacements, one for another, among the aliphatic amino acids Ala, Val, Leu and Ile; interchange of the hydroxyl residues Ser and Thr, exchange of the acidic residues Asp and Glu, substitution between the amide residues Asn and Gln, exchange of the basic residues Lys and Arg and replacements among the aromatic residues Phe, Tyr.

As indicated in detail above, further guidance concerning which amino acid changes are likely to be phenotypically silent (i.e., are not likely to have a significant deleterious effect on a function) can be found in Bowie, J.U., *et al.*, "Deciphering the Message in Protein Sequences: Tolerance to Amino Acid Substitutions," *Science 247:*1306-1310 (1990).

Thus, the fragment, derivative or analog of a polypeptide having the sequence shown in SEQ ID NO:2 may be one in which one or more of the amino acid residues are substituted with a conserved or non-conserved amino acid

residue (preferably a conserved amino acid residue) and such substituted amino acid residue may or may not be one encoded by the genetic code, or (ii) one in which one or more of the amino acid residues includes a substituent group, or (iii) one in which the polypeptide is fused with another compound, such as a compound to increase the half-life of the polypeptide (for example, polyethylene glycol), or (iv) one in which the additional amino acids are fused to the H11 polypeptide, such as an IgG Fc fusion region peptide or a sequence which is employed for purification of the H11 polypeptide. Such fragments, derivatives and analogs are deemed to be within the scope of those skilled in the art from the teachings herein.

The polypeptides of the present invention are preferably provided in an isolated form. By "isolated polypeptide" is intended a polypeptide removed from its native environment. Thus, a polypeptide produced and/or contained within a recombinant host cell is considered isolated for purposes of the present invention. Also intended as an "isolated polypeptide" are polypeptides that have been purified, partially or substantially, from a recombinant host cell or a native source.

The polypeptides of the present invention include the polypeptide having the amino acid sequence shown in SEQ ID NO:2, as well as polypeptides which are at least 95% identical, and more preferably at least 96%, 97%, 98% or 99% identical to the polypeptide comprising the amino acid sequence shown in SEQ ID NO:2, and also include portions of such polypeptides with at least 30 amino acids and more preferably at least 50 amino acids.

By a polypeptide having an amino acid sequence at least, for example, 95% "identical" to a reference amino acid sequence of an H11 polypeptide is intended that the amino acid sequence of the polypeptide is identical to the reference sequence except that the polypeptide sequence may include up to five amino acid alterations per each 100 amino acids of the reference sequence of the H11 polypeptide. In other words, to obtain a polypeptide having an amino acid sequence at least 95% identical to a reference amino acid sequence, up to 5% of

the amino acid residues in the reference sequence may be deleted or substituted with another amino acid, or a number of amino acids up to 5% of the total amino acid residues in the reference sequence may be inserted into the reference sequence. These alterations of the reference sequence may occur at the amino or carboxy terminal positions of the reference amino acid sequence or anywhere between those terminal positions, interspersed either individually among residues in the reference sequence or in one or more contiguous groups within the reference sequence.

As a practical matter, whether any particular polypeptide is at least 95%, 96%, 97%, 98% or 99% identical to, for instance, the amino acid sequence shown in SEQ ID NO:2 can be determined conventionally using known computer programs such the Bestfit program (Wisconsin Sequence Analysis Package, Version 8 for Unix, Genetics Computer Group, University Research Park, 575 Science Drive, Madison, WI 53711). When using Bestfit or any other sequence alignment program to determine whether a particular sequence is, for instance, 95% identical to a reference sequence according to the present invention, the parameters are set, of course, such that the percentage of identity is calculated over the full length of the reference amino acid sequence and that gaps in homology of up to 5% of the total number of amino acid residues in the reference sequence are allowed.

As described in detail below, the polypeptides of the present invention can also be used to raise polyclonal and monoclonal antibodies, which are useful in assays for detecting H11 protein expression as described below.

In another aspect, the invention provides a peptide or polypeptide comprising an epitope-bearing portion of a polypeptide of the invention. The epitope of this polypeptide portion is an immunogenic or antigenic epitope of a polypeptide of the invention. An "immunogenic epitope" is defined as a part of a protein that elicits an antibody response when the whole protein is the immunogen. These immunogenic epitopes are believed to be confined to a few loci on the molecule. On the other hand, a region of a protein molecule to which

an antibody can bind is defined as an "antigenic epitope." The number of immunogenic epitopes of a protein generally is less than the number of antigenic epitopes. See, for instance, Geysen *et al., Proc. Natl. Acad. Sci. USA 81*:3998-4002 (1983).

As to the selection of peptides or polypeptides bearing an antigenic epitope (i.e., that contain a region of a protein molecule to which an antibody can bind), it is well known in that art that relatively short synthetic peptides that mimic part of a protein sequence are routinely capable of eliciting an antiserum that reacts with the partially mimicked protein. See, for instance, Sutcliffe, J. G., Shinnick, T. M., Green, N. and Learner, R.A., "Antibodies that react with predetermined sites on proteins," *Science 219:*660-666 (1983). Peptides capable of eliciting protein-reactive sera are frequently represented in the primary sequence of a protein, can be characterized by a set of simple chemical rules, and are confined neither to immunodominant regions of intact proteins (i.e., immunogenic epitopes) nor to the amino or carboxyl terminals. Peptides that are extremely hydrophobic and those of six or fewer residues generally are ineffective at inducing antibodies that bind to the mimicked protein; longer, peptides, especially those containing proline residues, usually are effective. Sutcliffe *et al., supra*, at 661. For instance, 18 of 20 peptides designed according to these guidelines, containing 8-39 residues covering 75% of the sequence of the influenza virus hemagglutinin HA1 polypeptide chain, induced antibodies that reacted with the HA1 protein or intact virus; and 12/12 peptides from the MuLV polymerase and 18/18 from the rabies glycoprotein induced antibodies that precipitated the respective proteins.

Antigenic epitope-bearing peptides and polypeptides of the invention are therefore useful to raise antibodies, including monoclonal antibodies, that bind specifically to a polypeptide of the invention. Thus, a high proportion of hybridomas obtained by fusion of spleen cells from donors immunized with an antigen epitope-bearing peptide generally secrete antibody reactive with the native protein. Sutcliffe *et al., supra*, at 663. The antibodies raised by antigenic

epitope-bearing peptides or polypeptides are useful to detect the mimicked protein, and antibodies to different peptides may be used for tracking the fate of various regions of a protein precursor which undergoes post-translational processing. The peptides and anti-peptide antibodies may be used in a variety of qualitative or quantitative assays for the mimicked protein, for instance in competition assays since it has been shown that even short peptides (e.g., about 9 amino acids) can bind and displace the larger peptides in immunoprecipitation assays. See, for instance, Wilson *et al.*, *Cell 37*:767-778 (1984) at 777. The anti-peptide antibodies of the invention also are useful for purification of the mimicked protein, for instance, by adsorption chromatography using methods well known in the art.

Antigenic epitope-bearing peptides and polypeptides of the invention designed according to the above guidelines preferably contain a sequence of at least seven, more preferably at least nine and most preferably between about 15 to about 30 amino acids contained within the amino acid sequence of a polypeptide of the invention. However, peptides or polypeptides comprising a larger portion of an amino acid sequence of a polypeptide of the invention, containing about 30 to about 50 amino acids, or any length up to and including the entire amino acid sequence of a polypeptide of the invention, also are considered epitope-bearing peptides or polypeptides of the invention and also are useful for inducing antibodies that react with the mimicked protein. Preferably, the amino acid sequence of the epitope-bearing peptide is selected to provide substantial solubility in aqueous solvents (i.e., the sequence includes relatively hydrophilic residues and highly hydrophobic sequences are preferably avoided); and sequences containing proline residues are particularly preferred.

Non-limiting examples of antigenic polypeptides or peptides that can be used to generate H11-specific antibodies include, for example, polypeptides comprising amino acid residues 10-29 or amino acid residues 181-193 of the sequence shown in SEQ ID NO:2.

The epitope-bearing peptides and polypeptides of the invention may be produced by any conventional means for making peptides or polypeptides including recombinant means using nucleic acid molecules of the invention. For instance, a short epitope-bearing amino acid sequence may be fused to a larger polypeptide which acts as a carrier during recombinant production and purification, as well as during immunization to produce anti-peptide antibodies. Epitope-bearing peptides also may be synthesized using known methods of chemical synthesis. For instance, Houghten has described a simple method for synthesis of large numbers of peptides, such as 10-20 mg of 248 different 13 residue peptides representing single amino acid variants of a segment of the HA1 polypeptide which were prepared and characterized (by ELISA-type binding studies) in less than four weeks. Houghten, R. A., "General method for the rapid solid-phase synthesis of large numbers of peptides: specificity of antigen-antibody interaction at the level of individual amino acids," *Proc. Natl. Acad. Sci. USA 82*:5131-5135 (1985). This "Simultaneous Multiple Peptide Synthesis (SMPS)" process is further described in U.S. Patent No. 4,631,211 to Houghten *et al.* (1986). In this procedure the individual resins for the solid-phase synthesis of various peptides are contained in separate solvent-permeable packets, enabling the optimal use of the many identical repetitive steps involved in solid-phase methods. A completely manual procedure allows 500-1000 or more syntheses to be conducted simultaneously. Houghten *et al.*, *supra*, at 5134.

Epitope-bearing peptides and polypeptides of the invention are used to induce antibodies according to methods well known in the art. See, for instance, Sutcliffe *et al.*, *supra*; Wilson *et al.*, *supra*; Chow, M. *et al.*, *Proc. Natl. Acad. Sci. USA 82*:910-914; and Bittle, F. J. *et al.*, *J. Gen. Virol. 66*:2347-2354 (1985). Generally, animals may be immunized with free peptide; however, anti-peptide antibody titer may be boosted by coupling of the peptide to a macromolecular carrier, such as keyhole limpet hemacyanin (KLH) or tetanus toxoid. For instance, peptides containing cysteine may be coupled to carrier using a linker such as m-maleimidobenzoyl-N-hydroxysuccinimide ester (MBS), while other

peptides may be coupled to carrier using a more general linking agent such as glutaraldehyde. Animals such as rabbits, rats and mice are immunized with either free or carrier-coupled peptides, for instance, by intraperitoneal and/or intradermal injection of emulsions containing about 100 μg peptide or carrier protein and Freund's adjuvant. Several booster injections may be needed, for instance, at intervals of about two weeks, to provide a useful titer of anti-peptide antibody which can be detected, for example, by ELISA assay using free peptide adsorbed to a solid surface. The titer of anti-peptide antibodies in serum from an immunized animal may be increased by selection of anti-peptide antibodies, for instance, by adsorption to the peptide on a solid support and elution of the selected antibodies according to methods well known in the art.

Immunogenic epitope-bearing peptides of the invention, i.e., those parts of a protein that elicit an antibody response when the whole protein is the immunogen, are identified according to methods known in the art. For instance, Geysen *et al.*, *supra*, discloses a procedure for rapid concurrent synthesis on solid supports of hundreds of peptides of sufficient purity to react in an enzyme-linked immunosorbent assay. Interaction of synthesized peptides with antibodies is then easily detected without removing them from the support. In this manner a peptide bearing an immunogenic epitope of a desired protein may be identified routinely by one of ordinary skill in the art. For instance, the immunologically important epitope in the coat protein of foot-and-mouth disease virus was located by Geysen *et al. supra* with a resolution of seven amino acids by synthesis of an overlapping set of all 208 possible hexapeptides covering the entire 213 amino acid sequence of the protein. Then, a complete replacement set of peptides in which all 20 amino acids were substituted in turn at every position within the epitope were synthesized, and the particular amino acids conferring specificity for the reaction with antibody were determined. Thus, peptide analogs of the epitope-bearing peptides of the invention can be made routinely by this method. U.S. Patent No. 4,708,781 to Geysen (1987) further describes this method of identifying a peptide bearing an immunogenic epitope of a desired protein.

Further still, U.S. Patent No. 5,194,392 to Geysen (1990) describes a general method of detecting or determining the sequence of monomers (amino acids or other compounds) which is a topological equivalent of the epitope (i.e., a "mimotope") which is complementary to a particular paratope (antigen binding site) of an antibody of interest. More generally, U.S. Patent No. 4,433,092 to Geysen (1989) describes a method of detecting or determining a sequence of monomers which is a topographical equivalent of a ligand which is complementary to the ligand binding site of a particular receptor of interest. Similarly, U.S. Patent No. 5,480,971 to Houghten, R. A. *et al.* (1996) on Peralkylated Oligopeptide Mixtures discloses linear C_1-C_7-alkyl peralkylated oligopeptides and sets and libraries of such peptides, as well as methods for using such oligopeptide sets and libraries for determining the sequence of a peralkylated oligopeptide that preferentially binds to an acceptor molecule of interest. Thus, non-peptide analogs of the epitope-bearing peptides of the invention also can be made routinely by these methods.

III. Diagnostic Applications

The present invention is directed to methods of detecting melanoma and other cancers in a mammal by utilizing nucleic acid probes hybridizable to the H11 gene or transcripts, or antibodies specific for the H11 protein.

Nucleic Acid Hybridization Assays

In one embodiment of the invention, polynucleotides encoding H11 may be used for diagnostic purposes. The polynucleotides which may be used include oligonucleotide sequences, antisense RNA and DNA molecules. The polynucleotides may be used to detect and quantitate gene expression in biopsied tissues in which expression of H11 may be correlated with disease (*e.g.*, melanoma). The diagnostic assay may be used to distinguish between absence,

presence, and excess expression of H11, and to monitor regulation of H11 expression levels during therapeutic intervention.

In one aspect, hybridization with PCR probes which are capable of detecting polynucleotide sequences, including genomic sequences, may be used to identify nucleic acid sequences which encode H11. The specificity of the probe, whether it is made from a highly specific region or a less specific region, and the stringency of the hybridization or amplification (maximal, high, intermediate, or low) will determine whether the probe identifies only naturally occurring sequences encoding H11, alleles, or related sequences.

Probes may also be used for the detection of related sequences, and should preferably contain at least 50% of the nucleotides from an H11 encoding sequence. The hybridization probes of the subject invention may be DNA or RNA and may be derived from the nucleotide sequence of SEQ ID NO:1 or from a genomic sequence including promoter, enhancer elements, and introns of the naturally occurring H11 gene.

Means for producing specific hybridization probes for DNAs encoding H11 include the cloning of nucleic acid sequences encoding H11 polypeptide or H11 polypeptide derivatives into vectors for the production of mRNA probes. Such vectors are known in the art, commercially available, and may be used to synthesize RNA probes in vitro by means of the addition of the appropriate RNA polymerases and the appropriate labeled nucleotides. Hybridization probes may be labeled by a variety of reporter groups, for example, radionuclides such as ^{32}P or ^{35}S, or enzymatic labels, such as alkaline phosphatase coupled to the probe via avidin/biotin coupling systems, and the like.

Polynucleotide sequences encoding H11 may be used for the diagnosis of conditions or diseases which are associated with expression or over-expression of H11. Examples of such conditions or diseases include melanoma, as well as other specific cancers such as estrogen-dependent breast cancer and ovarian cancer. The polynucleotide sequences encoding H11, or their complements, may be used in Southern or northern analysis, dot blot, or other membrane-based

technologies; in PCR technologies; or in dip stick, pIN, ELISA or chip assays utilizing fluids or tissues from patient biopsies to detect altered H11 expression. Such qualitative or quantitative methods are well known in the art.

In a particular aspect, the nucleotide sequences encoding an H11 polypeptide ("H11") may be useful in assays that detect activation or induction of the cancers mentioned above. The nucleotide sequences encoding H11 may be labeled by standard methods, and added to a fluid or tissue sample from a patient under conditions suitable for the formation of hybridization complexes. After a suitable incubation period, the sample is washed and the signal is quantitated and compared with a standard value. If the amount of signal in the biopsied or extracted sample is significantly altered from that of a comparable control sample, the nucleotide sequences have hybridized with nucleotide sequences in the sample, and the presence of altered levels of nucleotide sequences encoding H11 in the sample indicates the presence of the associated disease. Such assays may also be used to evaluate the efficacy of a particular therapeutic treatment regimen in animal studies, in clinical trials, or in monitoring the treatment of an individual patient.

In order to provide a basis for the diagnosis of disease associated with expression of H11, a normal or standard profile for expression is established. This may be accomplished by combining cell extracts taken from normal subjects, either animal or human, with a sequence, or a fragment thereof, which encodes H11, under conditions suitable for hybridization or amplification. Standard hybridization may be quantified by comparing the values obtained from normal subjects with those from an experiment where a known amount of a substantially purified polynucleotide is used. Standard values obtained from normal samples may be compared with values obtained from samples from patients who are symptomatic for disease. Deviation between standard and subject values is used to establish the presence of disease.

Once disease is established and a treatment protocol is initiated, hybridization assays may be repeated on a regular basis to evaluate whether the

level of expression in the patient begins to approximate that which is observed in the normal patient. The results obtained from successive assays may be used to show the efficacy of treatment over a period ranging from several days to months.

With respect to cancer, the presence of a relatively high amount of transcript in biopsied tissue from an individual may indicate a predisposition for the development of the disease, or may provide a means for detecting the disease prior to the appearance of actual clinical symptoms. A more definitive diagnosis of this type may allow health professionals to employ preventative measures or aggressive treatment earlier thereby preventing the development or further progression of the cancer.

The nucleic acid sequence or portions thereof, of this invention are useful as probes for the detection of expression of the H11 gene in normal and diseased tissue. Therefore, one aspect of the present invention relates to a bioassay for detecting messenger RNA encoding the H11 protein in a biological sample comprising the steps of (a) contacting a biological sample with all or part of the nucleic acid sequence of this invention under conditions allowing a complex to form between said nucleic acid sequence and said messenger RNA, and (b) detecting said complexes. This method may further comprise a step (c) of determining the level of said messenger RNA.

RNA can be isolated as whole cell RNA or as poly(A)$^+$ RNA. Whole cell RNA can be isolated by a variety of methods known to those skilled in the art. (See, for example, "Current Protocols in Molecular Biology", Ausubel *et al.*, eds., John Wiley and Sons, New York (1992)). Such methods include extraction of RNA by differential precipitation (Birnboim, H. C., *Nucleic Acids Res. 16*:1487-1497 (1988)), extraction of RNA by organic solvents (Chomczynski, P. *et al.*, *Anal. Biochem. 162*:156-159 (1987)) and the extraction of RNA with strong denaturants (Chirgwin, J. M. *et al.*, *Biochemistry 18*:5294-5299 (1979)). Poly(A)$^+$ RNA can be selected from whole cell RNA by affinity chromatography on oligo-d(T) columns (Aviv, H. *et al.*, *Proc. Natl. Acad. Sci. 69*:1408-1412

(1972)). Examples of methods for determining cellular messenger mRNA levels for step (c) include, but are not limited to Northern blotting (Alwine, J. C. *et al.*, *Proc. Natl. Acad. Sci. 74*:5350-5354 (1977)), dot and slot hybridization (Kafatos, F. C. *et al.*, *Nucleic Acids Res. 7*:1541-1522 (1979)), filter hybridization (Hollander, M. C. *et al.*, *Biotechniques 9*:174-179 (1990)), RNase protection (Sambrook *et al.*, "Molecular Cloning, A Laboratory Manual", Cold Spring Harbor Press, Plainview, N.Y. (1989)), polymerase chain reaction (Watson, J. D. *et al.*, "Recombinant DNA" Second Edition, W. H. Freeman and Company, New York (1992)) and nuclear run-off assays ("Current Protocols in Molecular Biology" Supplement 9 (1990), Ausubel *et al.*, eds., John Wiley and Sons, New York, N.Y. (1992)).

Detection of complexes in Step (b) of the bioassay can also be carried out by a variety of techniques. Detection of the complexes by signal amplification can be achieved by several conventional labeling techniques including radiolabels and enzymes (Sambrook *et al.*, "Molecular Cloning, A Laboratory Manual", Cold Spring Harbor Press, Plainview, N.Y. (1989); "Current Protocols in Molecular Biology", Ausubel *et al.*, eds., John Wiley and Sons, New York (1992)). Radiolabeling kits are also commercially available. The H11 nucleic acid sequence used as a probe in step (a) of the bioassay may be RNA or DNA. Preferred methods of labeling the DNA sequences are with ^{32}P using Klenow enzyme or polynucleotide kinase. Preferred methods of labeling RNA or riboprobe sequences are with ^{32}P or ^{35}S using RNA polymerases. In addition, there are known non-radioactive techniques for signal amplification including methods for attaching chemical moieties to pyrimidine and purine rings (Dale, R. N. K. *et al.*, *Proc. Natl. Acad. Sci. 70*:2238-2242 (1973); Heck, R. F. S., *Am. Chem. Soc. 90*:5518-5523 (1968)), methods which allow detection by chemiluminescence (Barton, S. K. *et al.*, *J. Am. Chem. Soc. 114*:8736-8740 (1992)) and methods utilizing biotinylated nucleic acid probes (Johnson, T. K. *et al.*, *Anal. Biochem. 133*:125-131 (1983); Erickson, P. F. *et al.*, *J. Immunology Methods 51*:241-249 (1982); Matthaei, F. S. *et al.*, *Anal. Biochem. 157*:123-128

(1986)) and methods which allow detection by fluorescence using commercially available products. Non-radioactive labeling kits are also commercially available.

