Title: DISRUPTION OF EXPRESSION OF MULTIPLE ALLELES OF MAMMALIAN GENES

Abstract

Methods are provided for identifying a gene at a random chromosomal locus in the genome of a mammalian cell. The method involves inactivating one copy of the gene by integrating one DNA construct (knockout construct) in that gene copy. The knockout construct includes a positive selection marker region sequence and, in a 5' direction from the selection marker region sequence, a transcription initiation region sequence responsive to a transactivation factor, said transcription initiation region oriented for antisense RNA transcription in the direction away from the selection marker region sequence. The second copy of the gene is inactivated by transforming the cells with a second DNA construct (transactivation construct) containing a gene sequence for the transactivation factor which initiates antisense RNA transcription extending from the knockout construct into the chromosomal locus flanking the knockout construct at its 5' end. Inactivation of both gene copies may result in a change in cell phenotype distinguishable from the wild-type phenotype. Optionally, the wild-type phenotype can be regained by introducing a third construct that can inhibit antisense RNA transcription. A gene is provided associated with tumor susceptibility of mammalian cells, tsg 101.
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Disruption of expression of multiple alleles of mammalian genes

INTRODUCTION

5 Background

There has been considerable interest in the development of a method for identifying mammalian cell genes whose concurrent homozygous inactivation de novo leads to a defined phenotype, where multiple alleles of a gene have been inactivated and where it is easy to confirm that the inactivation results in a phenotype distinguishable from the wild-type. One use of this method is the identification of genes involved in tumor susceptibility.

15 Tumor susceptibility genes may be oncogenes, which are typically upregulated in tumor cells, or tumor suppressor genes, which are down-regulated or absent in tumor cells. Malignancies may arise when a tumor suppressor is lost and/or an oncogene is inappropriately activated. When such mutations occur in somatic cells, they result in the growth of sporadic tumors. Familial predisposition to cancer may occur when there is a mutation, such as loss of an allele encoding a tumor suppressor gene, present in the germline DNA of an individual. In the best characterized familial cancer syndromes, the primary mutation is a loss of function consistent with viability, but resulting in neoplastic change consequent to the acquisition of a second somatic mutation at the same locus.

25 Extensive studies of the early-onset breast cancer families have led to the recent identification of two candidate breast cancer suppressor genes, BRCA1 and BRCA2.
Although frequent mutations of BRCA1 or BRCA2 have been demonstrated in familial early onset breast cancer, this type of cancer represents only about 5-10% of all breast malignancies, and the possible role(s) of BRCA1 and BRCA2 in the remaining 90-95% of sporadic breast cancers has not been determined.

Deletion and loss of heterozygosity (LOH) of markers in human chromosome band 11p15 have been shown in a variety of human cancers, including lung cancer, testicular cancer and male germ cell tumor, stomach cancer, Wilms' tumor, ovarian cancer, bladder cancer, myeloid leukemia, malignant astrocytomas and other primitive neuroectodermal tumors, and infantile tumors of adrenal and liver. About 30% of sporadic breast carcinomas show a LOH in this region.

Since LOH is believed to indicate inactivation of a tumor suppressor gene at the location where LOH occurs, the frequent LOH found at 11p15 in a variety of human cancers suggests the presence of either a cluster of tumor suppressor genes or a single pleiotropic gene in this region.

The clinical importance of these cancers makes the identification of this putative tumor suppressor gene of great interest for diagnosis, therapy, and drug screening.

Relevant Literature

associated with the expression of antisense RNAs in different cell types. Giese et al. (1992) *Cell* 71:565-576 describes the inactivation of both copies of a gene in a transgenic mouse.


**SUMMARY OF THE INVENTION**

Mammalian tumor susceptibility genes and methods for their identification are provided, including the complete nucleotide sequences of human *TSG101* and mouse *tsg101* cDNA. Deletions in *TSG101* are associated with the occurrence of human cancers, for example breast carcinomas. The cancers may be familial, having as a component of risk an inherited genetic predisposition, or may be sporadic. The *TSG101* nucleic acid compositions find use in identifying homologous or related proteins and the DNA sequences encoding such proteins; in producing compositions that modulate the expression or function of the protein; and in studying associated physiological pathways. In addition, modulation of the gene activity in vivo is used for prophylactic and therapeutic purposes, such as treatment of cancer, identification of cell type based on expression, and the like. The DNA is further used as a diagnostic for a genetic predisposition to cancer, and to identify specific cancers having mutations in this gene.

The subject invention, in another aspect, provides a method for identifying de novo a gene at a random
chromosomal locus of a mammalian cell based on the phenotype produced by interfering with expression of multiple alleles of the gene corresponding to this locus. The method involves inactivating all copies of the gene and any of its alleles which have substantial sequence similarity.