Examples of biological samples that can be used in this bioassay include, but are not limited to, primary mammalian cultures, continuous mammalian cell lines, such as melanocyte cell lines, mammalian organs such as skin or retina, tissues, biopsy specimens, neoplasms, pathology specimens, and necropsy specimens.

Thus, in one embodiment of this aspect of the present invention, labeled polynucleotide sequences encoding H11 or portions of H11 can be used in Northern blot analysis of H11 mRNA expression in tissues. Northern analysis can be performed as described herein.

In another embodiment all or parts thereof of the nucleic acid of this invention can be used in *in situ* hybridization, in particular *in situ* PCR, on mammalian tissues to determine the precise site or subcellular site of expression of the H11 gene within a tissue. Labeled DNA or labeled RNA probes may be used. A preferred method of labeling the H11 nucleic acid sequence is synthesizing a ^{35}S-labeled RNA probe by in vitro transcription utilizing polymerases known to those skilled in the art. Conventional methods for preparation of tissues for *in situ* hybridization, synthesis of probes and detection of signal can be found in Chapter 14 of "Current Protocols in Molecular Biology," Ausubel *et. al.*, eds., John Wiley and Sons, New York, New York (1992), and Vander Ploeg, M., and Raap, A. K., in "New Frontiers in Cytology" Goerttler, K., Feichter, G.E., Witte. S., eds., pp 13-21 Springer-Verlag, New York (1988). The probe is then contacted with mammalian tissue sections and in situ analyses performed by conventional methods. Examples of tissues that can be used include, but are not limited to, mammalian embryos, adult mammalian tissues, such as skin, lymph nodes and retina, biopsy specimens, pathology specimens and necropsy specimens.

Thus, in another embodiment of this aspect of the invention, H11 polynucleotide probes may be used to evaluate H11 RNA expression *in situ* in

diseased tissue to examine for early development of neoplastic changes, to characterize radial and vertical growth phases of the melanoma lesion, or to assess the margins of the disease within the tissue in a manner known to those skilled in the art. Alternatively, H11 polynucleotide probes can be used to verify whether the margins of removed melanoma tumors are free of cancerous cells *in situ*.

Methods of the present invention can also be used to determine the efficacy of treatment for metastatic melanoma. Thus, in one aspect of the invention, labeled H11 polynucleotide probes may be used to detect melanoma cells in serum from an individual, and thus to prognose the progression of metastatic melanoma after treatment by standard methods such as chemotherapy, *etc.* See, for example, Keilhotz, U., *Eur. J. Cancer 34(Suppl. 3)*:537-541 (1998). In this aspect of the invention, RT-PCR (reverse-transcriptase PCR) can be used to detect the presence of H11-encoding polynucleotides from any melanoma cells present in serum from the affected individual. Protocols for the use of RT-PCR are well known in the art, and can be found, for example, in Chapter 15 of "Current Protocols in Molecular Biology," Ausubel *et. al.*, eds., John Wiley and Sons, New York, New York (1992).

A major consideration associated with hybridization analysis of DNA or RNA sequences is the degree of relatedness the probe has with the sequences present in the specimen under study. This is important with the blotting technique, since a moderate degree of sequence homology under nonstringent conditions of hybridization can yield a strong signal even though the probe and sequences in the sample represent non-homologous genes.

The particular hybridization technique is not essential to the invention, any technique commonly used in the art being within the scope of the present invention. Typical probe technology is described in U.S. Pat. No. 4,358,535 to Falkow et al., incorporated by reference herein. For example, hybridization can be carried out in a solution containing 6 x SSC (10 x SSC: 1.5 M sodium chloride, 0.15 M sodium citrate, pH 7.0), 5 x Denhardt's (1 Denhardt's: 0.2%

bovine serum albumin, 0.2% polyvinylpyrrolidone, 0.02% Ficoll 400), 10 mM EDTA, 0.5% SDS and about 10.sup.7 cpm of nick-translated DNA for 16 hours at 65 C. Alternatively, the conditions outlined in Example 1 below can be used.

The labeled probes, as described above, provide a general diagnostic method for detection of the H11 expression in tissue. The method is reasonably rapid, has a simple protocol, has reagents which can be standardized and provided as commercial kits, and allows for rapid screening of large numbers of samples.

In one method for carrying out the procedure, a clinical isolate containing RNA transcripts is fixed to a support. The affixed nucleic acid is contacted with a labeled polynucleotide having a base sequence complementary or homologous to the coding strand of the H11 gene.

The hybridization assays of the present invention are particularly well suited for preparation and commercialization in kit form, the kit comprising a carrier such as a box or carton compartmentalized to receive one or more containers (vials, test tubes, etc.) in close confinement, each of said containers comprising one of the separate elements to be used in hybridization assay.

For example, there may be a container containing H11 cDNA molecules suitable for labeling by "nick translation" (see, for example, Sambrook *et al.*, supra, for standard methodology), or labeled H11 cDNA or RNA molecules. Further containers may contain standard solutions for nick translation of H11 cDNA comprising DNA polymerase I/DNase I and unlabeled deoxyribonucleotides (i.e., dCTP, dTTP, dGTP, and dATP).

Additional diagnostic uses for oligonucleotides designed from the sequences encoding H11 may involve the use of PCR. Such oligomers may be chemically synthesized, generated enzymatically, or produced from a recombinant source. Oligomers will preferably consist of two nucleotide sequences, one with sense orientation (5' to 3') and another with antisense (3' to 5'), employed under optimized conditions for identification of H11 polynucleotides. The same two oligomers, nested sets of oligomers, or even a degenerate pool of oligomers may be employed under less stringent conditions for detection and/or quantitation of

closely related DNA or RNA sequences. For example, the H11 sense (5′-CCATGGCTGACGGTCAGATGCCCTTCTCCT-3′) (SEQ ID NO:3) and antisense (5′-TCCATGCCAAAGCCATCATCCAGCAG-3′) (SEQ ID NO:4) primers may be used.

Additional examples of H11 sense primers include 5'- GCCA CCATGG CTGACG GTCAGA TGCCCT TC-3' (SEQ ID NO: 17); 5'-AG CAGCCA CCATGG CTGACG GTCAGA TGCC-3' (SEQ ID NO: 18); 5'-GCTGAG CAGCCA CCATGG CTGACG GTCAGA-3' (SEQ ID NO: 19); 5'-CTGA GCTGAG CAGCCA CCATGG CTGACG GT-3' (SEQ ID NO: 20); 5'-CT CTCTGA GCTGAG CAGCCA CCATGG CTGA-3' (SEQ ID NO: 21); and 5'- GTTTCT CTCTGA GCTGAG CAGCCA CCATGG -3' (SEQ ID NO: 22).

Additional examples of H11 antisense primers include 3'-TG CCAAAG CCATCA TCCAGC AGGCGA-5' (SEQ ID NO: 23); 3'-AAAG CCATCA TCCAGC AGGCGA GAGG-5' (SEQ ID NO: 24); 3'-CCATCA TCCAGC AGGCGA GAGGAG AG-5' (SEQ ID NO: 25); 3'-CA TCCAGC AGGCGA GAGGAG AGGGGA-5' (SEQ ID NO: 26); 3'-CAGC AGGCGA GAGGAG AGGGGA GAGT-5' (SEQ ID NO: 27); and 3'-AGGCGA GAGGAG AGGGGA GAGTCC CG-5' (SEQ ID NO: 11).

In another embodiment of this aspect of the invention, H11 oligonucleotide primers (such as any of the primer sequences above) may be used in combination with primers for other relevant genes in a form of "multiplex PCR" to detect melanoma cells. See, for example, Hatta, N., *et al.*, *J. Clin. Pathol. 51*:597-601 (1998). In this embodiment, H11 primers can be used with primers for other genes everexpressed in specific tissues. For example, primers for the S100 gene (Allore, R.J., *et al.*, *J. Biol. Chem. 265*:15537-15543 (1990)) can be used in combination with H11 primers for the detection of melanoma.

Methods which may also be used to quantitate the expression of H11 include radiolabeling or biotinylating nucleotides, coamplification of a control nucleic acid, and standard curves onto which the experimental results are interpolated (Melby, P. C. *et al.*, *J. Immunol. Methods 159*:235-244 (1993);

Duplaa, C. *et al.*, *Anal. Biochem.* 229-236 (1993)). The speed of quantitation of multiple samples may be accelerated by running the assay in an ELISA format where the oligomer of interest is presented in various dilutions and a spectrophotometric or calorimetric response gives rapid quantitation.

H11-specific Antibodies

In another embodiment of the invention, antibodies directed against H11 protein can be used to detect melanoma, as well as other cancers in which H11 has been demonstrated to be expressed or in which H11 has been demonstrated to be overexpressed relative to normal, noncancerous tissue. Various histological staining methods, including immunohistochemical staining methods, may also be used effectively according to the teaching of the invention. Silver stain is but one method of visualizing the H11 protein. Other staining methods useful in the present invention will be obvious to the artisan, the determination of which would not involve undue experimentation (*see generally*, for example, *A Textbook of Histology*, Eds. Bloom and Fawcett, W.B. Saunders Co., Philadelphia (1964)).

One screening method for determining the existence of the H11 protein in a biological sample comprises, for example, immunoassays employing radioimmunoassay (RIA), enzyme-linked immunosorbant assay (ELISA), or immunhistochemistry methodologies, based on the production of specific antibodies (monoclonal or polyclonal) to H11. For these assays, biological samples of tissue are obtained (*e.g.*, biopsied tissue). For example, in one form of RIA, the substance under test is mixed with diluted antiserum in the presence of radiolabeled antigen. In this method, the concentration of the test substance will be inversely proportional to the amount of labeled antigen bound to the specific antibody and directly related to the amount of free labeled antigen. Other suitable screening methods will be readily apparent to those of skill in the art.

The present invention also relates to methods of detecting H11 or functional derivatives in a sample or subject. For example, antibodies specific

for H11, or a functional derivative, may be detectably labeled with any appropriate marker, for example, a radioisotope, an enzyme, a fluorescent label, a paramagnetic label, or a free radical.

Alternatively, antibodies specific for H11, or a functional derivative, may be detectably labeled with DNA by the technique of immuno-polymerase chain reaction (Sano *et al., Science 258*: 120-122 (1992)). The polymerase chain reaction (PCR) procedure amplifies specific nucleic acid sequences through a series of manipulations including denaturation, annealing of oligonucleotide primers, and extension of the primers with DNA polymerase (*see*, for example, Mullis *et al.*, U.S. Patent No. 4,683,202; Mullis *et al.*, U.S. Patent No. 4,683,195; Loh *et al., Science 243*:217 (1988)). The steps can be repeated many times, resulting in a large amplification of the number of copies of the original specific sequence. As little as a single copy of a DNA sequence can be amplified to produce hundreds of nanograms of product (Li *et al., Nature 335*:414 (1988)). Other known nucleic acid amplification procedures include transcription-based amplification systems (Kwoh *et al., Proc. Natl. Acad. Sci. USA 86*:1173 (1989); Gingeras *et al.*, WO 88/10315), and the "ligase chain reaction" in which two (or more) oligonucleotides are ligated in the presence of a nucleic acid target having the sequence of the resulting "di-oligonucleotide" thereby amplifying the di-oligonucleotide (Wu *et al., Genomics 4*:560 (1989); Backman *et al.*, EP 320,308; Wallace, EP 336,731; Orgel, WO 89/09835).

For example, the immuno-PCR assay can be carried out by immobilizing various amounts of the test material on the surface of microtiter wells (*see* Sanzo *et al., supra*, page 122, footnote 7). The wells are subsequently incubated with an H11 monoclonal antibody, washed, and then incubated with biotinylated H11 DNA molecules which have been conjugated to streptavidin-protein chimera (*Id.*). This chimera binds biotin (via the streptavidin moiety) and the Fc portion of an immunoglobulin G molecule (via the protein A moiety) (*Id.*, at 120; Sanzo *et al., Bio/Technology 9*:1378 (1991)). The wells are then washed to remove unbound conjugates. Any H11 present in the test material will be bound by the

H11 monoclonal antibody, which in turn, is bound by the protein A moiety of the biotinylated H11 DNA - streptavidin-protein A conjugate. Then, the H11 DNA sequences are amplified using PCR. Briefly, the microtiter wells are incubated with deoxyribonucleoside triphosphates, H11 oligonucleotide primers, and Taq DNA polymerase (*see* Sanzo *et al., supra*, page 122, footnote 11). An automated thermal cycler (such as the PTC-100-96 Thermal Cycler, MJ Research, Inc.) can be used to perform PCR under standard conditions (*Id.*). The PCR products are then analyzed by agarose gel electrophoresis after staining with ethidium bromide.

Methods of making and detecting such detectably labeled antibodies or their functional derivatives are well known to those of ordinary skill in the art, and have been described in more detail above. Standard reference works setting forth the general principles of immunology include the work of Klein (*Immunology: The Science of Self-Nonself Discrimination*, John Wiley & Sons, New York (1982)); Kennett *et al.* (*Monoclonal Antibodies and Hybridomas: A New Dimension in Biological Analyses*, Plenum Press, New York (1980)); Campbell ("Monoclonal Antibody Technology," In: *Laboratory Techniques in Biochemistry and Molecular Biology*, Volume 13 (Burdon, R., *et al.*, eds.), Elsevier, Amsterdam (1984)); and Eisen (In: *Microbiology*, 3rd Ed. (Davis, *et al.*, Harper & Row, Philadelphia (1980)).

The antibodies used in the present invention (*e.g.*, AB-10 and AB-181) may be prepared as previously described (Aurelian, L., *et al.*, Cancer *Cells* 7:187-191 (1989)). H11-specific polyclonal and monoclonal antibodies can also be generated against substantially pure H11 protein or H11 protein fragments isolated from recombinant hosts (for example, *see* Carroll *et al.*, "Production and Purification of Polyclonal Antibodies to the Foreign Segment of ß-Galactosidase Fusion Proteins," in *DNA Cloning: A Practical Approach*, Volume III, IRL Press, Washington, D.C., pp. 89-111 (1987); Mole *et al.*, "Production of Monoclonal Antibodies Against Fusion Proteins Produced in *Escherichia coli*," in *DNA Cloning: A Practical Approach*, Volume III, IRL Press, Washington, D.C., pp.

113-1139 (1987)). Alternatively, H11-specific polyclonal and monoclonal antibodies can be generated against substantially pure H11 protein or protein fragments (peptides) isolated from biological material such as melanoma tissue and cell lines (*e.g.*, SKMEL31 cells), by using well known techniques.

It will be appreciated that Fab and $F(ab')_2$ and other fragments of the antibodies useful in the present invention may be used for the detection and quantitation of H11 according to the methods disclosed herein in order to detect and diagnose melanoma in the same manner as an intact antibody. Such fragments are typically produced by proteolytic cleavage, using enzymes such as papain (to produce Fab fragments) or pepsin (to produce $F(ab')_2$ fragments).

An antibody is said to be "capable of binding" a molecule if it is capable of specifically reacting with the molecule to thereby bind the molecule to the antibody. The term "epitope" is meant to refer to that portion of any molecule capable of being bound by an antibody which can also be recognized by that antibody. Epitopic determinants usually consist of chemically active surface groupings of molecules such as amino acids or sugar side chains and have specific three dimensional structural characteristics as well as specific charge characteristics.

An "antigen" is a molecule capable of being bound by an antibody which is additionally capable of inducing an animal to produce antibody capable of binding to an epitope of that antigen. An antigen may have one, or more than one epitope. The specific reaction referred to above is meant to indicate that the antigen will react, in a highly selective manner, with its corresponding antibody and not with the multitude of other antibodies which may be evoked by other antigens.

The antibodies, or fragments of antibodies, useful in the present invention may be used to quantitatively or qualitatively detect the presence of cells which contain the H11 antigens. Thus, the antibodies (or fragments thereof) useful in the present invention may be employed histologically to detect or visualize the presence of an H11.

A particularly preferred embodiment of the invention is the use of H11-specific antibodies to detect the presence of H11 protein in histological samples of biopsied tissue, to detect the presence of melanoma cells.

The antibodies (or fragments thereof) useful in the present invention are thus particularly suited for use in *in vitro* immunoassays to detect the presence of an H11 in body tissue or cellular extracts. In such immunoassays, the antibodies (or antibody fragments) may be utilized in liquid phase or, preferably, bound to a solid-phase carrier, as described above.

Such an assay for H11 would comprise incubating a biological sample from said subject, said subject being suspected of having a condition in which H11 is expressed, in the presence of a detectably labeled binding molecule (e.g., antibody) capable of identifying H11, and detecting said binding molecule which is bound in a sample.

Specifically, in this embodiment of the invention, a biological sample may be treated with nitrocellulose, or other solid support which is capable of immobilizing cells, cell particles or soluble proteins. The support may then be washed with suitable buffers followed by treatment with the detectably labeled H11-specific antibody. The solid phase support may then be washed with the buffer a second time to remove unbound antibody. The amount of bound label on said solid support may then be detected by conventional means.

By "solid phase support" is intended any support capable of binding antigen or antibodies. Well-known supports, or carriers, include glass, polystyrene, polypropylene, polyethylene, dextran, nylon, amylases, natural and modified celluloses, polyacrylamides, agaroses, and magnetite. The nature of the carrier can be either soluble to some extent or insoluble for the purposes of the present invention. The support material may have virtually any possible structural configuration so long as the coupled molecule is capable of binding to an antigen or antibody. Thus, the support configuration may be spherical, as in a bead, or cylindrical, as in the inside surface of a test tube, or the external surface of a rod. Alternatively, the surface may be flat such as a sheet, test strip, etc.

Preferred supports include polystyrene beads. Those skilled in the art will note many other suitable carriers for binding monoclonal antibody or antigen, or will be able to ascertain the same by use of routine experimentation.

One embodiment for carrying out the diagnostic assay of the present invention on a biological sample containing an H11, comprises:

(a) contacting a detectably labeled H11-specific antibody with a solid support to effect immobilization of said H11-specific antibody or a fragment thereof;

(b) contacting a sample suspected of containing H11 with said solid support;

(c) incubating said detectably labeled H11-specific antibody with said support for a time sufficient to allow the immobilized H11-specific antibody to bind to the H11;

(d) separating the solid phase support from the incubation mixture obtained in step (c); and

(e) detecting the bound label and thereby detecting and quantifying H11.

Alternatively, labeled H11-specific antibody/H11 complexes in a sample may be separated from a reaction mixture by contacting the complex with an immobilized antibody or protein which is specific for an immunoglobulin, e.g., *Staphylococcus* protein A, *Staphylococcus* protein G, anti-IgM or anti-IgG antibodies. Such anti-immunoglobulin antibodies may be polyclonal, but are preferably monoclonal. The solid support may then be washed with a suitable buffer to give an immobilized H11/labeled H11-specific antibody complex. The label may then be detected to give a measure of an H11.

This aspect of the invention relates to a method for detecting an H11 or a fragment thereof in a sample comprising:

(a) contacting a sample suspected of containing an H11 with an H11-specific antibody or fragment thereof which binds to H11; and

(b) detecting whether a complex is formed.

The invention also relates to a method of detecting an H11 in a sample, further comprising:

(c) contacting the mixture obtained in step (a) with an Fc binding molecule, such as an antibody, *Staphylococcus* protein A, or *Staphylococcus* protein G, which is immobilized on a solid phase support and is specific for the H11-specific antibody to give a H11/H11-specific antibody immobilized antibody complex;

(d) washing the solid phase support obtained in step (c) to remove unbound H11/H11-specific antibody complex;

(e) and detecting the label bound to said solid support.

Of course, the specific concentrations of detectably labeled antibody and H11, the temperature and time of incubation, as well as other assay conditions may be varied, depending on various factors including the concentration of an H11 in the sample, the nature of the sample, and the like. The binding activity of a given lot of anti-H11 antibody may be determined according to well known methods. Those skilled in the art will be able to determine operative and optimal assay conditions for each determination by employing routine experimentation.

Other such steps as washing, stirring, shaking, filtering and the like may be added to the assays as is customary or necessary for the particular situation.

One of the ways in which the H11-specific antibody can be detectably labeled is by linking the same to an enzyme. This enzyme, in turn, when later exposed to its substrate, will react with the substrate in such a manner as to produce a chemical moiety which can be detected, for example, by spectrophotometric, fluorometric or by visual means. Enzymes which can be used to detectably label the H11-specific antibody include, but are not limited to, malate dehydrogenase, staphylococcal nuclease, delta-V-steroid isomerase, yeast alcohol dehydrogenase, α-glycerophosphate dehydrogenase, triose phosphate isomerase, horseradish peroxidase, alkaline phosphatase, asparaginase, glucose oxidase, ß-galactosidase, ribonuclease, urease, catalase, glucose-VI-phosphate dehydrogenase, glucoamylase and acetylcholinesterase.

Detection may be accomplished using any of a variety of immunoassays. For example, by radioactively labeling the H11-specific antibodies or antibody fragments, it is possible to detect H11 through the use of radioimmune assays. A good description of a radioimmune assay may be found in *Laboratory Techniques and Biochemistry in Molecular Biology*, by Work, *et al.*, North Holland Publishing Company, NY (1978), with particular reference to the chapter entitled "An Introduction to Radioimmune Assay and Related Techniques" by Chard, incorporated by reference herein.

The radioactive isotope can be detected by such means as the use of a gamma counter or a scintillation counter or by autoradiography. Isotopes which are particularly useful for the purpose of the present invention are: ^{3}H, ^{125}I, ^{131}I, ^{35}S, ^{14}C, and preferably ^{125}I.

It is also possible to label the H11-specific antibody with a fluorescent compound. When the fluorescently labeled antibody is exposed to light of the proper wave length, its presence can then be detected due to fluorescence. Among the most commonly used fluorescent labeling compounds are fluorescein isothiocyanate, rhodamine, phycoerytherin, phycocyanin, allophycocyanin, *o*-phthaldehyde and fluorescamine.

The H11-specific antibody can also be detectably labeled using fluorescence emitting metals such as ^{152}Eu, or others of the lanthanide series. These metals can be attached to the H11-specific antibody using such metal chelating groups as diethylenetriaminepentaacetic acid (DTPA) or ethylenediaminetetraacetic acid (EDTA).

The H11-specific antibody also can be detectably labeled by coupling it to a chemiluminescent compound. The presence of the chemiluminescent-tagged H11-specific antibody is then determined by detecting the presence of luminescence that arises during the course of a chemical reaction. Examples of particularly useful chemiluminescent labeling compounds are luminol, isoluminol, theromatic acridinium ester, imidazole, acridinium salt and oxalate ester.

The H11-specific antibody may also be labeled with biotin and then reacted with avidin. A biotin-labeled DNA fragment will be linked to the H11-biotinylated monoclonal antibody by an avidin bridge. H11 molecules are then detected by polymerase chain reaction (PCR) amplification of the DNA fragment with specific primers (Sano *et al., Science 258*: 120-122 (1992)).

Likewise, a bioluminescent compound may be used to label the H11-specific antibody of the present invention. Bioluminescence is a type of chemiluminescence found in biological systems in which a catalytic protein increases the efficiency of the chemiluminescent reaction. The presence of a bioluminescent protein is determined by detecting the presence of luminescence. Important bioluminescent compounds for purposes of labeling are luciferin, luciferase and aequorin.

Detection of the H11-specific antibody may be accomplished by a scintillation counter, for example, if the detectable label is a radioactive gamma emitter, or by a fluorometer, for example, if the label is a fluorescent material. In the case of an enzyme label, the detection can be accomplished by colorimetric methods which employ a substrate for the enzyme. Detection may also be accomplished by visual comparison of the extent of enzymatic reaction of a substrate in comparison with similarly prepared standards.

The detection of foci of such detectably labeled antibodies is indicative of a disease or dysfunctional state as previously described. For the purposes of the present invention, the H11 which is detected by this assay may be present in a biological sample. Any sample containing H11 can be used. One of ordinary skill in the art will be able to determine suitable conditions which allow the use of specific samples.

Preferably, the detection of cells which express an H11 may be accomplished by *in vivo* imaging techniques, in which the labeled antibodies (or fragments thereof) are provided to a subject, and the presence of H11 is detected without the prior removal of any tissue sample. Such *in vivo* detection procedures have the advantage of being less invasive than other detection methods.

Using *in vivo* imaging techniques, it will be possible to differentiate between normal melanocytes (normal non-cancerous tissue) and melanoma tissue because H11 will be detected only in the melanoma tissue. Thus, using this aspect of the invention, one would be able to distinguish the borders of cancerous tissue, or to characterize the radial and vertical growth phases of the melanoma lesion. For example, one could determine, prior to treatment (*e.g.*, surgery), the exact location of cancerous melanoma tissue. Similarly, after treatment (*e.g.*, excision of the melanoma tissue), one would be able to determine whether all cancerous tissue had been removed.

There are many different *in vivo* labels and methods of labeling known to those of ordinary skill in the art. Examples of the types of labels which can be used in the present invention include radioactive isotopes and paramagnetic isotopes. Those of ordinary skill in the art will know of other suitable labels for binding to the antibodies used in the invention, or will be able to ascertain such, using routine experimentation. Furthermore, the binding of these labels to the antibodies can be done using standard techniques common to those of ordinary skill in the art.

An important factor in selecting a radionuclide for *in vivo* diagnosis is that the half-life of a radionuclide be long enough so that it is still detectable at the time of maximum uptake by the target, but short enough so that deleterious radiation upon the host is minimized. Ideally, a radionuclide used for *in vivo* imaging will lack a particulate emission, but produce a large number of photons in the 140-200 keV range, which maybe readily detected by conventional gamma cameras.

For *in vivo* diagnosis radionuclides may be bound to antibody either directly or indirectly by using an intermediary functional group. Intermediary functional groups which are often used in binding radioisotopes which exist as metallic ions to immunoglobulins are DTPA and EDTA. Typical examples of ions which can be bound to immunoglobulins are ^{99m}Tc, ^{123}I, ^{111}In, ^{131}I, ^{97}Ru, ^{67}Cu, ^{67}Ga, ^{125}I, ^{68}Ga, ^{72}As, ^{89}Zr, and ^{201}Tl.

For diagnostic *in vivo* imaging, the type of detection instrument available is a major factor in selecting a given radionuclide. The radionuclide chosen must have a type of decay which is detectable for a given type of instrument. In general, any conventional method for visualizing diagnostic imaging can be utilized in accordance with this invention. For example, PET, gamma, beta, and MRI detectors can be used to visualize diagnostic imaging.

The antibodies useful in the invention can also be labeled with paramagnetic isotopes for purposes of *in vivo* diagnosis. Elements which are particularly useful, as in Magnetic Resonance Imaging (MRI), include ^{157}Gd, ^{55}Mn, ^{162}Dy, and ^{56}Fe.

Those of ordinary skill in the art will know of other suitable labels which may be employed in accordance with the present invention. The binding of these labels to antibodies or fragments thereof can be accomplished using standard techniques commonly known to those of ordinary skill in the art. Typical techniques are described by Kennedy, *et al.* (*Clin. Chim. Acta 70*:1-31 (1976)) and Schurs, *et al.* (*Clin. Chim. Acta 81*:1-40 (1977)). Coupling techniques mentioned in the latter are the glutaraldehyde method, the periodate method, the dimaleimide method, and the m-maleimidobenzyl-N-hydroxy-succinimide ester method.

In situ detection may be accomplished by removing a histological specimen from a patient, and providing the combination of labeled antibodies of the present invention to such a specimen. The antibody (or fragment) is preferably provided by applying or by overlaying the labeled antibody (or fragment) to a biological sample. Through the use of such a procedure, it is possible to determine not only the presence of an H11, but also the distribution of an H11 on the examined tissue. Using the present invention, those of ordinary skill will readily perceive that any of a wide variety of histological methods (such as staining procedures) can be modified in order to achieve such *in situ* detection.

The binding molecules of the present invention may be adapted for utilization in an immunometric assay, also known as a "two-site" or "sandwich"

assay. In a typical immunometric assay, a quantity of unlabeled antibody (or fragment of antibody) is bound to a solid support that is insoluble in the fluid being tested (*i.e.*, cell lysate, or extract from biopsied tissue) and a quantity of detectably labeled soluble antibody is added to permit detection and/or quantitation of the ternary complex formed between solid-phase antibody, antigen, and labeled antibody.

Typical, and preferred, immunometric assays include "forward" assays in which the antibody bound to the solid phase is first contacted with the sample being tested to extract the antigen from the sample by formation of a binary solid phase antibody-antigen complex. After a suitable incubation period, the solid support is washed to remove the residue of the fluid sample, including unreacted antigen, if any, and then contacted with the solution containing an unknown quantity of labeled antibody (which functions as a "reporter molecule"). After a second incubation period to permit the labeled antibody to complex with the antigen bound to the solid support through the unlabeled antibody, the solid support is washed a second time to remove the unreacted labeled antibody. This type of forward sandwich assay may be a simple "yes/no" assay to determine whether antigen is present or may be made quantitative by comparing the measure of labeled antibody with that obtained for a standard sample containing known quantities of antigen. Such "two-site" or "sandwich" assays are described by Wide at pages 199-206 of *Radioimmune Assay Method*, edited by Kirkham and Hunter, E. & S. Livingstone, Edinburgh, 1970.

In another type of "sandwich" assay, which may also be useful with the antigens of the present invention, the so-called "simultaneous" and "reverse" assays are used. A simultaneous assay involves a single incubation step as the antibody bound to the solid support and labeled antibody are both added to the sample being tested at the same time. After the incubation is completed, the solid support is washed to remove the residue of fluid sample and uncomplexed labeled antibody. The presence of labeled antibody associated with the solid support is then determined as it would be in a conventional "forward" sandwich assay.

In the "reverse" assay, stepwise addition first of a solution of labeled antibody to the fluid sample followed by the addition of unlabeled antibody bound to a solid support after a suitable incubation period is utilized. After a second incubation, the solid phase is washed in conventional fashion to free it of the residue of the sample being tested and the solution of unreacted labeled antibody. The determination of labeled antibody associated with a solid support is then determined as in the "simultaneous" and "forward" assays.

The above-described *in vitro* or *in vivo* detection methods may be used in the detection and diagnosis of melanoma without the necessity of removing tissue. Such detection methods may be used to assist in the determination of the stage of progression of melanoma by evaluating and comparing the concentration of H11 in the biological sample.

As used herein, an effective amount of a diagnostic reagent (such as an antibody or antibody fragment) is one capable of achieving the desired diagnostic discrimination and will vary depending on such factors as age, condition, sex, the extent of disease of the subject, counterindications, if any, and other variables to be adjusted by the physician. The amount of such materials which are typically used in a diagnostic test are generally between 0.1 to 5 mg, and preferably between 0.1 to 0.5 mg.

The assay of the present invention is also ideally suited for the preparation of a kit. Such a kit may comprise a carrier being compartmentalized to receive in close confinement therewith one or more containers such as vials, tubes and the like, each of said container means comprising the separate elements of the immunoassay.

For example, there may be a container containing a first antibody immobilized on a solid phase support, and a further container containing a second detectably labeled antibody in solution. Further containers may contain standard solutions comprising serial dilutions of the H11 to be detected. The standard solutions of an H11 may be used to prepare a standard curve with the concentration of H11 plotted on the abscissa and the detection signal on the

ordinate. The results obtained from a sample containing an H11 may be interpolated from such a plot to give the concentration of the H11.

IV. Therapeutic Applications

Gene Therapy

Further, the subject invention can employ a gene therapy approach utilizing molecular biology techniques or procedures whereby a target cell or tissue is genetically altered to increase its rate of growth or to stimulate growth by transgenically providing the target cell or tissue with a gene encoding the H11 protein. The target cells can thus be genetically engineered to encode and produce H11. The H11 gene can be cloned or otherwise amplified, isolated and inserted (either in a sense or antisense orientation) into appropriate vectors for transfer into the target cells. Further, the target cells can be provided with promoter sequences which activate the appropriate gene sequence in the genome of the target cell to instruct the cell to produce the desired proteins or promoters.

The gene therapy approach of the present invention can be used as a method to stimulate or enhance wound healing *in vivo* or the growth of skin graft plates *in vitro*. Estrogen is one example of an activator which could be used to activate the introduced H11 gene.

In this preferred embodiment of the invention, H11 DNA (e.g., the nucleic acid sequence shown in SEQ ID NO:1) can be incorporated into a polynucleotide construct suitable for introducing the nucleic acid molecule into cells of the animal to be treated, to form a transfection vector. The transfection vector is then introduced into selected target tissues of the cells of the animal *in vivo* (or in cultured skin cells in vitro) using any of a variety of methods known to those skilled in the art. Alternatively, naked DNA may be transfected into the cells, with or without cationic lipids.

Techniques for the construction of transfection vectors containing H11 DNA are well-known in the art, and are generally described in "Working Toward Human Gene Therapy," Chapter 28 in *Recombinant DNA, 2nd Ed.*, Watson, J.D. *et al.* (eds.), Scientific American Books: New York (1992), pp. 567-581, or Sambrook *et al.*, *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor, New York (1989).

Gene therapy approaches that may be used to deliver an H11 gene include injection of plasmid DNA (Horton, H.M., *et al.*, *Proc. Natl. Acad. Sci. USA 96(4)*:1553-1558 (1999)); transduction using adenoviral vectors (Waugh, J.M., *et al.*, *Proc. Natl. Acad. Sci. USA 96(3)*:1065-1070 (1999)); transduction using retrovial vectors (Axelrod, J.H., *et al.*, *Proc. Natl. Acad. Sci. USA 87*:5173-5177 (1990); Drumm, M.L., *et al.*, *Cell 62*:1227-1233 (1990); Krueger, G.G., *et al.*, *J. Invest. Dermatol. 112*:233-239 (1999); Palmer, T.D., *et al.*, *Blood 73*:438-445 (1989); and Rosenberg, S.A., *et al.*, *N. Eng. J. Med. 323*:570-578 (1990)); and gene transfer using liposomes (Mason, C.A.E., *et al.*, *Nature Medicine 5(2)*:176-182 (1999)). In addition, general methods for construction of gene therapy vectors and the introduction of such vectors into a mammal for therapeutic purposes may be obtained in the above-referenced publications, the disclosures of which are specifically incorporated herein by reference in their entirety. In one such general method, vectors containing H11 DNA of the present invention are directly introduced into the cells or tissues of the mammal to be treated, preferably by injection, inhalation, ingestion, topical application, or introduction into a mucous membrane via solution, but more preferably topical application. Such an approach is generally referred to as "*in vivo*" gene therapy.

Alternatively, cells or tissues may be removed from the mammal to be treated and placed into culture according to methods that are well-known to one of ordinary skill in the art. Transfection vectors or naked DNA containing the H11 DNA may then be introduced into these cells or tissues by any of the methods described generally above for introducing isolated polynucleotides into a cell or tissue. After a sufficient amount of time to allow incorporation of the

H11 DNA, the cells or tissues may then be re-inserted into the mammal to be treated. Since introduction of the H11 gene is performed outside of the body of the mammal, this approach is generally referred to as "*ex vivo*" gene therapy. See U.S. Patent No. 5,399,346. Gene transfer through transfection of cells *ex vivo* can be performed by a variety of methods, including, for example, calcium phosphate precipitation, diethylaminoethyl dextran, electroporation, lipofection, or viral infection. Such methods are well known in the art (see, for example, Sambrook *et al.*).

For both *in vivo* and *ex vivo* gene therapy, the H11 DNA of the invention may be operatively linked to the regulatory DNA sequence, or "promoter," for human H11 to form a genetic construct as described above. This construct, containing both the human H11 promoter and the H11 DNA, may be subcloned into a suitable vector such as a plasmid, adenovirus vector, retrovirus vector, or the like, and introduced into the animal to be treated in an in vivo gene therapy approach, or into the cells or tissues of the mammal in an ex vivo approach.

Alternatively, the H11 DNA of the invention may be operatively linked to a heterologous regulatory DNA sequence, or promoter, to form a genetic construct as described above. The heterologous regulatory sequence may be tissue specific. The vector containing the genetic construct is then directly introduced into the animal to be treated or into the cells or tissues of the animal, as described.

The term "operably linked," as used herein, denotes a relationship between a regulatory region (typically a promoter element, but may include an enhancer element) and the gene, whereby the transcription of the gene is under the control of the regulatory region.

The term "heterologous" means a DNA sequence not found in the native genome. That is, two nucleic acid elements are said to be "heterologous" if the elements are derived from two different genes, or alternatively, two different species. Thus, "heterologous DNA regulatory sequence" indicates that the

regulatory sequence is not naturally ligated to the DNA sequence for the H11 gene.

The term "promoter" is used according to its art-recognized meaning. It is intended to mean the DNA region, usually upstream to the coding sequence of a gene, which binds RNA polymerase and directs the enzyme to the correct transcriptional start site.

In general, a promoter may be functional in a variety of tissue types and in several different species of organisms, or its function may be restricted to a particular species and/or a particular tissue. Further, a promoter may be constitutively active, or it may be selectively activated by certain substances (e.g., a tissue-specific factor), under certain conditions (e.g., in the presence of an enhancer element, if present, in the genetic construct containing the promoter), or during certain developmental stages of the organism (e.g., active in fetus, silent in adult).

Promoters useful in the practice of the present invention are preferably "tissue-specific"--that is, they are capable of driving transcription of a gene in one tissue while remaining largely "silent" in other tissue types. An example of a tissue-specific promoter that would be useful in the present invention is the human tyrosinase promoter, which has been shown to target gene expression at the transcriptional level to melanoma cells (Park, B.J., *et al.*, *Hum. Gene Ther. 10*:889-898 (1999)). Alternatively, the keratin tissue-specific promoter (Vassar *et al.*, *Proc. Natl. Acad. Sci. U.S.A. 86*:8565 (1989), or perhaps the stromelysin 3 or c-erbB3 promoters (specific for breast cancer cells) (Basset, P., *et al.*, *Nature 348*: 699; and Huber, B. E., *PNAS 88*:8099 (1991) would be useful in the present invention.

Examples of other tissue-specific promoters that could be used in the present invention are provided in the following references: (1) Shani, *Mol. Cell. Biol., 6:*2624 (1986); (2) Swift *et al.*, *Cell 38:*639 (1984); (3) Krumlauf *et al.*, *Nature 319*:224 (1985); (4) Townes *et al.*, *EMBO J. 4:*1715 (1985); (5) Lacy *et al.*, *Cell 34*:343 (1983); (6) Wagner *et al.*, *Proc. Natl. Acad. Sci. U.S.A., 78*:6376

(1981); (7) Brinster *et al.*, *Nature 283*:499 (1980); (8) Rusconi *et al.*, in "The Impact of Gene Transfer Techniques in Eukaryotic Cell Biology," ed. J. S. Schell *et al.*, pp. 134-152, Berlin: Springer Verlag (1984); (9) Behringer *et al.*, *Genes Dev. 2*:453 (1988); (10) Storb *et al.*, *Nature 310*:238 (1984); (11) Grosschedl *et al.*, *Cell 38*:647 (1984); (12) Selden *et al.*, *Nature 321*:545 (1986); (13) Shani, *Nature 314*:283 (1985); (14) Peschon *et al.*, Ann. N. York Acad, Sci., 564:186 (1989); (15) Breitman *et al.*, *Dev. 106:*457 (1989); (16) Crenshaw *et al.*, *Genes and Development 3*:959 (1989); (17) Tremblay *et al.*, *Proc. Natl. Acad. Sci. U.S.A. 85*:8890 (1988); (18) Tatsumi *et al.*, *Nippon Rinsho 47*:2213 (1989); (19) Muller *et al.*, *Cell 54*:105 (1988); (20) Palmiter *et al.*, *Ann. Rev. Genet. 20*:465 (1986); (21) Vassar *et al.*, *Proc. Natl. Acad. Sci. U.S.A. 86*:8565 (1989); (22) McVey *et al.*, *J. Biol. Chem. 263*:11 (1988); (23) Allison *et al.*, *Mol. Cell. Biol. 9*:2254 (1989); (24) Danciger *et al.*, *Proc. Natl. Acad. Sci. U.S.A. 86*:8565 (1989); (25) Forss-Petter *et al.*, *J. Neurosci. Res. 16*:141 (1986); (26) Sutcliffe, *Trends in Genetics 3*:73 (1987); (27) Nathans *et al.*, *Proc. Natl. Acad. Sci. U.S.A. 81*:4851 (1984); (28) Brenner, M., *et al.*, *J. Neurosci. 14*:1030 (1994); (29) Kim, L. S., *et al.*, *J. Biol. Chem 268*:15689 (1993); (30) Kaneda, N., *et al.*, *Neuron 6*:583 (1991); (31) Salbaum, J. M., *et al.*, *EMBO J.7*:2807 (1988); (32) Wirak, D. O., *et al.*, *EMBO J. 10*:289 (1990); (33) Mercer E. H., *et al.*, *Neuron 7*:703 (1991); (34) Hcyle, G. W., *et al.*, *J. Neurosci. 14*:2455 (1994); (35) Miura, M., *et al.*, *Gene 75*:31 (1989); (36) Reeben, M., *et al.*, *J. Neurosci. Res. 40*:177 (1995); (37) Boularand, S., *et al.*, *J. Biol. Chem 270*:3757 (1995); (38) Stoll, J. and Goldman, D., *J. Neurosci. Res. 28*:457 (1991); (39) Vandaele, S., *et al.*, *Genes & Dev. 5*:1136 (1991); (40) Oberdick, J., *et al.*, *Science 248*:223 (1990); (41) Maue, R. A., *et al.*, *Neuron 4*:223 (1990); (42) Hersh, L. B., *et al.*, *J. Neurochem. 61*:306 (1993); (43) Ibanex, C. F. and Persson, H., *Eur. J. Neurosci. 3*:1309 (1991); (44) Forss-Petter, S., *et al.*, *Neuron 5*:187 (1990); (45) Thai, A. L. V., *et al.*, *Mol. Brain Res. 17*:227 (1993); (46) Peschon, J. J., *et al.*, *Proc. Natl. Acad. Sci. U.S.A. 84*:5316 (1987); (47) Borsook, D., *et al.*, *Mol. Endocrinol. 6*:1502 (1992); (48) Joshi, J. and Sabol, S. L., *Mol. Endocrinol. 5*:1069 (1991); (49) Watanabe, T., *et*

al., J. Biol. Chem. 265:7432 (1990); (50) Campos, R. V., *et al., Mol. Rnfovtinol. 6*:1642 (1992); (51) Basset, P., *et al., Nature 348*: 699 (1990); (52) Bombardieri, E. *et al., Eur. J. Cancer 31A*:184 (1995); Koh, T. *et al., Int. J. Cancer 60*:843 (1995); (53) Thai, A. L. V., *et al., Mol. Brain Res. 17*:227 (1993); (54) Huber, B. E., *PNAS 88*:8099 (1991); (55) Zuibel, I., *et al., J. Cell Physiol. 162*:36 (1995); (56) Watanabe, T., *et al., J. Biol. Chem. 265*:7432 (1990).