In another aspect, the subject invention provides a rapid method for establishing the function of a gene in a mammalian cell of which at least a portion of the sequence has been previously isolated. In this aspect, the construct integrated in the genome includes two homologous recombination sites which allow for the integration of the construct at the target site. Additionally, the subject invention provides for DNA constructs, vectors, and mammalian cells containing the DNA constructs in their genome.

DESCRIPTION OF THE SPECIFIC EMBODIMENTS

Mammalian ts101 gene compositions and methods for their isolation are provided. Of particular interest are the human and mouse homologs. Certain human cancers show deletions at the TSG101 locus. Many such cancers are sporadic, where the tumor cells have a somatic mutation in TSG101. The TSG101 genes and fragments thereof, encoded protein, and anti-TSG101 antibodies are useful in the identification of individuals predisposed to development of such cancers, and in characterizing the phenotype of sporadic tumors that are associated with this gene. Tumors may be typed or staged as to the TSG101 status, e.g., by detection of mutated sequences, antibody detection of abnormal protein products, and functional assays for altered TSG101 activity. The encoded TSG101 protein is useful in drug screening for compositions that mimic TSG101 activity or expression, particularly with respect to TSG101 function as a tumor suppressor in oncogenesis. TSG101 can
be used to investigate the interactions with stathmin and the role the complex plays in the regulation of the cell. The human TSG101 and mouse tsg101 gene sequences and isolated nucleic acid compositions are provided. In identifying the human and mouse TSG101/tsg101 genes, the novel gene discovery approach "random homozygous knock out" was utilized. A retroviral gene search vector carrying a reporter gene was used to select and identify cells containing the vector integrated into target transcriptionally active chromosomal DNA regions, behind chromosomal promoters. 5' to and in reverse orientation to the reporter gene was a regulated promoter with no transcription activity, but which could be highly activated by a transactivator. The system generates large amounts of antisense RNA, which interacts with both alleles of the target gene. Cells transfected with the search vector were further transfected with a plasmid encoding a transactivator. The cells were plated to select for genes whose inactivation led to cellular transformation. While control cell populations formed no colonies in soft agar, the transactivated cells produced 20 colonies. One of these clones was shown to be highly tumorigenic in nude mice. mRNA selection, using a primer specific for the reporter gene, was used to isolate mRNA from the target gene. The mRNA was then used to generate a cDNA clone, which was further used in hybridization screening to isolate the full-length mouse tsg101 cDNA.

To obtain the human homolog of mouse tsg101, the mouse cDNA sequence was used to query dbEST. Ten human partial cDNA sequences included in the database showed 85% to 95% identity to mouse tsg101. A conserved sequence was used to design primers that amplify segments of human TSG101 cDNA, employing total DNA isolated from a human cDNA library as template. The TSG101 gene has been mapped to human chromosome sub-bands 11p15.1-15.2, and is closely
linked to the Sequence Tagged Site (STS) markers D11S921 through D11S1308 (a detailed map of human genome markers may be found in Dib et al. (1996) *Nature* **280**:152; http://www.genethon.fr).

The full length human cDNA contains an 1140 bp open reading frame, encoding a 380 amino acid protein. The human and mouse cDNAs are 86% identical at the nucleotide level. The predicted proteins are 94% identical and are distinguished by 20 amino acid mismatches and one gap. A coiled-coil domain (human TSG101 aa 231-302) and a proline-rich domain (human TSG101 aa 130-205, 32% proline) typical of the activation domains of transcription factors are highly conserved between the human and mouse proteins, with only one amino acid mismatch in each of the two domains. The leucine zipper motif in the coiled-coil domain of the human TSG101 protein is identical to the one in the mouse protein.

DNA from a tumor that is suspected of being associated with TSG101 is analyzed for the presence of an oncogenic mutation in the TSG101 gene. Sporadic tumors associated with loss of TSG101 function include a number of carcinomas known to have deletions in the region of human chromosome 11p15, e.g. carcinomas of the breast, lung cancer, testicular cancer and male germ cell tumor, stomach cancer, Wilms’ tumor, ovarian cancer, bladder cancer, myeloid leukemia, malignant astrocytomas and other primitive neuroectodermal tumors, and infantile tumors of adrenal and liver.