For additional examples of tissue-specific promoters, see U.S. Patent Nos. 5,834,306 and 5,416,027, and references cited therein.

In addition to a promoter, the genetic construct may also contain other genetic control elements, such as enhancers, repressible sequences, and silencers, which may be used to regulate replication of the vector in the target cell. The only requirement is that the genetic element be activated, derepressed, enhanced, or otherwise genetically regulated by factors in the host cell and, with respect to methods of treatment, not in the non-target cell.

An "element," when used in the context of nucleic acid constructs, refers to a region of the construct or a nucleic acid fragment having a defined function. For example, a enhancer element, as used herein, is a region of DNA that, when associated with the H11 gene operably linked to a promoter, enhances the transcription of that gene.

The term "enhancer" is used according to its art-recognized meaning. It is intended to mean a sequence found in eukaryotes which can increase transcription from a gene when located (in either orientation) up to several kilobases from the gene being studied. These sequences usually act as enhancers when on the 5′ side (upstream) of the gene in question. However, some enhancers are active when placed on the 3′ side (downstream) of the gene. In some cases, enhancer elements can activate transcription from a gene with no (known) promoter.

Preferred enhancers include the DF3 breast cancer-specific enhancer and enhancers from viruses and the steroid receptor family. Other preferred transcriptional regulatory sequences include NF1, SP1, AP1, and FOS/JUN.

Any of a variety of methods known to those skilled in the art may be used to introduce transfection vectors of the present invention into selected target tissue cells. Such methods include, for example, viral-mediated gene transfer using retroviruses, adeno-associated virus (AAV), herpes virus, vaccinia virus, or RNA viruses (*e.g.*, Grunhaus and Horowitz, *Semin. Virol. 3*:237-252 (1992); Herz and Gerard, *Proc. Nat. Acad. Sci. USA 90*:2812-2816 (1993); and Rosenfeld *et al.*, *Cell 68*:143-155 (1992)); liposome-mediated gene transfer (Morishita *et al.*, *J. Clin. Invest. 91*:2580 (1993); Felgner *et al.*, U.S. Patent Nos. 5,703,055 (1997) and 5,858,784 (1999)); injection of naked DNA directly into a target tissue (*e.g.*, Felgner *et al.*, U.S. Patent No. 5,589,466 (1996); Wolff *et al.*, U.S. Patent No. 5,693,622 (1997)); and receptor-mediated gene transfer (Wu and Wu, *Biochemistry 27*:887-892 (1988); Wagner *et al.*, *PNAS USA 87*:3410-3414 (1990); Curiel *et al.*, U.S. Patent 5,547,932 (1996); and Beug *et al.*, U.S. Patent No. 5,354,844 (1994)).

In any of these methods, where a vector may be targeted to selectively transfect a specific population of cells, it will be understood that in addition to local administration (such as may be achieved by injection into the target tissue), the vector may be administered systemically (e.g., intravenously) in a biologically-compatible solution or pharmaceutically acceptable delivery vehicle. Vector constructs administered in this way may selectively infect the target tissue. According to the present invention, the presence of a target tissue-specific promoter on the construct provides an independent means of restricting expression of the therapeutic gene.

Antisense Oligonucleotides

Antisense oligonucleotides have been described as naturally occurring biological inhibitors of gene expression in both prokaryotes (Mizuno *et al.*, *Proc. Natl. Acad. Sci. USA 81*:1966-1970 (1984)) and eukaryotes (Heywood, *Nucleic Acids Res. 14*:6771-6772 (1986)), and these sequences presumably function by

hybridizing to complementary mRNA sequences, resulting in hybridization arrest of translation (Paterson, *et al.*, *Proc. Natl. Acad. Sci. USA, 74*:4370-4374 (1987)).

Antisense oligonucleotides are short synthetic DNA or RNA nucleotide molecules formulated to be complementary to a specific gene or RNA message. Through the binding of these oligomers to a target DNA or mRNA sequence, transcription or translation of the gene can be selectively blocked and the disease process generated by that gene can be halted (*see*, for example, Jack Cohen, *Oligodeoxynucleotides, Antisense Inhibitors of Gene Expression*, CRC Press (1989)). The cytoplasmic location of mRNA provides a target considered to be readily accessible to antisense oligodeoxynucleotides entering the cell; hence much of the work in the field has focused on RNA as a target. Currently, the use of antisense oligodeoxynucleotides provides a useful tool for exploring regulation of gene expression *in vitro* and in tissue culture (Rothenberg, *et al.*, *J. Natl. Cancer Inst. 81*:1539-1544 (1989)).

Antisense therapy is the administration of exogenous oligonucleotides which bind to a target polynucleotide located within the cells. For example, antisense oligonucleotides may be administered systemically for anticancer therapy (WO 90/09180; U.S. Pat. No. 5,271,941). The H11 protein is produced by malignant melanoma cells, and in relatively high concentrations (*i.e.*, relative to controls). Thus, H11 antisense oligonucleotides of the present invention may be active in treatment against melanoma, as well as other cancers, such as estrogen-dependent breast cancer, in which H11 has been demonstrated to be overexpressed in cancerous tissue.

Examples of antisense oligonucleotides which have been demonstrated to inhibit H11 expression in melanoma cells include the following:

(a) 3'-GGTACCGACTGCCAGT-5' (aODN-1); and (SEQ ID NO:7)

(b) 3'-GGTGGTACCGACTGCC-5' (aODN-2) (SEQ ID NO:8)

Preferably, the antisense oligonucleotide sequence is a 10 to 30-mer, more preferably, a 12-16-mer. Also, the antisense oligonucleotide may be a phosphorothioate or one of the other oligonucleotide derivatives mentioned above, as well as pharmaceutical compositions comprising such oligonucleotides and a pharmaceutically acceptable carrier.

Included as well in the present invention are pharmaceutical compositions comprising an effective amount of at least one of the H11 antisense oligonucleotides of the invention in combination with a pharmaceutically acceptable carrier. In one embodiment, a single H11 antisense oligonucleotide is utilized. In another embodiment, two H11 antisense oligonucleotides are utilized which are complementary to adjacent regions of the H11 DNA. Administration of two H11 antisense oligonucleotides which are complementary to adjacent regions of the DNA or corresponding mRNA may allow for more efficient inhibition of H11 genomic transcription or mRNA translation, resulting in more effective inhibition of H11 production.

Preferably, the H11 antisense oligonucleotide is coadministered with an agent which enhances the uptake of the antisense molecule by the cells. For example, the H11 antisense oligonucleotide may be combined with a lipophilic cationic compound which may be in the form of liposomes. The use of liposomes to introduce nucleotides into cells is taught, for example, in U.S. Patent Nos. 4,897,355 and 4,394,448. *See also* U.S. Patent Nos. 4,235,871, 4,231,877, 4,224,179, 4,753,788, 4,673,567, 4,247,411, 4,814,270 for general methods of preparing liposomes comprising biological materials.

Alternatively, the H11 antisense oligonucleotide may be combined with a lipophilic carrier such as any one of a number of sterols including cholesterol, cholate and deoxycholic acid. A preferred sterol is cholesterol.

In addition, the H11 antisense oligonucleotide may be conjugated to a peptide that is ingested by cells. Examples of useful peptides include peptide hormones, antigens or antibodies, and peptide toxins. By choosing a peptide that is selectively taken up by the neoplastic cells, specific delivery of the antisense

agent may be effected. The H11 antisense oligonucleotide may be covalently bound via the 5'OH group by formation of an activated aminoalkyl derivative. The peptide of choice may then be covalently attached to the activated H11 antisense oligonucleotide via an amino and sulfhydryl reactive hetero bifunctional reagent. The latter is bound to a cysteine residue present in the peptide. Upon exposure of cells to the H11 antisense oligonucleotide bound to the peptide, the peptidyl antisense agent is endocytosed and the H11 antisense oligonucleotide binds to the target H11 mRNA to inhibit translation (Haralambid *et al.*, WO 8903849; Lebleu *et al.*, EP 0263740).

Antisense H11 oligonucleotides can also be attached to radionuclides to be used for the *in vivo* imaging of tumor cells (e.g., melanoma cells overexpressing the H11 protein) and diagnosis of melanoma tumors by radiography (see, for example, Dewanjee, M.K. *et al.*, *J. Nucl. Med. 35*:1054-1063 (1994) and Urbain, J.L., *et al.*, *Eur. J. Nucl. Med. 22*:499-504 (1995)), as well as for therapy due to cell killing by the radionuclide (see, for example, Kairemo, K.J., *et al.*, *Cancer Gene Ther. 5*:408-412 (1998) and Watanabe, N., *et al.*, *Nucl. Med. Biol. 26*:239-243 (1999)). Alternatively, antisense H11 oligonucleotides may also be conjugated to toxin molecules for uptake by cells, leading to cytotoxicity or death of the target cell.

An antisense H11 polynucleotide may also be linked to the HSV TK gene for preferential delivery to melanoma cells, allowing specific localized therapy with ganciclovir. See, for example, Hall, S.J., *et al.*, *Cancer Research 58*:3221-3226 (1998). The antisense H11 oligonucleotide would be used to specifically target melanoma cells.

The H11 antisense oligonucleotides and the pharmaceutical compositions of the present invention may be administered by any means that achieve their intended purpose. For example, administration may be by parenteral, subcutaneous, intravenous, intramuscular, intra-peritoneal, or transdermal routes, but preferably by subcutaneous routes. The dosage administered will be

dependent upon the age, health, and weight of the recipient, kind of concurrent treatment, if any, frequency of treatment, and the nature of the effect desired.

Compositions within the scope of this invention include all compositions wherein the H11 antisense oligonucleotide is contained in an amount effective to achieve inhibition of proliferation and/or stimulate differentiation of the subject cancer cells. While individual needs vary, determination of optimal ranges of effective amounts of each component is with the skill of the art. Typically, the H11 antisense oligonucleotide may be administered to mammals, e.g. humans, at a dose of 0.005 to 1 mg/kg/day, or an equivalent amount of the pharmaceutically acceptable salt thereof, per day of the body weight of the mammal being treated.

Antisense oligonucleotides can be prepared which are designed to interfere with transcription of the H11 gene by binding transcribed regions of duplex DNA (including introns, exons, or both) and forming triple helices (U.S. 5,594,121, U.S. 5,591,607, WO96/35706, WO96/32474, WO94/17091, WO94/01550, WO 91/06626, WO 92/10590). Preferred oligonucleotides for triple helix formation are oligonucleotides which have inverted polarities for at least two regions of the oligonucleotide (*Id.*). Such oligonucleotides comprise tandem sequences of opposite polarity such as 3'---5'-L-5'---3', or 5'---3'-L-3'---5', wherein L represents a 0-10 base oligonucleotide linkage between oligonucleotides. The inverted polarity form stabilizes single-stranded oligonucleotides to exonuclease degradation (Froehler *et al., supra*). The invention is related as well to pharmaceutical compositions comprising such oligodeoxynucleotides and a pharmaceutically acceptable carrier. An example of a pharmaceutically acceptable carrier is polyethylene glycol, which can be used for topical administration of the pharmaceutical composition (see, for example, Kulka, M., *et al.*, *Antiviral Research 20*:115-130 (1993)).

In therapeutic application, the triple helix-forming oligonucleotides can be formulated in pharmaceutical preparations for a variety of modes of

administration, including systemic or localized administration, as described above.

The antisense oligonucleotides and triple helix-forming oligonucleotides of the present invention may be prepared according to any of the methods that are well known to those of ordinary skill in the art, including methods of solid phase synthesis and other methods as disclosed in the publications, patents and patent applications cited herein.

Vaccines

In another aspect of the present invention, the recombinant or natural H11 protein, peptides, or analogs thereof, or modified H11 peptides, or analogs thereof may be used as a vaccine either prophylactically or therapeutically. When provided prophylactically the vaccine is provided in advance of any evidence of melanoma. The prophylactic administration of the H11 vaccine should serve to prevent or attenuate melanoma in a mammal. In a preferred embodiment mammals, preferably humans, at high risk for melanoma are prophylactically treated with the vaccines of this invention. Examples of such mammals include, but are not limited to, humans with a family history of melanoma, humans with a history of atypical moles, humans with a history of FAM-M syndrome or humans afflicted with melanoma previously resected and therefore at risk for reoccurrence. When provided therapeutically, the vaccine is provided to enhance the patient's own immune response to the tumor antigen present on the melanoma or metastatic melanoma. The vaccine, which acts as an immunogen, may be a cell, cell lysate from cells transfected with a recombinant expression vector, cell lysates from cells transfected with an H11 recombinant expression vector, or a culture supernatant containing the expressed protein. Alternatively, the immunogen is a partially or substantially purified recombinant H11 protein, peptide or analog thereof or modified peptides or analogs thereof. The proteins

or peptides may be conjugated with lipoprotein or administered in liposomal form or with adjuvant.

While it is possible for the immunogen to be administered in a pure or substantially pure form, it is preferable to present it as a pharmaceutical composition, formulation or preparation.

The formulations of the present invention, both for veterinary and for human use, comprise an immunogen as described above, together with one or more pharmaceutically acceptable carriers and, optionally, other therapeutic ingredients. The carrier(s) must be "acceptable" in the sense of being compatible with the other ingredients of the formulation and not deleterious to the recipient thereof. The formulations may conveniently be presented in unit dosage form and may be prepared by any method well-known in the pharmaceutical art.

Formulations suitable for intravenous intramuscular, subcutaneous, or intraperitoneal administration conveniently comprise sterile aqueous solutions of the active ingredient with solutions which are preferably isotonic with the blood of the recipient. Such formulations may be conveniently prepared by dissolving solid active ingredient in water containing physiologically compatible substances such as sodium chloride (e.g. 0.1-2.0M), glycine, and the like, and having a buffered pH compatible with physiological conditions to produce an aqueous solution, and rendering said solution sterile. These may be present in unit or multi-dose containers, for example, sealed ampoules or vials.

The formulations of the present invention may incorporate a stabilizer. Illustrative stabilizers are polyethylene glycol, proteins, saccharides, amino acids, inorganic acids, and organic acids which may be used either on their own or as admixtures. These stabilizers are preferably incorporated in an amount of 0.11-10,000 parts by weight per part by weight of immunogen. If two or more stabilizers are to be used, their total amount is preferably within the range specified above. These stabilizers are used in aqueous solutions at the appropriate concentration and pH. The specific osmotic pressure of such aqueous solutions is generally in the range of 0.1-3.0 osmoles, preferably in the range of

0.8-1.2. The pH of the aqueous solution is adjusted to be within the range of 5.0-9.0, preferably within the range of 6-8. In formulating the immunogen of the present invention, anti-adsorption agent may be used.

Additional pharmaceutical methods may be employed to control the duration of action. Controlled release preparations may be achieved through the use of polymer to complex or absorb the proteins or their derivatives. The controlled delivery may be exercised by selecting appropriate macromolecules (for example polyester, polyamino acids, polyvinyl, pyrrolidone, ethylenevinylacetate, methylcellulose, carboxymethylcellulose, or protamine sulfate) and the concentration of macromolecules as well as the methods of incorporation in order to control release. Another possible method to control the duration of action by controlled-release preparations is to incorporate the H11 protein, peptides and analogs thereof into particles of a polymeric material such as polyesters, polyamino acids, hydrogels, poly(lactic acid) or ethylene vinylacetate copolymers. Alternatively, instead of incorporating these agents into polymeric particles, it is possible to entrap these materials in microcapsules prepared, for example, by coacervation techniques or by interfacial polymerization, for example, hydroxy-methylcellulose or gelatin-microcapsules and poly(methylmethacylate) microcapsules, respectively, or in colloidal drug delivery systems, for example, liposomes, albumin microspheres, microemulsions, nanoparticles, and nanocapsules or in macroemulsions.

When oral preparations are desired, the compositions may be combined with typical carriers, such as lactose, sucrose, starch, talc magnesium stearate, crystalline cellulose, methyl cellulose, carboxymethyl cellulose, glycerin, sodium alginate or gum arabic, among others, or with components that increase mucosal immunity, such as cholera toxin, for example.

Vaccination can be conducted by conventional methods. For example, the immunogen can be used in a suitable diluent such as saline or water, or complete or incomplete adjuvants. Further, the immunogen may or may not be bound to a carrier to make the protein immunogenic. Examples of such carrier molecules

include but are not limited to bovine serum albumin (BSA), keyhole limpet hemocyanin (KLH), tetanus toxoid, and the like. The immunogen also may be coupled with lipoproteins or administered in liposomal form or with adjuvants. The immunogen can be administered by any route appropriate for antibody production such as intravenous, intraperitoneal, intramuscular, subcutaneous, and the like. The immunogen may be administered once or at periodic intervals until a significant titer of anti-H11 immune cells or anti-H11 antibody is produced. The presence of anti-H11 immune cells may be assessed by measuring the frequency of precursor CTL (cytoxic T-lymphocytes) against H11 antigen prior to and after immunization by a CTL precursor analysis assay (Coulie, P. et al., (1992) International Journal Of Cancer 50:289-297). The antibody may be detected in the serum using the immunoassay described above.

The administration of the vaccine or immunogen of the present invention may be for either a prophylactic or therapeutic purpose. When provided prophylactically, the immunogen is provided in advance of any evidence or in advance of any symptom due to melanoma. The prophylactic administration of the immunogen serves to prevent or attenuate melanoma in a mammal. When provided therapeutically, the immunogen is provided at (or shortly after) the onset of the disease or at the onset of any symptom of the disease. The therapeutic administration of the immunogen serves to attenuate the disease.

A preferred embodiment is a vaccine prepared using recombinant H11 protein or peptide expression vectors. To provide a vaccine to an individual a genetic sequence which encodes for all or part of the H11 nucleic acid sequence is inserted into a expression vector, as described above, and introduced into the mammal to be immunized. Examples of vectors that may be used in the aforementioned vaccines include, but are not limited to, defective retroviral vectors, adenoviral vectors vaccinia viral vectors, fowl pox viral vectors, or other viral vectors (Mulligan, R. C., *Science 260*:926-932 (1993)). The viral vectors carrying all or part of the H11 nucleic sequence can be introduced into a mammal

either prior to any evidence of melanoma or to mediate regression of the disease in a mammal afflicted with melanoma.

Examples of methods for administering the viral vector into the mammals include, but are not limited to, exposure of cells to the virus ex vivo, or injection of the retrovirus or a producer cell line of the virus into the affected tissue or intravenous administration of the virus. Alternatively the viral vector carrying all or part of the H11 nucleic acid sequence may be administered locally by direct injection into the melanoma lesion or topical application in a pharmaceutically acceptable carrier. Local administration is the preferred route.

The quantity of viral vector, carrying all or part of the H11 nucleic acid sequence, to be administered is based on the titer of virus particles. A preferred range of the immunogen to be administered may be about 10^6 to about 10^{11} virus particles per mammal, preferably a human. After immunization the efficacy of the vaccine can be assessed by production of antibodies or immune cells that recognize the antigen, as assessed by specific lytic activity or specific cytokine production or by tumor regression. One skilled in the art would know the conventional methods to assess the aforementioned parameters. If the mammal to be immunized is already afflicted with melanoma or metastatic melanoma the vaccine can be administered in conjunction with other therapeutic treatments. Examples of other therapeutic treatments includes, but are not limited to, adoptive T cell immunotherapy, coadministration of cytokines or other therapeutic drugs for melanoma.

Alternatively all or parts thereof of a substantially or partially purified the H11 protein may be administered as a vaccine in a pharmaceutically acceptable carrier. Ranges of H11 protein that may be administered are about 0.001 to about 100 mg per patient, preferred doses are about 0.01 to about 100 mg per patient. Preferred doses may be about 0.001 mg to about 100 mg, most preferred are about 0.01 mg to about 100 mg. The peptide may be synthetically or recombinantly produced. Immunization is repeated as necessary, until a sufficient titer of anti-immunogen antibody or immune cells has been obtained.

In yet another alternative embodiment a viral vector, such as a retroviral vector, can be introduced into mammalian cells. Examples of mammalian cells into which the retroviral vector can be introduced include, but are not limited to, primary mammalian cultures or continuous mammalian cultures, COS cells, NIH3T3, or 293 cells (ATCC #CRL 1573). The means by which the vector carrying the gene may be introduced into a cell includes, but is not limited to, microinjection, electroporation, transfection or transfection using DEAE dextran, lipofection, calcium phosphate or other procedures known to one skilled in the art (Sambrook et al. (Eds.) in "Molecular Cloning. A laboratory manual", Cold Spring Harbor Press Plainview, N.Y. (1989)). The mammalian cells expressing the H11 antigen can be administered to mammals and serve as a vaccine or immunogen. Examples of how the cells expressing H11 antigens can be administered include, but is not limited to, intravenous, intraperitoneal or intralesional. Alternatively, a nucleic acid sequence corresponding to H11 peptides which have been modified to enhance their binding to MHC molecules may be used.

The vaccine formulation of the present invention comprise an immunogen that induces an immune response directed against the melanoma associated antigens such as the melanoma associated H11 antigen. The vaccine formulations may be evaluated first in animal models, initially rodents, and in nonhuman primates and finally in humans. The safety of the immunization procedures is determined by looking for the effect of immunization on the general health of the immunized animal (weight change, fever, appetite behavior etc.) and looking for pathological changes on autopsies. After initial testing in animals, melanoma cancer patients can be tested. Conventional methods would be used to evaluate the immune response of the patient to determine the efficiency of the vaccine.

In yet another embodiment of this invention all, part, or parts of the H11 protein or H11 peptides or analogs thereof, or modified H11 peptides or analogs thereof, may be exposed to dendritic cells cultured in vitro. The cultured dendritic cells provide a means of producing T-cell dependent antigens comprised

of dendritic cell modified antigen or dendritic cells pulsed with antigen, in which the antigen is processed and expressed on the antigen activated dendritic cell. The H11 antigen activated dendritic cells or processed dendritic cell antigens may be used as immunogens for vaccines or for the treatment of melanoma. The dendritic cells should be exposed to antigen for sufficient time to allow the antigens to be internalized and presented on the dendritic cells surface. The resulting dendritic cells or the dendritic cell process antigens can than be administered to an individual in need of therapy. Such methods are described in Steinman et al. (WO93/208185) and in Banchereau et al. (EPO Application 0563485A1) which are incorporated herein by reference.

In yet another embodiment of this invention T-cells isolated from individuals can be exposed to the H11 protein or portions thereof, or H11 peptides or analogs thereof or H11 modified peptides or analogs thereof in vitro and then administered to a patient in need of such treatment in a therapeutically effective amount. Examples of where T-lymphocytes can be isolated, include but are not limited to, peripheral blood cells lymphocytes (PBL), lymph nodes, or tumor infiltrating lymphocytes (TIL). Such lymphocytes can be isolated from the individual to be treated or from a donor by methods known in the art and cultured in vitro (Kawakami, Y. *et al.*, *J. Immunol. 142*:2453-3461 (1989)). Lymphocytes are cultured in media such as RPMI or RPMI 1640 or AIM V for 1-10 weeks. Viability is assessed by trypan blue dye exclusion assay. The lymphocytes are exposed to all or part of the H11 protein for part or all of the culture duration. Examples of how these sensitized T-cells can be administered to the mammal include but are not limited to, intravenously, intraperitoneally or intralesionally. Parameters that may be assessed to determine the efficacy of these sensitized T-lymphocytes include, but are not limited to, production of immune cells in the mammal being treated or tumor regression. Conventional methods are used to assess these parameters. Such treatment can be given in conjunction with cytokines or gene modified cells (Rosenberg, S. A. *et al.*, *Human Gene Therapy 3*:75-90 (1992); Rosenberg, S. A. *et al.*, *Human Gene Therapy 3*:57-73 (1992)).

The following examples are illustrative, but not limiting, or the method and compositions of the present invention. Other suitable modifications and adaptations of the variety of conditions and parameters normally encountered and obvious to those skilled in the art are within the spirit and scope of the invention.

Example 1

Materials and Methods

Cells and tissues

Human melanoma cell lines SK-MEL-31, SK-MEL-2 and G361 as well as HeLa (human cervical cancer cells) and 293 cells (adenovirus E1a-immortalized human embryonic kidney) were obtained from American Type Culture Collection. SK-MEL-31, SK-MEL-2 and 293 cells were grown in Eagle's modified minimal essential medium (EMEM) supplemented with 1mM non-essential amino acids, 1% sodium pyruvate and 10% fetal calf serum (FCS). HeLa cells were grown in EMEM with 10% FCS. G361 cells were grown in McCoy's medium with 10% FCS. Normal human melanocyte cultures were purchased from Clonetics (San Diego, CA). Biopsies of primary melanoma and common benign nevi (three patients each) were obtained from Dr. J. Burnett (Department of Dermatology, University of Maryland).

Antibodies

Polyclonal antibodies to peptides in the RR1 PK oncoprotein (Aurelian, L., *et al., Cancer Cells* 7:187-191 (1989)) and to peptides that respectively represent H11 amino acids 10-29 (CHYPSRLRRDPFRDSPLSSR (SEQ ID NO: 5); anti-H11-10 antibody, or AB-10) or 181-194 (SFNNELPQDSQEVT (SEQ ID NO: 6); anti-H11-181 antibody, or AB-181) were conjugated to keyhole limpet hemocyanin (KLH), purified and injected into rabbits, as previously

described (Aurelian, L, *et al.*, *Cancer Cells* 7:187-191 (1989). Antibody to heat shock protein 27 (hsp27) was the gift of Dr. Nose, Showa University, Japan.

Library screening and plasmid construction

An established pre-amplified HeLa cDNA (UniZap^{tm} XR) library (Stratagene, LaJolla, CA) constructed in a lambda based vector, was screened with antibodies to RR1 PK peptides. The preparation of host bacterial cultures (*E. coli* XL1 blue MRF'), lambda library infection/plating and cDNA expression (on IPTG impregnated nitrocellulose filters) as well as antibody-based screening (immunoblotting) were performed as per manufacturer's instructions. A total of $1x10^6$ plaques were screened and positive plaques were subjected to 3 successive rounds of purification through plaque isolation and re-screening. Eight pure plaque isolates were obtained which contained cDNA sequences. Single-stranded cDNA contained within a ssDNA Bluescript II phagemid (Stratagene) was rescued, isolated and purified from lambda clones using freshly titered helper phage VCM13 according to manufacturer's instructions. ssDNA was sequenced (UMAB Biopolymer Laboratory) using primers specific for either Bluescript II or cDNA sequences. cDNA was rescued as a double stranded Bluescript II phagemid using the ExAssist/SOLR System (Stratagene) according to manufacturer's instructions. The resulting clone was designated H11. H11 was subsequently cloned from a custom prepared library constructed from a primary human melanoma using oligo(dT) primed cDNA and UniZaptm XR (Stratagene) by screening with the 1.8kb EcoRI/XhoI H11 cDNA fragment labeled with [γ-^{32}P]dCTP using an oligonucleotide kit (Pharmacia, Uppsala, Sweden) as per manufacturer's instructions. A total of 10 pure plaques containing cDNA sequences were obtained from $1x10^6$ screened plaques.

Northern blot hybridization

PolyA$^+$ mRNA from adult and fetal tissues was obtained from Clontech (San Diego, CA). Northern blot hybridization was done as previously described

(Feng, C.P., *et al., Antisense Res. & Nucleic Acid Drug Dev. 6*:25-35 (1996)). Briefly, prehybridization was in a solution containing 50% formamide, 5x SSC (1x is 0.15M NaCl, 0.01M $NaH_2PO_4.H_2O$, 1mM EDTA, pH7.4), 10x Denhardt's (1x is 2% polyvinylpyrolidone, 2% Ficoll, 2% BSA), 1% sodium dodecyl sulfate (SDS) and 100 μg/ml heat denatured salmon sperm DNA for 5 hrs at 42°C. Hybridization was for 20 hrs at 42°C with [^{32}P]-labeled probes in the prehybridization solution. The H11 probe was the 1.8kb EcoRI/XhoI cDNA fragment. A γ-actin cDNA probe (Leader, D.P., *et al., Gene 36*:369-374 (1985)) served as control. Probes were [γ-^{32}P]dCTP labeled by the random priming method using an oligonucleotide kit (Pharmacia) as per manufacturer's instructions. Blots were washed four times in 2x SSC with 0.05% SDS (40 min; 20 °C) followed by 2 washes with 0.1x SSC and 0.1% SDS (40 min; 50 °C) and visualized by autoradiography.

RNA isolation and RT-PCR

Total cellular RNA was isolated from cells and tissues with RNAzol (Tel-Test, Friendswood, Texas) as per manufacturer's instructions. Additionally, cDNA was obtained from a panel of normal human tissues which included colon, ovary, prostate and testis [Human Multiple Tissue cDNA Panel (Clontech, Palo Alto, CA)] and from a panel of human tumor tissues [Human Tumor Tissue cDNA Panels (Clontech)] which included a breast carcinoma (G1-101), two lung carcinomas (LX-1 and G1-117), one ovarian carcinoma (G1-102) and one adenocarcinoma each of the colon (CX-1), prostate (PC3), and pancreas (G1-103). RT-PCR was done as previously described (Imafuku, S., *et al., J. Invest. Dermatol. 109*:550-556 (1997); Kokuba, S., *et al., Brit. J. Dermatol. 138*:952-964 (1998)). Briefly, RNA was first incubated with 250 units of DNAse I (Boehringer Mannheim, Indianapolis, IN), for 1 hr at 37 °C. To generate cDNA, 2 μg DNAse I-treated RNA was incubated with 200 units MMTV reverse transcriptase (Gibco-BRL, Gaithersburg, MD), 400 μM deoxynucleoside triphosphates (dNTP), RT buffer (10mM Tris-HCl pH 8.5, 15mM KCl, 0.6mM

$MgCl_2$) 10 mM dithiothreitol (DTT) and 10 pmol (0.5 μM) of antisense H11 or actin primers for 1 hr at 42 °C. Each PCR reaction was done with either one-tenth of the generated cDNA or 1ng of the cDNA from the Clontech tissue panels. PCR reactions were with 10pmoles (0.5 μM) of the H11 sense and antisense primers (5'-CCATGGCTGACGGTCAGATGCCCTT CTCCT-3' (SEQ ID NO: 3) and 5'-TCCATGCCAAAGCCATCATCCAGCAG-3' (SEQ ID NO: 4) or β-actin sense and antisense primers (5'-GTGGGGCGCCCCAGGCACCA-3' (SEQ ID NO: 9) and 5'-CTCCTTAATGTCACGCACGATTTC-3'(SEQ ID NO: 10)) (Yamamura, M, *et al., Science 254*:277-279 (1991)) and the AdvanTaq Plus kit (Clontech). The PCR program included 1 min at 94 °C for 1 cycle, 94 °C for 30 seconds and 68 °C for 2 min. There were 26 cycles with a final extension of 5 min at 68 °C on a Perkin Elmer thermocycler (Model 9700). Products were analyzed by electrophoresis on 8% acrylamide gels and subsequent staining with 0.5 μg/ml ethidium bromide. The relative abundance of H11 mRNA was estimated by first normalizing to the value of actin mRNA in each sample by densitometric scanning using a BioRad GS-700 Imaging Densitometer, as described (Imafuku, S., *et al., J. Invest. Dermatol. 109*:550-556 (1997); Kokuba, S., *et al., Brit. J. Dermatol. 138*:952-964 (1998)).

Labeling of cell extracts, immunoprecipitation and immunoblotting

Labeling and preparation of whole cell extracts was as previously described (Smith, C.C., *et al., Virology 200*:598-612 (1994); Nelson, J.W., *et al., J. Biol. Chem. 271*:17021-17027 (1996)). Briefly, cells were labeled with [^{35}S]-methionine (100 μCi/ml; sp. act. 1120 Ci/mmol; Dupont NEN Research Products, Boston, MA) in methionine-free DMEM with 10% dialyzed FBS (4 hrs., 37°C). Labeled cells were resuspended in RIPA buffer (20mM Tris-HCl, pH 7.4, 150mM NaCl, 0.1% SDS, 1% Nonidet-P40 (NP-40), 1% sodium deoxycholate, 1mM phenylmethyl sulfonyl fluoride (PMSF) (Sigma Chemical Co. St. Louis MO), 10mM benzamidine (Sigma) and 100 Kallikrein units/ml aprotinin (Sigma) and incubated on ice for 15 min. The extracts were clarified

by centrifugation (30 min) at 20,000 x g and the supernatants were used in immunoprecipitation. In some experiments, cells were labeled with [^{32}P]-orthophosphate (500 μCi/ml; NEN Research Products) in phosphate-free EMEM with 10% dialyzed FBS for 4 hrs at 37°C. They were resuspended in a buffer consisting of 50mM HEPES (pH 7.5), 0.15M NaCl, 1% NP-40, 5mM EDTA, 10 mM sodium pyrophosphate, 1mM sodium vanadate, 100mM sodium fluoride, 1mM PMSF and 100 Kallikrein units/ml of aprotinin and incubated on ice for 15 min.

For immunoprecipitation, cell extracts were incubated (1 hr., 4°C) with 15 μl of antibody and (30 min., 4°C) with 100 μl of protein A-Sepharose CL4B beads (50% v/v, Sigma). Beads were washed three times with ice-cold RIPA buffer and bound proteins were eluted by boiling (5 min) in 50μl denaturing solution [150mM Tris-HCl (pH 7.0), 5.7% SDS, 14% 2-ß-mercaptoethanol, 17% sucrose, 0.04% bromthymol blue]. Proteins were resolved by SDS-PAGE on polyacrylamide gels and visualized by autoradiography. In some experiments, the resolved proteins were transferred to nitrocellulose membranes and immunoblotting was performed by incubation with the respective antibodies followed by protein A-peroxidase (Sigma) for 1 hr at room temperature each. Detection was with ECL reagents (Amersham, Arlington Heights, Ill) as per manufacturer's instructions.

Immunofluorescent and immunoperoxidase staining

Cells were stained by the indirect immunofluorescence (IFA) procedure, as previously described (Smith, C.C., *et al., Virology 200*:598-612 (1994)). Fixed (acetone, 5 min. room temperature) and unfixed (membrane staining) cells were studied. Thin (0.5 μm) optical sections were obtained using an epifluorescence confocal microscope fitted with an argon ion laser (Zeiss LSM410) and analyzed using Adobe Photoshop software package version 3.05, as described (Kieval, R.S., *et al., Am. J. Physiol. 263*:C545-C550 (1992)). For immunoperoxidase staining, cells fixed in frozen (-70 °C) methanol, were

blocked with normal goat serum (1:10 dilution in PBS) and exposed (4hrs, 4 °C) to primary antibody followed (1 hr) by secondary antibody. They were stained with a peroxidase kit (LSAB kit, DAKO) and counterstained with Gill's hematoxylin (Aurelian, L., *et al., Cancer Cells 7*:187-191 (1989); Kokuba, S., *et al., Brit. J. Dermatol. 138*:952-964 (1998)).

Cell cycle analysis

For cell cycle analysis, cells ($5x10^4$) were grown under the appropriate conditions, resuspended following trypsinization, counted and fixed in 1 ml of cold 70% ethanol. Cells were then washed twice with cold PBS and stored in 1ml of PBS at 4 °C. For DNA analysis, $1x10^6$ cells in 1 ml PBS were incubated (30 min, 37 °C) with 1 unit of DNAse free RNAse (Boehringer Mannheim). This was followed by addition of propidium iodide (100 μl of a 0.5 mg/ml stock solution) and the cells were analyzed within 60 min on a Becton Dickinson FACSscan cytometer equipped with a 15 mW laser (Aurelian, L., *et al., Analyt. & Quant. Cytol. 1*:207-216 (1979)). The percentage of cells in the various phases of the cell cycle was estimated by gating the G_1/G_0, S and G_2/M regions of the histogram.

Effect of antisense oligonucleotides

Cells ($5x10^3$) were incubated in triplicate in 96-well plates in 0.1 ml of culture medium supplemented with 10% heat-inactivated FCS for 30 min at 65 °C to destroy nuclease activity and cultured (24 hrs) in the presence or absence of antisense, sense or randomly scrambled phosphorothioate oligonucleotides (ODNs), as described (Feng, C.P., *et al., Antisense Res. & Nucleic Acid Drug Dev. 6*:25-35 (1996); Care, A., *et al., Mol. Cell Biol. 16*:4842-4851 (1996); Kulka, M., *et al., Proc. Natl. Acad. Sci. 86*:6868-6872 (1989)). The ODN sequences were first tested against sequences in Genbank database. Two distinct antisense ODNs complementary to sequences that encompass the H11 translation initiation site were studied. Their sequence was 3'-GGTACCGACTGCCAGT-5'·

(aODN-1) (SEQ ID NO:7) and 3'-GGTGGTACCGACTGCC-5' (aODN-2) (SEQ ID NO: 8) respectively. The scrambled ODN sequence was 3'-TACTAACGACCTCGTG-5' (scODN) (SEQ ID NO: 15). The sense ODN was for aODN-2 and its sequence was 5'-CCACCATGGCTGACGG-3' (sODN) (SEQ ID NO: 16). Dose response curves were determined with ODNs at 10, 20 and 30 μM concentrations. To measure DNA synthesis, cell cultures were labeled with [^{3}H]-thymidine ([^{3}H]-TdR, 2.5 μCi/ml; New England Nuclear) for 4 hrs and harvested as described (Wachsman, M., *et al., J. Inf. Dis. 159*:625-634 (1989)). The radioactivity on the filters was determined with a Beckman LS6800 liquid scintillation counter. Antisense inhibition was calculated relative to that of sc-ODN treated cells, whose inhibition did not exceed 15% of the proliferation rate shown by untreated cells. Growth inhibition was calculated as [1 -([^{3}H]-TdR incorporation of treated cells/[^{3}H]-TdR incorporation of control cells)] x 100 (Care, A., *et al., Mol. Cell Biol. 16*:4842-4851 (1996)).

Expression and purification of GST fusion proteins

The 1 kb NcoI fragment of H11 DNA which contains the open reading frame (ORF) was cloned into the Glutathione S transferase expression vector pGEXlambda which was used to transform *E. coli* XL1 Blue. To induce protein expression, bacterial cultures were grown to an optical density (600nm) of 1 and induced with 0.1mM isopropyl thiogalactopyranoside (IPTG) for 4 hrs. Cells were pelleted and resuspended in 1/20 the culture volume of lysis buffer [PBS (pH 7.2), 1% Triton X-100, 1mM EDTA, 0.1% ME, 0.2mM PMSF, 5mM benzamidine] containing 0.5mg/ml lyzozyme. Resuspended cells were sonicated 2x (30s each) on ice and cleared of cell debris by centrifugation. Cleared lysates were incubated with (50% v/v) glutathione-Sepharose beads (30 min; 4°C), washed 3x with cold PBS (pH 7.4), boiled 5 min in gel denaturing solution and analyzed by SDS-PAGE on % polyacrylamide gels.

Example 2

Isolation of H11 cDNA

H11 was first isolated from a commercially available HeLa cDNA library screened by immunoblotting with antibodies to peptides within the HSV-2 RR1 PK oncoprotein (Aurelian, L., *Frontiers in Bioscience 3*:D237-249 (1998)). This choice was predicated on previous findings that a cross-reactive protein is expressed in HeLa and melanoma cells (unpublished) and our original goal was to identify additional members of the RR1 PK subfamily (Aurelian, L., *Frontiers in Bioscience 3*:D237-249 (1998)). A total of $1x10^6$ plaques were screened and purified as described in materials and methods. Eight pure plaque isolates were obtained which contained cDNA sequences. Single-stranded cDNA contained within one phagemid was rescued, isolated and purified. This cDNA, designated H11, was subcloned and sequenced. The γ -^{32}P-dCTP labeled H11 was then used to screen the melanoma cDNA library. The cDNA in one subcloned plaque was sequenced and found to be identical to H11. As shown in FIG. 1, H11 cDNA consisted of 1835 bp (GenBank accession No. AF133207). This size is similar to that of RR1 PK (Aurelian, L., *Frontiers in Bioscience 3*:D237-249 (1998)). However, on computer analysis, H11 did not show significant homology to any sequence registered in the GenBank or European Molecular Biology Laboratory databases (releases 111.0 and 52.0, respectively), including RR1 PK.

Example 3

Computer-assisted predictions of H11 protein and its properties

H11 cDNA contains one open reading frame (ORF) that consists of 588 nucleotides. This would encode a protein of 196 amino acids with a calculated molecular weight of 21.5 kDa (FIG. 1) (SEQ ID NO:2). Comparison with known proteins in the public data bases using the FASTA program did not reveal significant identities. Direct alignment revealed a 33% identity to RR1 PK over

the 196 amino acids in the H11 ORF. However, the identical sequences were scattered and it is unclear whether such similarity is biologically relevant.

Computer-assisted analysis of the predicted amino acid sequence in the H11 ORF, aligned as described by Hanks, S.K., *et al., Science 241*:42-52 (1988), revealed the presence of potential PK catalytic motifs, when allowing for conservative substitutions. This includes a potential catalytic motif I followed by Val (amino acids 75-80), two sites consistent with catalytic motif II (invariant Lys; residues 104-106 and 110-113) and a catalytic motif III (Glu; residues 120 or 126,127), which are part of the stable scaffolding of PK active sites (Taylor, S.S., *et al., Ann. Rev. Cell Biol. 8*:492-462 (1992)). Catalytic motifs IV and V are poorly conserved in all PKs (Taylor, S.S., *et al., Ann. Rev. Cell Biol. 8*:492-462 (1992)), but there was a potential catalytic motif VI (residues 133-138) that retained Asn, which seems to be invariant (Montell, C., & Rubin, G.M., *Cell 52*:757-772 (1988)). There was also a triplet similar to the consensus Asp-Phe-Gly (catalytic motif VII) (amino acids 150-152) that retained the invariant (Gibbs, C.S. & Zoller, M.J., *J. Biol. Chem. 266*:8923-8931 (1991)) Asp residue. Further predictions from the computer-assisted analysis of the H11 sequence included: (i) a potential N-glycosylation site at amino acid 138, (ii) two elements consistent with the consensus pattern for myristylation Gly-X-X-X-Ser/Thr at residues 62 and 132 respectively, and (iii) a motif (PPPFPGEP (SEQ ID NO: 12); amino acids 88-95), consistent with the consensus proline-rich SH3-binding domain P-X-PXX-P that is involved in protein-protein interactions (Pawson, T., *Nature 373*:573-580 (1995)). Additional features in H11 included phosphorylation sites for casein kinase 2 (CKII) (residues 47 and 176) and for protein kinase C (residues 27, 63, 76, 104, 122 and 140). At amino acid residue 28 there was an active site signature for serine proteases (subtilisin family) that are involved in the processing of hormone precursors at sites comprised of basic amino acid pairs (Barr, P.J., *Cell 66*:1-3 (1991)). However, unlike these proteases, H11 had only one active site signature. The H11 sequence SPLSSR (SEQ ID NO: 13) (residues

24-29) is similar to an element (SPESER) (SEQ ID NO: 14) in the peptides used to generate the RR1 PK antibodies employed for screening the HeLa cell library.

Example 4

H11 RNA expression in human tissues

To examine whether H11 is expressed in human tissues and determine the size of the transcript, poly(A)$^+$ mRNA derived from various adult and fetal tissues were analyzed by Northern blot hybridization with H11 cDNA. To control for RNA quantity, blots were also hybridized with an actin cDNA probe. A single 1.8kb transcript was detected. In adult tissues, the largest band was seen in prostate and colon (FIG. 2B) closely followed by placenta (FIG. 2A). A small H11 band was also seen in the small intestine (FIG. 2B). The transcript was not seen in brain, lung, liver, pancreas, thymus, testes, ovary, spleen and peripheral blood leukocytes (PBL) (FIGs. 2A and 2B). In fetal tissues, expression was observed in lung and weakly in kidney (FIG. 2C). The different levels of H11 expression in the various tissues are not an artefact due to improper RNA levels in the various tissues, since the actin levels were virtually identical in all lanes (FIGs. 2A, 2B and 2C). The finding that H11 is expressed in fetal, but not adult, lung and kidney suggests that it may be involved during early lung and kidney development.

Example 5

H11 expression is increased in human melanoma

To examine whether H11 expression is altered in cancer tissues, we used RT-PCR with H11 and actin (control) primers. H11 RNA levels were expressed as the ratio of H11 to actin RNA as determined by densitometric scanning of the respective bands. As shown in FIGs. 3A and 3B, the levels of actin RNA were similar in all tissues and cell lines studied in these series. By contrast, H11 RNA

levels differed in the various samples and were maximal in melanoma. Thus, the H11/actin ratios were 9.2, 15 and 12 for G361, SK-MEL-31 and SK-MEL-2 cells respectively (FIG. 3A, lanes 2-4). Similar H11/actin ratios (4.7, 9.6 and 10 respectively) were also seen in the three studied primary melanoma tissues, as shown for two of these in FIG. 3B, lanes 6,9. By contrast, only minimal H11 RNA levels (H11/actin ratios = 0.1 to 0.2) were seen in normal melanocytes (FIG. 3B, lane 8) and in the three studied benign nevi, as shown for one of these in FIG. 3B, lane 7. Relatively low levels of H11 expression were also seen in HeLa cells (FIG. 4A, lane 5; H11/actin ratio = 1.1), ovarian cancer (FIG. 3B, lane 3; H11/actin ratio = 0.33) and testicular cancer (FIG. 3B, lane 5; H11/actin ratio = 0.23), while normal ovary and testis were negative (FIG. 3B, lanes 2,4). H11 expression was not seen in pancreas, lung, breast, colon and prostate cancer tissues (FIG. 3A, lanes 8-13), but it was seen in the CATES cell line established from a testicular cancer (FIG. 3A, lane 6; H11/actin ratio = 0.2). 293 cells which are immortalized kidney cells were negative (FIG. 3A, lane 7). While final conclusions must await the study of larger patient groups, the data indicate that H11 expression is increased in some (most notably melanoma), but not other (viz. prostate) cancers.

Example 6

A phosphoprotein consistent with H11 is constitutively expressed in melanoma cells

To examine whether a protein of the predicted H11 size is expressed in RNA positive cells, we used immunoprecipitation studies with antibodies to H11 peptides. Cells were labeled with [^{35}S]-methionine for 4 hrs and immunoprecipitated with H11 antibodies H11-10 and H11-181 that respectively recognize amino acids 10-29 (N-terminal) and 181-193 (C-terminal). Preimmune rabbit serum served as control. A 25kDa protein, consistent with H11, was precipitated by both H11 antibodies from G361 cells (FIG. 4A, lanes 1,2). The protein was also precipitated from SK-MEL-2, SK-MEL-31 and HeLa cells (data

not shown). It was not precipitated from the RNA negative 293 cells (FIG. 4A, lanes 4,5). Preimmune serum was negative (FIG. 4A, lanes 3,6).

Inasmuch as the predicted size of the H11 protein is lower than that of the protein precipitated by the H11 antibodies, the question arises whether H11 is post-translationally modified. Because potential phosphorylation sites were predicted by the computer-assisted analysis of the H11 protein, G361 cells were labeled with [^{32}P]-orthophosphate for 4 hrs and the cell extracts were used in immunoprecipitation with the H11 and preimmune sera. 293 cells served as control. A 25kDa phosphoprotein was precipitated from G361 cells by both H11 antibodies (FIG. 4B, lanes 1,2), but not by the preimmune serum (FIG. 4B, lane 3). To confirm the identity of the precipitated phosphoprotein, proteins were transferred to nitrocellulose paper and immunoblotted with H11-181 antibody or with antibody to an unrelated protein of a similar size (hsp27). The 25kDa phosphoprotein in the G361 precipitates was recognized by H11-181 antibody (FIG. 4C, lanes 1,2), but not by antibody to hsp27 (FIG. 4D, lanes 1,2). Proteins were not seen in the immunoblots of the precipitates obtained from G361 cells with preimmune serum (FIGs. 4C and 4D, lanes 3) or from 293 cells (FIGs. 4C and 4D, lanes 4-6), supporting the conclusion that the 25 kDa phosphoprotein precipitated from G361 cells is H11. Phosphoamino acid analysis, done as previously described (Chung, T.D., *et al., J. Virol. 63*:3389-3398 (1989)), indicated that the label is primarily on serine residues (data not shown).

Example 7

The H11 protein in melanoma cells is associated with the plasma membrane

Two series of experiments were done in order to examine the subcellular localization of the H11 protein in melanoma cells. In the first series, G361 cells were fixed and used in immunoperoxidase staining with H11-10 antibody. Preimmune serum served as control. Staining was seen only with the H11-10 antibody (FIG. 5, Panel 1). It was located throughout the cytoplasm but seemed

to concentrate around the cell periphery, suggesting that H11 may be associated with the plasma membrane. Preimmune serum was negative (FIG. 5, Panel 2).

The second series of experiments was designed to test the validity of the interpretation that H11 is associated with the plasma membrane. G361, SK-MEL-2 and SK-MEL-31 cells were stained with H11-10 antibody by IFA and examined by confocal epifluorescence microscopy. Thin optical sections at 0.5 μm intervals through the cells were analyzed. Preimmune serum served as negative control. FIG. 6A presents 6 successive optical sections at 0.2, 4, 6, 10 and 17 μm distances through a G361 cell labeled with H11 antibody. In panels 1 and 2, the focal plane is near the cell surface and discrete punctate nonhomogeneous pattern of labeling is evident. In FIG. 6A, Panels 3-5, as the focal plane passes through the cell, labeling of the cell perimeter becomes evident. The oval region largely devoid of fluorescence is the cell nucleus, indicating that H11 is not associated with the nuclear membrane. In FIG. 6A, Panel 6, the focal plane is near the opposite cell surface, and the staining again assumes a punctate appearance. Labeling of intracellular membranous structures or mitochondria is not evident.

To show more clearly the distribution of the label on the plasma membrane, a close-up view of one cell image (in this case a SK-MEL-31 cell) is shown in FIG. 6B. The staining is composed of discrete foci of different fluorescence intensity (arrows) that identify a two-dimensional pattern of cell-surface distribution. Consistent with the conclusion that H11 is associated with the plasma membrane, staining with the H11 antibody was also seen when using live (unfixed) cells resuspended by scraping or by trypsinization (FIG. 6C, Panel 1). Preimmune serum was negative (FIG. 6C, Panel 2).

Example 8

Antisense H11 ODNs inhibit melanoma cell growth

The RT-PCR studies indicated that H11 is over-expressed in melanoma relative to normal melanocytes or benign nevi, suggesting that expression may be related to melanoma cell proliferation. To test this possibility, we took advantage of previous findings from our (Feng, C.P., *et al., Antisense Res. & Nucleic Acid Drug Dev. 6*:25-35 (1996); Kulka, M., *et al., Proc. Natl. Acad. Sci. 86*:6868-6872 (1989)) and other (Care, A., *et al., Mol. Cell Biol. 16*:4842-4851 (1996)) laboratories, that antisense ODNs can specifically inhibit the translation of the targeted genes. G361, SK-MEL-31 and SK-MEL-2 melanoma cells were exposed (24 hrs) to different doses of antisense ODNs complementary to the H11 translation initiation site (aODN-1 and aODN-2) and analyzed for their ability to proliferate, as determined by [^{3}H]-TdR incorporation. Sense and scrambled ODNs served as controls. Both immunoprecipitation and immunoperoxidase staining with H11 antibody were used to verify abrogation/reduction of gene expression.

Representative results are shown in FIGs. 7A and 7B for treated G361 cells. Both antisense ODNs inhibited [^{3}H]-TdR incorporation. Inhibition was seen with as little as 10 μM of the ODNs and was maximal at 30 μM. [^{3}H]-TdR incorporation was minimally (10-15%) reduced by treatment with 30 μM of scrambled ODN (FIG. 7A). Growth inhibition (calculated relative to the scODN) was 98 and 86% for aODN-1 and aODN-2 respectively, while cell growth was not decreased in cells treated with the sODN (FIG. 7B). The difference between the proliferative rate of cells treated with the antisense and sense (or scrambled) ODNs was statistically significant ($p < 0.001$ by Student's t test).

To examine the relationship between H11 expression and inhibition of cell growth, duplicate cell cultures were treated with aODN-2 (30 μM; 24 hrs), labeled with [^{35}S]-methionine (4 hrs; 37 μC), and cell extracts were immunoprecipitated with H11-10 antibody. scODN and sODN served as controls.

H11 levels were significantly reduced in cells treated with aODN-2 (FIG. 8, lane 1) as also evidenced by immunoprecipitation with H11 antibody (FIG. 8, lane 4). The levels of H11 seen in extracts (FIG. 8, lane 2) and anti-H11-10 immunoprecipitates (FIG. 8, lane 5) from cells treated with sODN were similar to those seen in untreated cells (FIG. 8, lanes 3,6) or scODN-treated cells (data not shown). These findings support the interpretation that inhibition of melanoma cell growth by H11 antisense ODNs is specifically related to decreased expression of H11.

Additional evidence that cell growth inhibition by antisense ODNs is H11-specific includes: (i) the antisense ODNs did not inhibit the growth of 293 cells that do not express H11 (FIG. 7B), (ii) the growth of HeLa cells that express H11 was also inhibited by the antisense ODNs (FIG. 7B), and (iii) actin was expressed equally well in cells treated with the antisense (FIG. 8, lane 1) or sense (FIG. 8, lane 2) ODNs and in untreated cells (FIG. 8, lane 3). These findings are consistent with the established ability of antisense ODNs to inhibit translation of their cognate genes, and suggest that H11 gene expression is required for melanoma cell growth.

Example 9

Melanoma cells treated with H11 antisense ODNs are arrested at G_1

To further examine the role of H11 in cell growth, G361, SK-MEL-31 and SK-MEL-2 cells were treated (24 hrs, 37°C) with aODN-2, sc-ODN or s-ODN and stained with propidium iodide to examine the proportion of cells in different stages of the cell cycle. Untreated cells, as well as cells treated with sODN or scODN were almost equally distributed between the G_1 (43-46%) and S (37-39%) phases. By contrast the majority of the cells treated with aODN-2 were in the G_1 phase (69-71%), with only 18-19% of the cells in the S phase. Similar results were obtained for aODN-1 (data not shown). We interpret these findings to indicate that melanoma cells treated with H11 antisense ODNs are arrested

at/before the G_1/S proliferative control checkpoints, suggesting that H11 functions in the G_1 phase of the cell cycle.

Example 10

H11 protein expression and kinase activity

The H11 ORF consists of 588 nucleotides which would encode a protein of 196 amino acids with a calculated molecular weight of 21.5 kDa. Furthermore, computer-assisted analysis suggested that the H11 protein may have protein kinase activity. To examine the validity of these interpretations, we constructed a GST-H11 recombinant and used it in western blotting and immunocomplex PK assays. In a first series of experiments, extracts of bacteria, induced to express the GST-H11 fusion protein and uninduced controls were immunoblotted with the H11 antibodies AB-10 and AB-181 that respectively represent N-terminal and C-terminal H11 epitopes. The results are summarized in FIGs. 9A, 9B, and 9C. Both H11 antibodies failed to recognize proteins in the uninduced bacterial extracts (FIG. 9A, lanes 1,2). By contrast, a 50kDa protein that is consistent with the GST-H11 fusion protein (28 and 21.5 kDa for GST and H11 respectively) was recognized in the induced bacterial cell extracts by the AB-181 antibody (C-terminal H11) (FIG. 9A, lane 3). Consistent with the conclusion that it represents the GST-H11 fusion proteins, the 50 kDa species was also recognized by the AB-10 (N-terminal H11) antibody (FIG. 9A, lane 4) and by the GST antibody (FIG. 9A, lane 5). However, both of these latter antibodies also recognized a 29-34 kDa species (FIG. 9A, lanes 4,5) that is likely to represent a breakdown product of the GST-H11 fusion protein at a site downstream of the AB-10 epitope at position 1-181. The breakdown site in the fusion protein is somewhat variable as evidenced by the finding of two distinct species (34 and 29 kDa) in some induced bacterial cell lysates (FIG. 9C, lane 2). Similar results were obtained by western blotting assays with low affinity antibody to the entire H11 protein purified from the GST-H11 fusion protein (data not shown).

In a second series of experiments we used immunocomplex PK assays of the induced and uninduced bacterial cell lysate, in order to begin examining the question of the potential protein kinase activity of H11. Specifically, extracts of induced and uninduced bacteria were precipitated with AB-10 or AB-181 antibodies and the precipitates were used in PK assays in the absence of exogenously added protein substrates. One phosphorylated 50 kDa protein consistent with the GST-H11 fusion protein, was seen in the AB-181 precipitate from induced (FIG. 9B, lane 1), but not uninduced (FIG. 9B, lane 3) bacteria. Three phosphorylated proteins that are respectively consistent with the GST-H11 fusion protein (50 kDa) and its breakdown products (34 and 29 kDa) were seen in the precipitates obtained with AB-10 antibody from induced (FIG. 9B, lane 2) but not uninduced (FIG. 9B, lane 4) bacteria. The phosphorylated proteins are indeed H11 and its breakdown products as evidenced by western blotting of the gel in FIG. 9B with AB-10 antibody (FIG. 9C). Taken *in toto*, these data indicate that H11 codes for a protein of approximately 21 kDa that may have kinase activity. However, further studies, including protein purification and mutation analyses, are needed before final conclusions can be reached about the protein kinase activity of H11.

Example 11

H11 is involved in cell transformation: expression is required for anchorage independent growth

The finding that H11 expression is required for cell growth raises the possibility that it may be involved in cell transformation. To begin to address this question, 293 (immortalized human embryonic kidney) cells were transfected with an HA tagged H11 expression vector which expressed a neomycin selectable marker. Constitutive cell lines were established from several clones using 400 μg/ml G418 and were assayed for H11 expression by immunoblotting with anti-H11 AB-10 antibody. One of these cell lines which had the highest level of expression of H11 was assayed for anchorage independent growth and evidenced

significantly increased agarose cloning efficiency defined as the number of colonies/ $5x10^4$ cells (CF = 0.12%) as compared to untransfected 293 cells (CE = 0.006%). The data suggest that H11 may be involved in transformation.

Example 12

H11 is involved in melanocyte transformation: expression in dysplastic nevi in situ

If H11 is involved in melanocyte transformation, its expression should be increased in dysplastic nevi *in situ*. To begin testing this interpretation, serial sections of tissues from patients with melanoma as well as those with benign or dysplastic nevi were stained in immunohistochemistry with H11-181 antibody. Melanoma tissues (78%) stained with H11-181 antibody and staining was seen in melanocytes. The highest dilution of antibody that stained at least 50% of the cells was 1:800-1:1600. Staining was also seen in 75% of nevi with mild dysplastic changes but the highest dilution staining at least 50% of the cells was 1:200-1:400. This compares to 10% of the benign nevi and none of the normal skin tissues staining with H11-181 at a dilution of 1:200 (FIGs. 10A-10G).

Example 13

H11 expression in breast and prostate cancer

To examine whether H11 is expressed at least in some breast or prostate cancer cells, immunoblotting was performed with H11 antibody. The results shown in FIG. 11, lanes 1 and 3, indicate that H11 is expressed in cell lines established from hormone-responsive breast and prostate cancer cells. Preimmune serum is negative (FIG. 11, lanes 2 and 4).

It should be understood that the examples and embodiments described herein are for illustrative purposes only and that various modifications or changes

in light thereof will be suggested to persons skilled in the art and are to be included within the spirit and purview of this application and the scope of the appended claims. All publications, patents and patent applications cited herein are incorporated in their entirety by reference herein.

What Is Claimed Is:

1. An isolated nucleic acid molecule, comprising a polynucleotide that comprises a nucleotide sequence at least 90% identical to a sequence selected from the group consisting of:

(a) the nucleotide sequence of SEQ ID NO:1;

(b) a nucleotide sequence comprising the portion of the nucleotide sequence of SEQ ID NO:1 that encodes an H11 polypeptide; and

(c) a nucleotide sequence complementary to the nucleotide sequence in (a) or (b).

2. The nucleic acid molecule of claim 1 wherein said polynucleotide comprises a nucleotide sequence at least 95% identical to said reference sequence.

3. An isolated nucleic acid molecule, comprising a polynucleotide which hybridizes under stringent hybridization conditions to a polynucleotide that comprises a nucleotide sequence identical to a nucleotide sequence in (a), (b) or (c) of claim 1.

4. An isolated nucleic acid molecule, comprising a polynucleotide that comprises a sequence selected from the group consisting of:

(a) a nucleotide sequence of a fragment of the sequence shown in SEQ ID NO:1, wherein said fragment comprises at least 10 contiguous nucleotides from SEQ ID NO:1; and

(b) a nucleotide sequence complementary to a nucleotide sequence in (a).

5. An isolated nucleic acid molecule, comprising a polynucleotide which encodes the amino acid sequence of an epitope-bearing portion of an H11

polypeptide, said H11 polypeptide comprising an amino acid sequence at least 90% identical to the amino acid sequence depicted in SEQ ID NO:2.

6. An isolated polynucleotide of claim 4, wherein said polynucleotide is detectably labeled.

7. An isolated polypeptide comprising an amino acid sequence at least 95% identical to a sequence selected from the group consisting of:

(a) the amino acid sequence shown in SEQ ID NO:2;

(b) an amino acid sequence encoded by the nucleic acid sequence shown in SEQ ID NO:1; and

(c) an amino acid sequence of an epitope-bearing portion of any one of the polypeptides of (a) or (b).

8. An isolated polypeptide of claim 7, wherein said polypeptide is phosphorylated.

9. An isolated polypeptide of claim 7, comprising an epitope-bearing portion of an H11 polypeptide.

10. An isolated polypeptide of claim 9, which is produced or contained in a recombinant host cell.

11. A method for making a recombinant vector comprising inserting the isolated nucleic acid molecule of claim 1 into a vector.

12. The method of claim 11, wherein said vector is an expression vector.

13. A recombinant vector produced by the method of claim 11.

14. A recombinant vector comprising the nucleic acid molecule of claim 1.

15. A method of making a recombinant host cell, comprising introducing the nucleic acid molecule of claim 1 into a host cell.

16. A recombinant host cell produced by the method of claim 15.

17. A recombinant host cell comprising the nucleic acid molecule of claim 1.

18. A method for producing an isolated H11 polypeptide, comprising culturing the recombinant host cell of claim 16 under conditions such that said polypeptide is expressed, and isolating said polypeptide.

19. An isolated H11 polypeptide produced according to the method of claim 18.

20. An isolated polypeptide comprising an epitope-bearing portion of the H11 protein.

21. A method of producing an isolated H11-specific antibody comprising immunizing an animal with an isolated H11 polypeptide of claim 7, and isolating an H11-specific antibody from said animal.

22. An isolated H11-specific antibody produced according to the method of claim 21.

23. The isolated antibody of claim 22, wherein said antibody is detectably labeled.

24. A hybridoma cell that secretes an antibody specific for the polypeptide of claim 20.

25. A nucleic acid probe for the detection of the expression of an H11 polypeptide in a sample from an animal, comprising a nucleic acid molecule sufficient to specifically detect under stringent hybridization conditions the presence of a polynucleotide encoding said H11 polypeptide in said sample.

26. A method of detecting the presence of a polynucleotide encoding an H11 polypeptide in a sample, comprising:

(a) contacting said sample with the probe of claim 25 under conditions of hybridization; and

(b) detecting the formation of a hybrid of said probe and said polynucleotide.

27. A method of detecting an H11 polypeptide in cells or tissue, comprising:

(a) contacting a sample of said cells or said tissue with at least one antibody that specifically binds to an H11 polypeptide; and

(b) detecting said antibody which is bound in said sample.

28. A method of diagnosing or detecting cancer in an animal, comprising:

(a) contacting a sample of cells or tissue from said animal, with at least one antibody that specifically binds to an H11 polypeptide; and

(b) detecting said antibody which is bound in said sample, wherein detection of elevated levels of H11 polypeptide, compared to control cells or control tissue, indicates that said cells or said tissue are cancerous.

29. The method of claim 28, wherein said cancer is melanoma.

30. A method of treating a cancer in an animal, comprising introducing into cells of the animal in need thereof an antisense oligonucleotide that is complementary to a polynucleotide encoding an H11 protein, whereby said antisense oligonucleotide binds to said polynucleotide encoding an H11 protein and inhibits the growth of said cells, and said cancer is treated.

31. The method of 30, wherein said cancer is melanoma.

32. The method of 30, wherein said cancer is ovarian cancer or estrogen-dependent breast cancer.

33. A method of preventing or inhibiting melanoma in an animal, comprising administering to the animal in need thereof an antigenic H11 polypeptide, whereby said H11 polypeptide causes the production within said animal of antibodies that specifically bind to said H11 polypeptide and prevents or inhibits the growth of melanoma tumors.

34. A method of enhancing wound healing in an animal, comprising introducing into cells of the wound in need thereof a polynucleotide construct comprising a gene expressing an H11 polypeptide, or fragment or analog thereof, whereby said H11 polypeptide or fragment or analog thereof is produced and the healing of the wound is enhanced.

35. A method of treating a hyperproliferative skin disorder in an animal, comprising introducing into skin cells of the animal in need thereof an antisense oligonucleotide that is complementary to a polynucleotide encoding an H11 protein, whereby said antisense oligonucleotide binds to said polynucleotide encoding an H11 protein and inhibits the growth of said skin cells and treats said disorder.

36. The method of claim 35, wherein said hyperproliferative skin disorder is psoriasis.

37. An antisense oligonucleotide comprising a nucleotide sequence which is complementary to a polynucleotide encoding an amino acid sequence at least 95% identical to a sequence selected from the group consisting of:

(a) the amino acid sequence shown in SEQ ID NO:2;

(b) an amino acid sequence encoded by the nucleic acid sequence shown in SEQ ID NO:1; and

(c) an amino acid sequence of an epitope-bearing portion of any one of the polypeptides of (a) or (b).

```
   1 CTCGCTGGGTTTATTAAGCTCCTGGCTCCGCTCTAGACCTCAGCGGTTCTGGCTGCCAGC
  61 CTGGGCAGCCTGGGAAGCCTGGGAGGACGGTGGCTTGCCGGTCTGTCGTCAGGCAGTGCG
 121 GACGGGGACCCTCTGGGATTCTGCTGGATCTGCCCCGGGGGTTACCTTTGGGGGCTGGGA
 181 CCCCAGTCGAGGGGACACAACCGTCCCTGGCAGTGGTTGGTTCTGCTTCTCCCTGCAGAA
 241 AAGCAGCATTTTCGGAAGCTGAAGAATAAGCTAGCCCAGCCACACCACCTTGTTGTGTGA
 301 CCTTGGGCAGGTGGTTCTGTCTCTCTGAGCCTCTGTTTCTCTCTGAGCTGAGCAGCCACC
 361 atggctgacggtcagatgcccttctcctgccactacccaagccgcctgcgccgagacccc
     M  A  D  G  Q  M  P  F  S  C  H  Y  P  S  R  L  R  R  D  P
 421 ttccgggactctcccctctcctctcgcctgctggatgatggctttggcatggacccttc
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     P  G  T  L  R  S  G  M  V  P  R  G  P  T  A  T  A  R  F  G
 601 gtgcctgccgagggcaggacccccccaccttccctggggagccctggaaagtgtgtgtg
     V  P  A  E  G  R  T  P  P  P  F  P  G  E  P  W  K  V  C  V
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     N  V  H  S  F  K  P  E  E  L  M  V  K  T  K  D  G  Y  V  E
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     K  K  I  Q  L  P  A  E  V  D  P  V  T  V  F  A  S  L  S  P
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     E  G  L  L  I  I  E  A  P  Q  V  P  P  Y  S  T  F  G  E  S
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     S  F  N  N  E  L  P  Q  D  S  Q  E  V  T  C  T  *
 961 ACTGGCCCATCCTTGTTTTGTCCCCAACCCTAGGGCTTCTCTGATTCCAGGATACATTAC
1021 TTTAGCTGAACTCAGATTTAGTGCAAGTAAAATGTTAGAGGGTGCGGGGGTGAGGACTGA
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1561 TAAGGACGCTGGGACCCTGTCAGTTTATGATCTGACCTAGGTCCCCCCTTTCTTCTGTCC
1621 CCTGTTTTTTAAGTCCGGATTTTTACAGAAGGAACTGTCTCCAGACAGCTCATCAAGGAAC
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FIG. 1

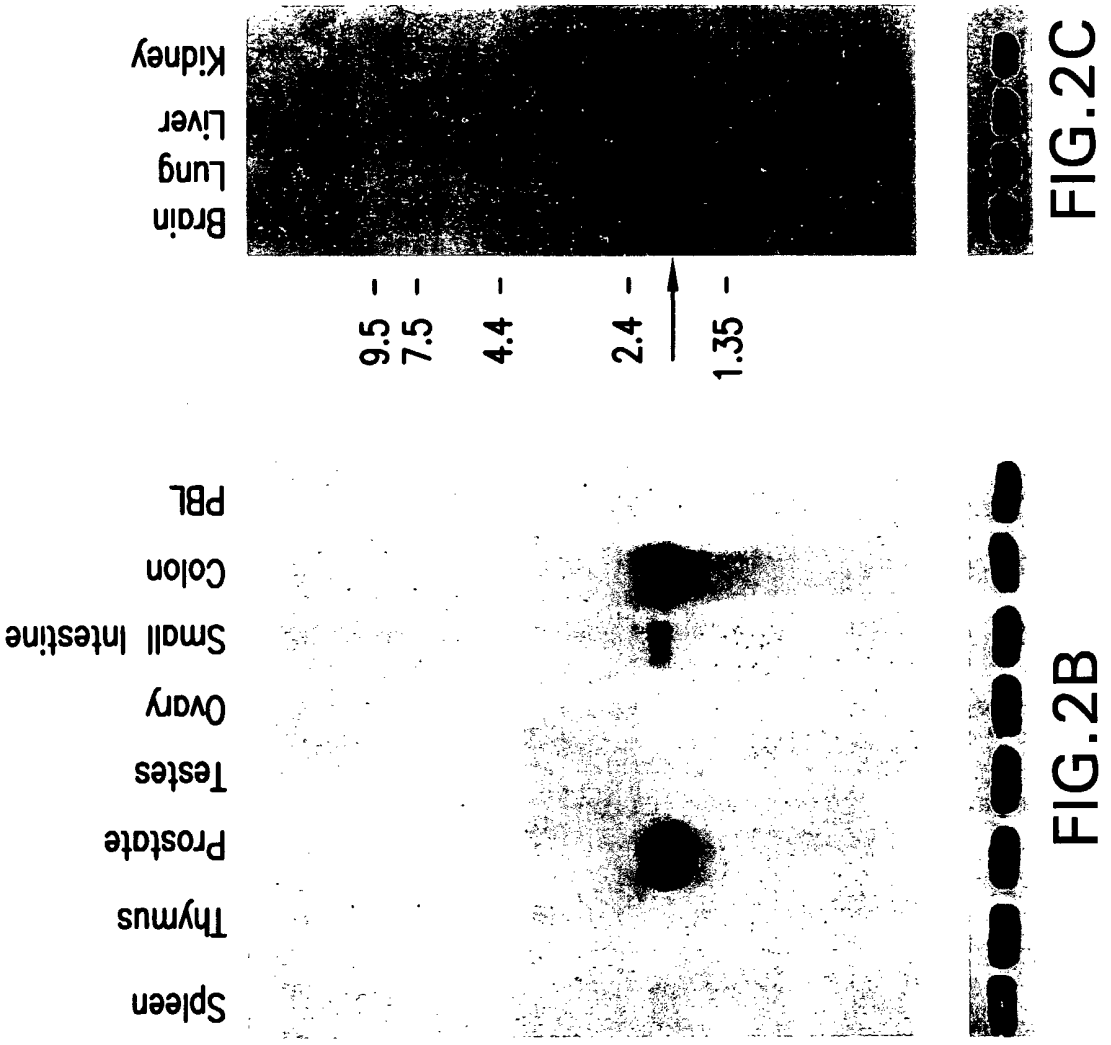


FIG.2A

FIG.2B

FIG.2C

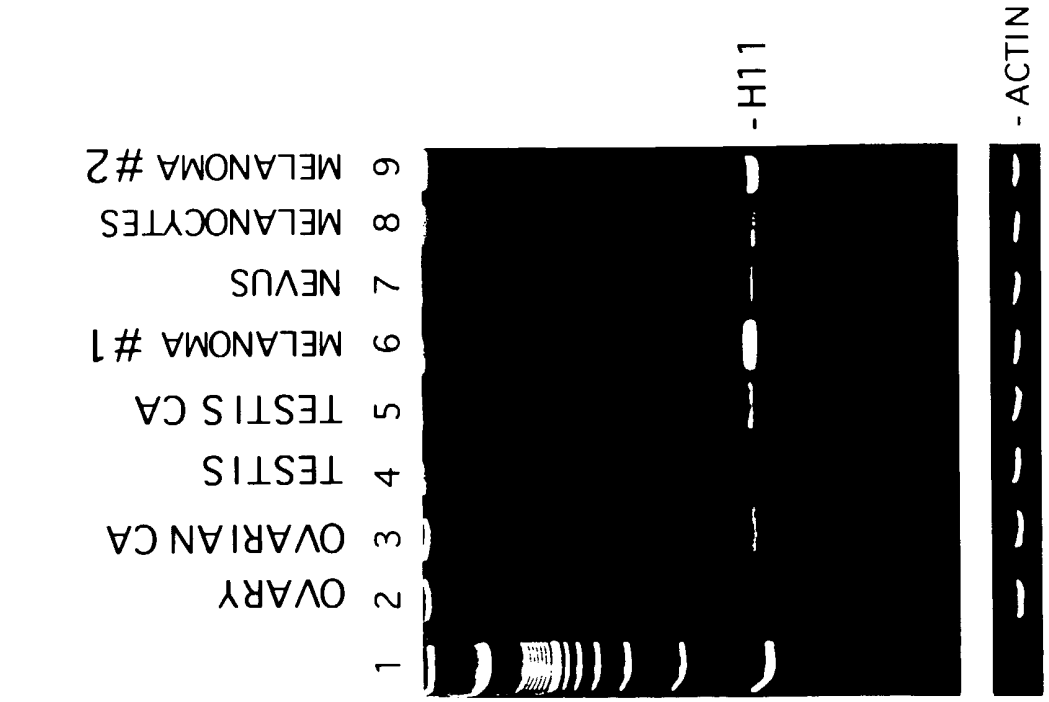

- H11
- ACTIN
1 OVARY
2 OVARIAN CA
3 TESTIS
4 TESTIS CA
5 MELANOMA #1
6 NEVUS
7 MELANOCYTES
8 MELANOMA #2
9

FIG.3B

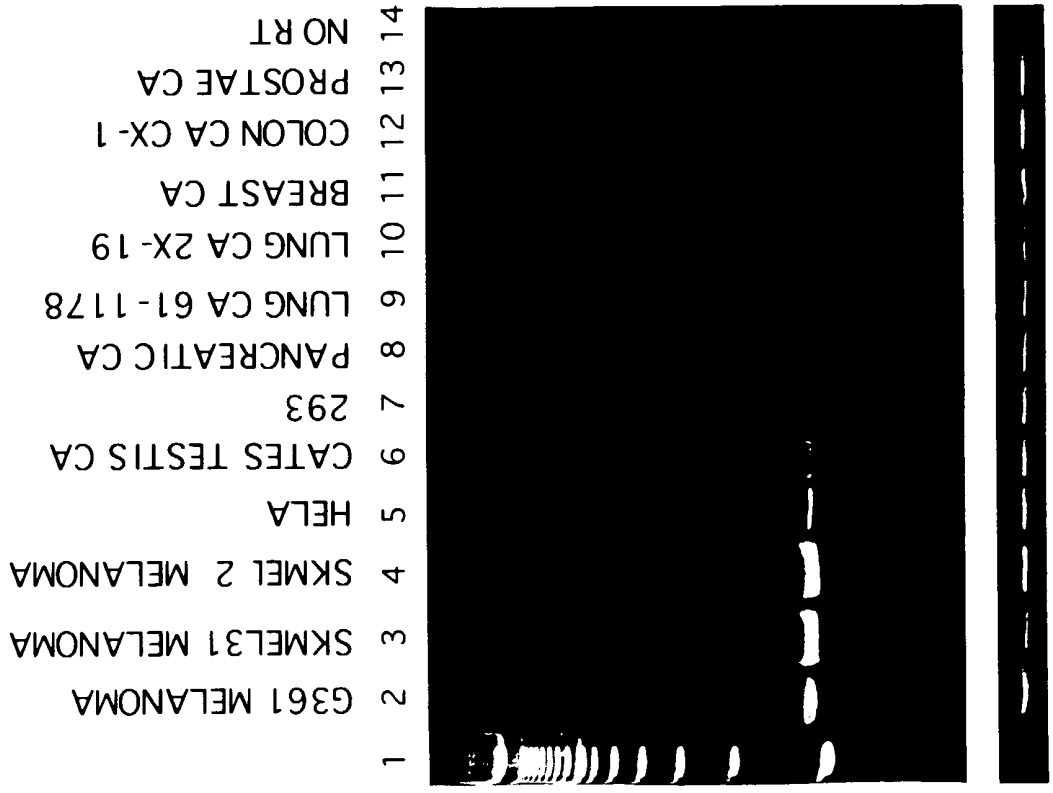

1 G361 MELANOMA
2 SKMEL31 MELANOMA
3 SKMEL 2 MELANOMA
4 HELA
5 CATES TESTIS CA
6 293
7 PANCREATIC CA
8 LUNG CA 61-1178
9 LUNG CA 2X-19
10 BREAST CA
11 COLON CA CX-1
12 PROSTAE CA
13 NO RT
14

FIG.3A

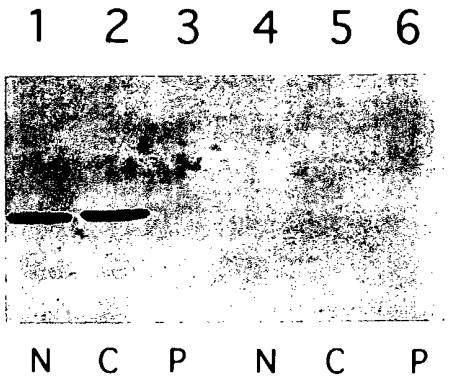


FIG.4A

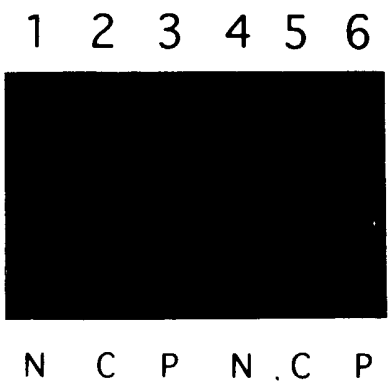


FIG.4B

FIG.4C

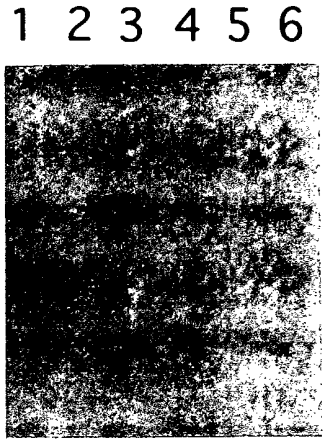


FIG.4D

FIG.5A

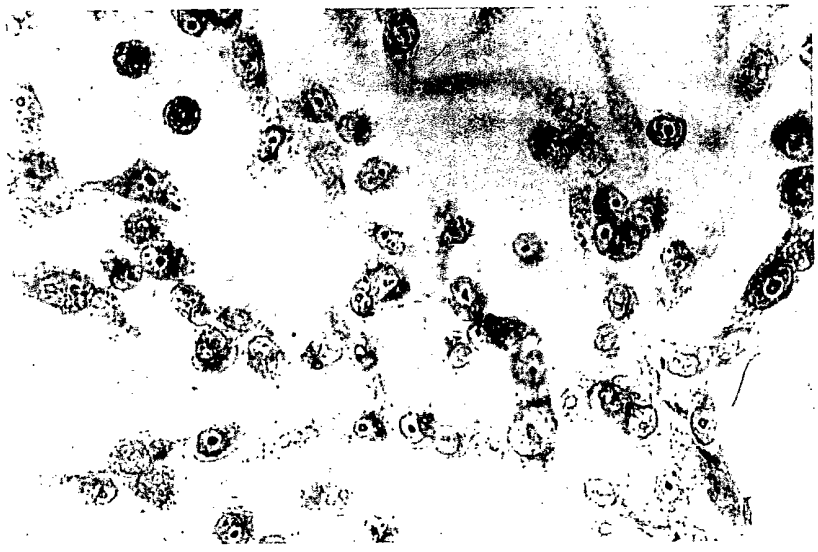


FIG.5B

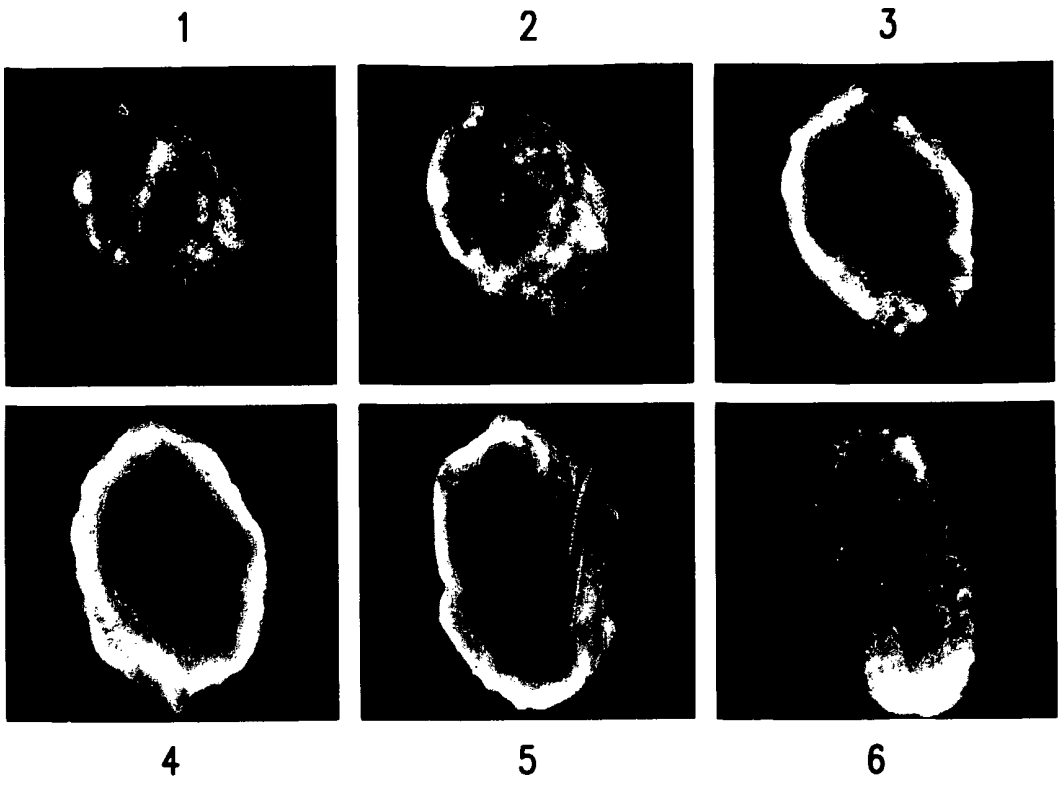


FIG.6A

FIG.6B

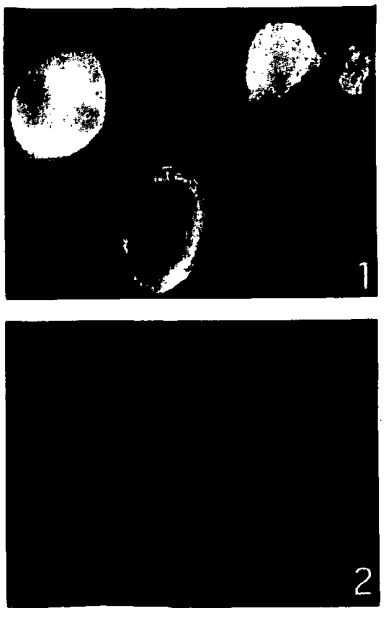
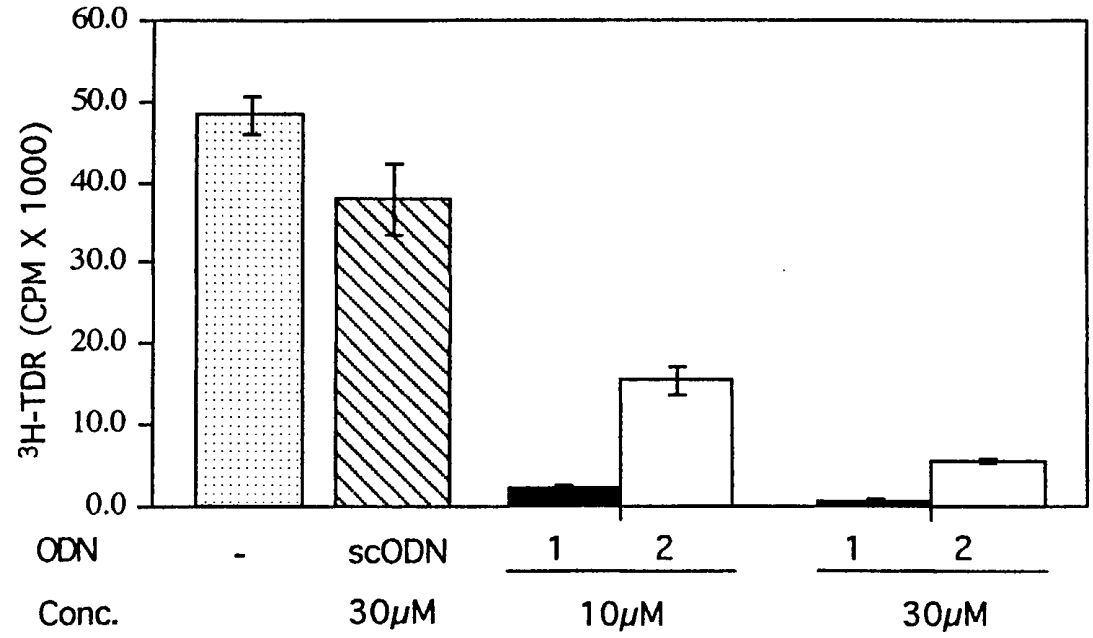


FIG.6C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30.0
20.0
10.0
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ODN
-
scODN
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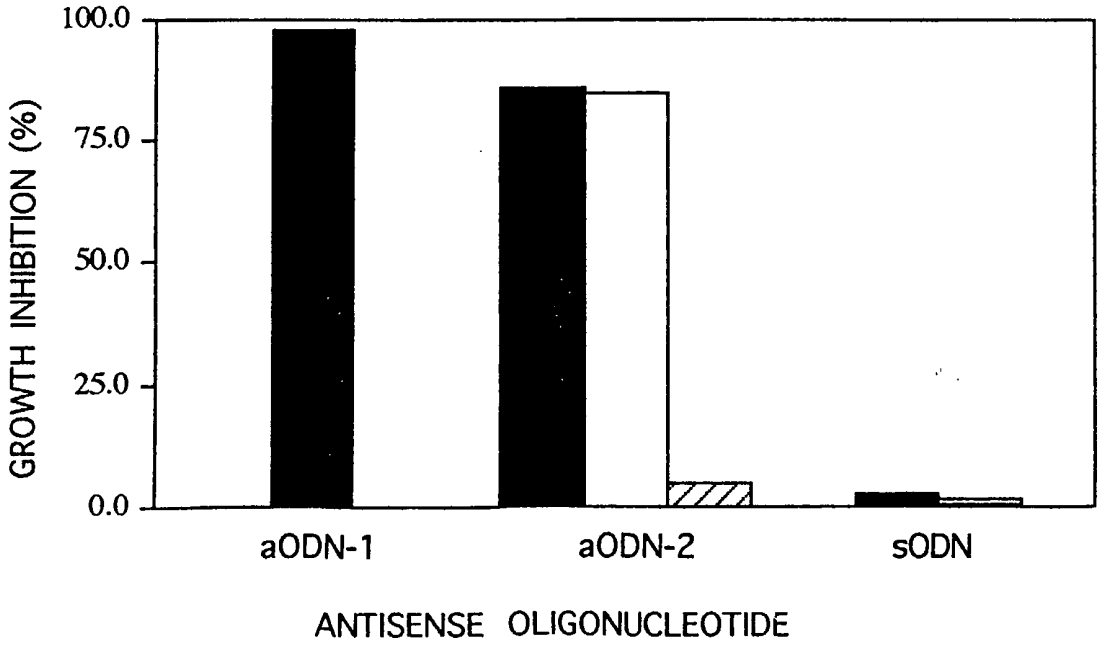
2
1
2
Conc.
30μM
10μM
30μM

FIG.7A


100.0
75.0
50.0
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GROWTH INHIBITION (%)
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aODN-2
sODN
ANTISENSE OLIGONUCLEOTIDE

FIG.7B

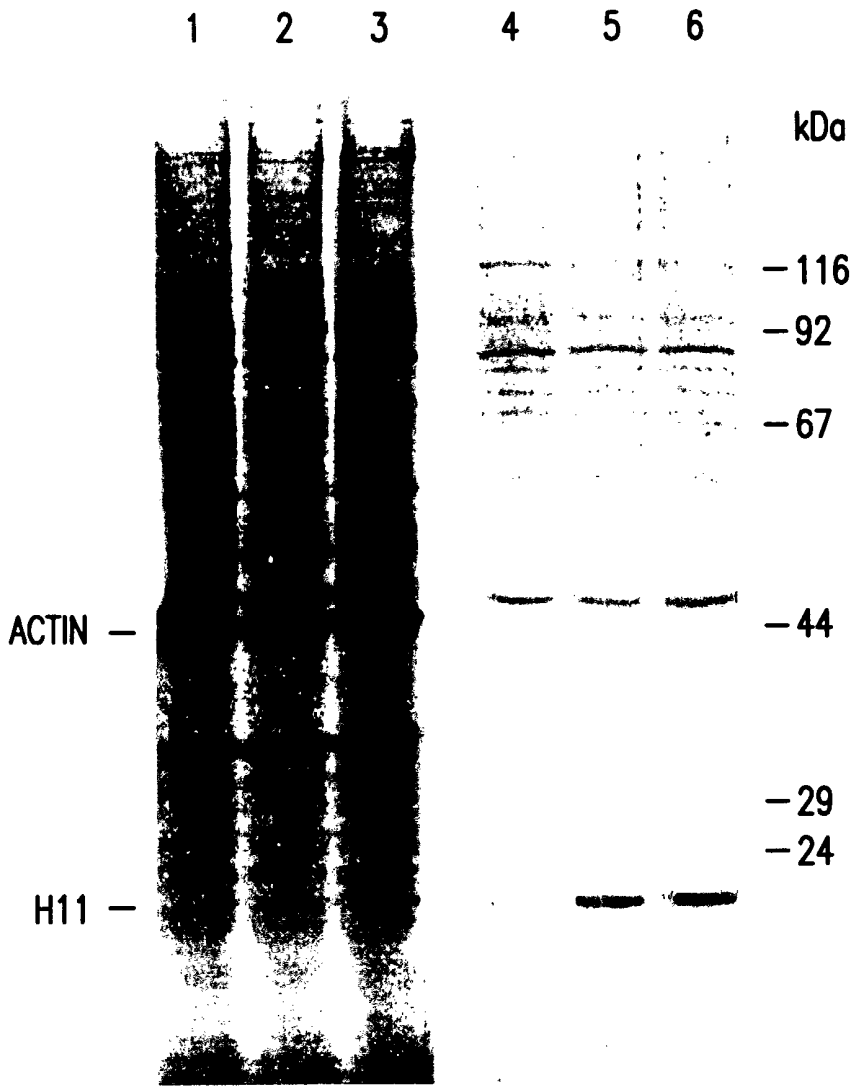

FIG.8

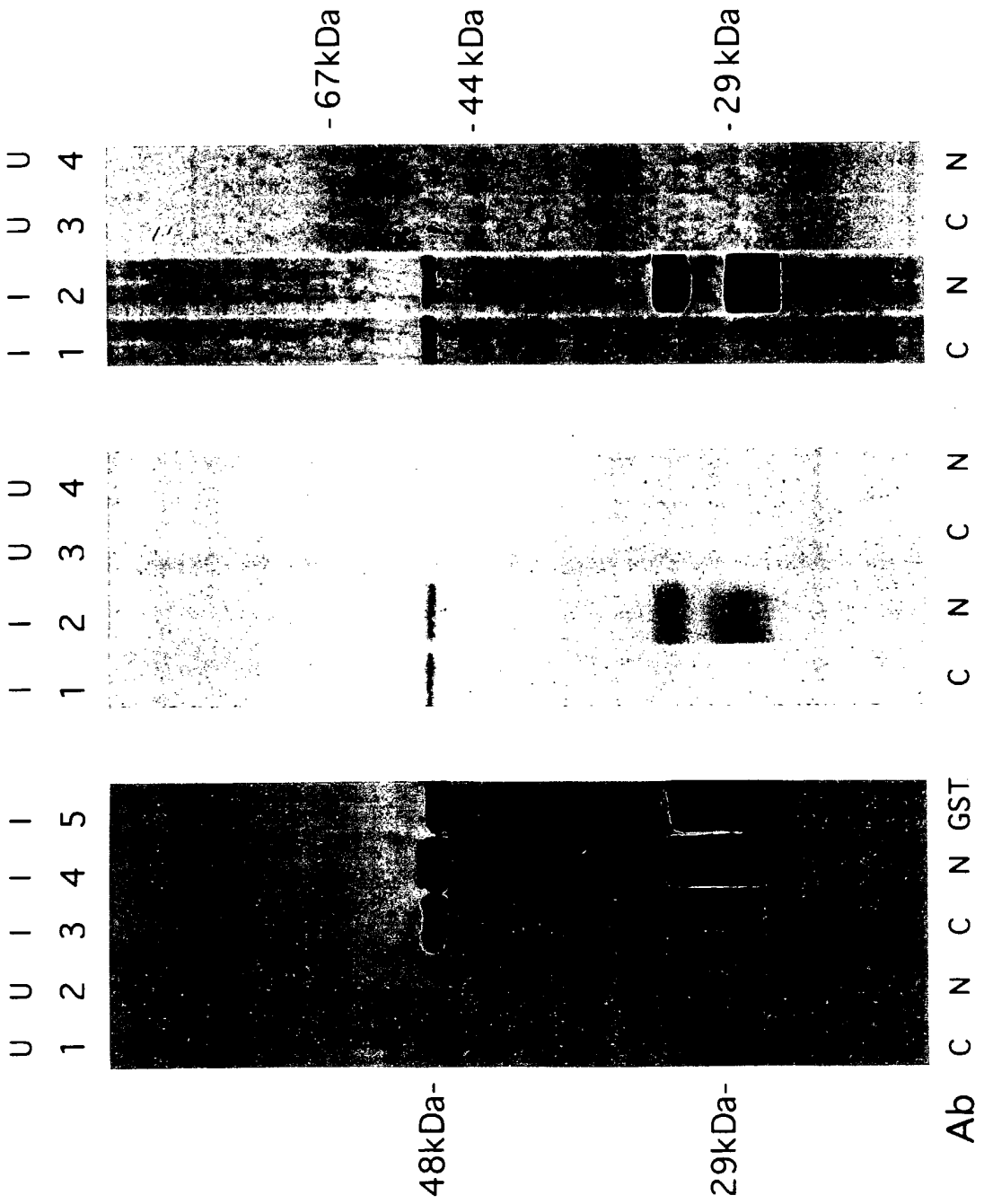

FIG.9C

FIG.9B

FIG.9A

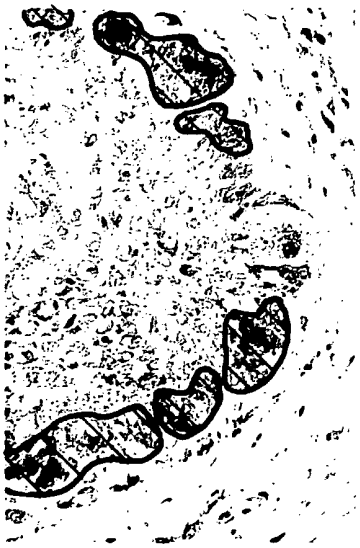

FIG.10C

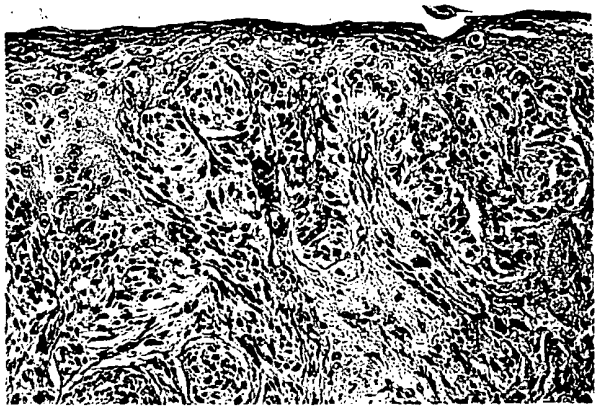

FIG.10B

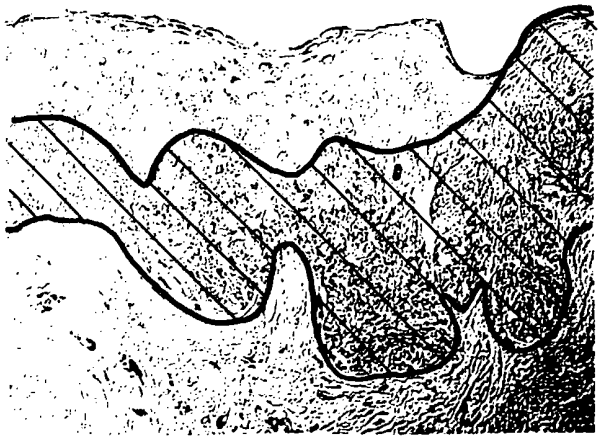

FIG.10A

FIG.10D

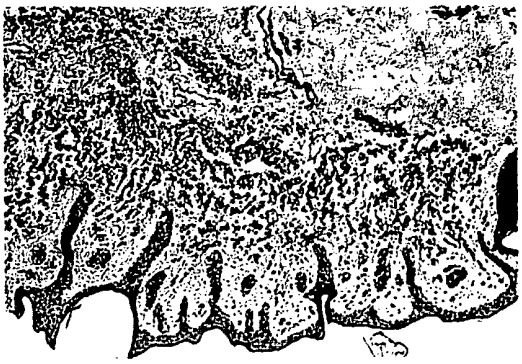

FIG.10E

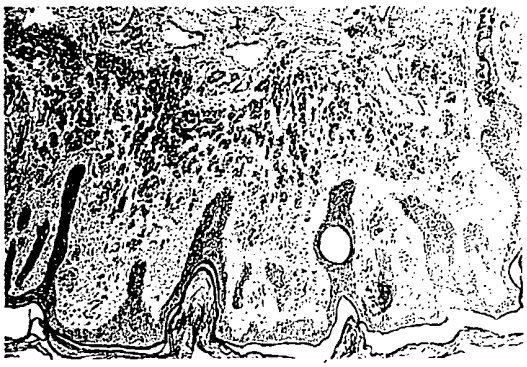

FIG.10F

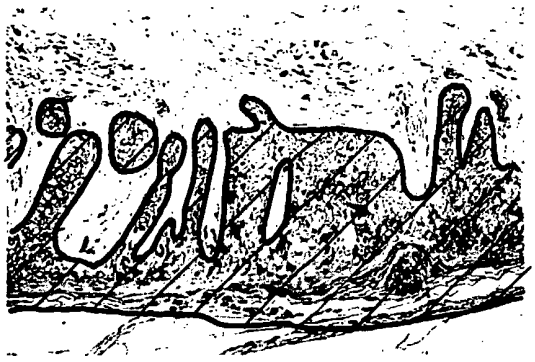

FIG.10G

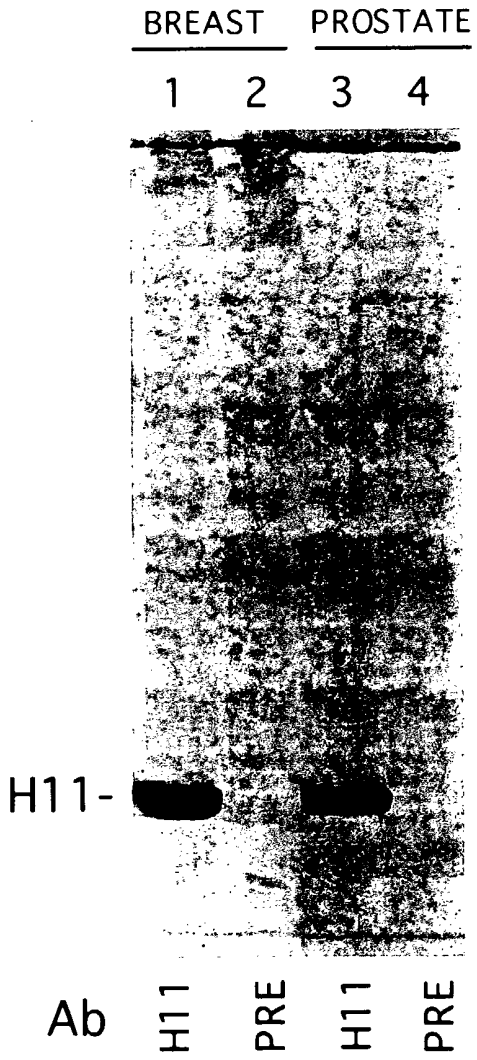

FIG.11

SEQUENCE LISTING

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Met Ala Asp Gly Gln Met Pro Phe Ser Cys His Tyr Pro Ser Arg Leu
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cgc cga gac ccc ttc cgg gac tct ccc ctc tcc tct cgc ctg ctg gat 456
Arg Arg Asp Pro Phe Arg Asp Ser Pro Leu Ser Ser Arg Leu Leu Asp
20 25 30

gat ggc ttt ggc atg gac ccc ttc cca gac gac ttg aca gcc tct tgg 504
Asp Gly Phe Gly Met Asp Pro Phe Pro Asp Asp Leu Thr Ala Ser Trp
35 40 45

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ccc gac tgc gct ctg cct cgt ctc tcc tcc gcc tgg cca ggc acc cta   552
Pro Asp Cys Ala Leu Pro Arg Leu Ser Ser Ala Trp Pro Gly Thr Leu
     50                  55                  60

agg tcg ggc atg gtg ccc cgg ggc ccc act gcc acc gcc agg ttt ggg   600
Arg Ser Gly Met Val Pro Arg Gly Pro Thr Ala Thr Ala Arg Phe Gly
 65                  70                  75                  80

gtg cct gcc gag ggc agg acc ccc cca ccc ttc cct ggg gag ccc tgg   648
Val Pro Ala Glu Gly Arg Thr Pro Pro Pro Phe Pro Gly Glu Pro Trp
                 85                  90                  95

aaa gtg tgt gtg aat gtg cac agc ttc aag cca gag gag ttg atg gtg   696
Lys Val Cys Val Asn Val His Ser Phe Lys Pro Glu Glu Leu Met Val
            100                 105                 110

aag acc aaa gat gga tac gtg gag gtg tct ggc aaa cat gaa gag aaa   744
Lys Thr Lys Asp Gly Tyr Val Glu Val Ser Gly Lys His Glu Glu Lys
        115                 120                 125

cag caa gaa ggt ggc att gtt tct aag aac ttc aca aag aaa atc cag   792
Gln Gln Glu Gly Gly Ile Val Ser Lys Asn Phe Thr Lys Lys Ile Gln
    130                 135                 140

ctt cct gca gag gtg gat cct gtg aca gta ttt gcc tca ctt tcc cca   840
Leu Pro Ala Glu Val Asp Pro Val Thr Val Phe Ala Ser Leu Ser Pro
145                 150                 155                 160

gag ggt ctg ctg atc atc gaa gct ccc cag gtc cct cct tac tca aca   888
Glu Gly Leu Leu Ile Ile Glu Ala Pro Gln Val Pro Pro Tyr Ser Thr
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            180                 185                 190

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Val Thr Cys Thr
        195

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gttctcaaag tttccacatt agcactccct aaggacgctg ggagcctgtc agtttatgat 1591

ctgacctagg tccccccttt cttctgtccc ctgtttttaa gtccggattt ttacagaagg 1651

aactgtctcc agacagctca tcaaggaacc aagcaaaggc cagatagcct gacagatagg 1711

ctagtggtaa ttgtgtatat gggcgggacg tgtgtgtcat tattatttga gtttatgctg 1771

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Arg Arg Asp Pro Phe Arg Asp Ser Pro Leu Ser Ser Arg Leu Leu Asp
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Asp Gly Phe Gly Met Asp Pro Phe Pro Asp Asp Leu Thr Ala Ser Trp
         35                  40                  45

Pro Asp Cys Ala Leu Pro Arg Leu Ser Ser Ala Trp Pro Gly Thr Leu
     50                  55                  60

Arg Ser Gly Met Val Pro Arg Gly Pro Thr Ala Thr Ala Arg Phe Gly
 65                  70                  75                  80

Val Pro Ala Glu Gly Arg Thr Pro Pro Pro Phe Pro Gly Glu Pro Trp
                 85                  90                  95

Lys Val Cys Val Asn Val His Ser Phe Lys Pro Glu Glu Leu Met Val
            100                 105                 110

Lys Thr Lys Asp Gly Tyr Val Glu Val Ser Gly Lys His Glu Glu Lys
        115                 120                 125

Gln Gln Glu Gly Gly Ile Val Ser Lys Asn Phe Thr Lys Lys Ile Gln
    130                 135                 140
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Leu Pro Ala Glu Val Asp Pro Val Thr Val Phe Ala Ser Leu Ser Pro
145 150 155 160

Glu Gly Leu Leu Ile Ile Glu Ala Pro Gln Val Pro Pro Tyr Ser Thr
165 170 175

Phe Gly Glu Ser Ser Phe Asn Asn Glu Leu Pro Gln Asp Ser Gln Glu
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Val Thr Cys Thr
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<220>
<223> Description of Artificial Sequence: sense H11 primer

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<210> 4
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<220>
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<400> 4
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Leu Ser Ser Arg
20

<210> 6
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primer

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<210> 25
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