Characterization of sporadic tumors will generally require analysis of tumor cell DNA, conveniently with a biopsy sample. Where metastasis has occurred, tumor cells may be detected in the blood. Of particular interest is the detection of deletions in the TSG101 gene, e.g. by amplification of the region and size fractionation of the amplification product; restriction mapping, etc. Screening
of tumors may also be based on the functional or antigenic characteristics of the protein. Immunoassays designed to detect the normal or abnormal TSG101 protein may be used in screening. Alternatively, functional assays, e.g. assays based on detecting changes in the stathmin pathway mediated by TSG101, may be performed.

A wide range of mutations are found, up to and including deletion of the entire short arm of chromosome 11. Specific mutations of interest include any mutation that leads to oncogenesis, including insertions, substitutions and deletions in the coding region sequence, introns that affect splicing, promoter or enhancer that affect the activity and expression of the protein. A "normal" sequence of TSG101 is provided in SEQ ID NO:3 (human). In many cases, mutations disrupt the coiled coil domain, resulting in a protein that is truncated or has a deletion in this region. Other mutations of interest may affect the proline rich domain, or other conserved regions of the protein. The leucine zipper within the coiled coil domain is also of particular interest. Biochemical studies may be performed to confirm whether a candidate sequence variation in the TSG101 coding region or control regions is oncogenic. For example, oncogenicity activity of the mutated TSG101 protein may be determined by its ability to complement a loss of TSG101 activity in 3T3 cells, by binding studies with stathmin, etc.

The TSG101 gene may also be used for screening of patients suspected of having a genetic predisposition to TSG101-associated tumors, where the presence of a mutated TSG101 sequence confers an increased susceptibility to cancer. Diagnosis is performed by protein, DNA sequence, PCR screening, or hybridization analysis of any convenient sample from a patient, e.g. biopsy material, blood sample, scrapings from cheek, etc. A typical patient genotype will have an oncogenic mutation on one chromosome. When the
normal copy of TSG101 is lost, leaving only the reduced function mutant copy, abnormal cell growth is the result.

Prenatal diagnosis may be performed, particularly where there is a family history of the disease, e.g. an affected parent or sibling. A sample of fetal DNA, such as an amniocentesis sample, fetal nucleated or white blood cells isolated from maternal blood, chorionic villus sample, etc. is analyzed for the presence of the predisposing mutation. Alternatively, a protein based assay, e.g. functional assay or immunoassay, is performed on fetal cells known to express TSG101.

The DNA sequence encoding TSG101 may be cDNA or genomic DNA or a fragment thereof. The term "TSG101 gene" shall be intended to mean the open reading frame encoding specific TSG101 polypeptides, as well as adjacent 5' and 3' non-coding nucleotide sequences involved in the regulation of expression, up to about 1 kb beyond the coding region, in either direction. The gene may be introduced into an appropriate vector for extrachromosomal maintenance or for integration into the host.

The term "cDNA" as used herein is intended to include all nucleic acids that share the arrangement of sequence elements found in native mature mRNA species, where sequence elements are exons, 3' and 5' non-coding regions. Normally mRNA species have contiguous exons, with the intervening introns deleted, to create a continuous open reading frame encoding TSG101.

The genomic TSG101 sequence has non-contiguous open reading frames, where introns interrupt the coding regions. A genomic sequence of interest comprises the nucleic acid present between the initiation codon and the stop codon, as defined in the listed sequences, including all of the introns that are normally present in a native chromosome. It may further include the 3' and 5' untranslated regions found in the mature mRNA. It may further include specific
transcriptional and translational regulatory sequences, such as promoters, enhancers, etc., including about 1 kb of flanking genomic DNA at either the 5' or 3' end of the coding region. The genomic DNA may be isolated as a fragment of 50 kbp or smaller; and substantially free of flanking chromosomal sequence.

The nucleic acid compositions of the subject invention encode all or a part of the subject polypeptides. Fragments may be obtained of the DNA sequence by chemically synthesizing oligonucleotides in accordance with conventional methods, by restriction enzyme digestion, by PCR amplification, etc. For the most part, DNA fragments will be of at least 15 nt, usually at least 18 nt, more usually at least about 50 nt. Such small DNA fragments are useful for hybridization screening, etc. Larger DNA fragments, i.e. greater than 100 bp, usually greater than 500 bp, are useful for production of the encoded polypeptide. Single stranded oligonucleotides of from about 18 to 35 nt in length are useful for PCR amplifications. For use in amplification reactions, such as PCR, a pair of primers will be used. The exact composition of the primer sequences is not critical to the invention, but for most applications the primers will hybridize to the subject sequence under stringent conditions, as known in the art. It is preferable to chose a pair of primers that will generate an amplification product of at least about 50 nt, preferably at least about 100 nt. Algorithms for the selection of primer sequences are generally known, and are available in commercial software packages. Amplification primers hybridize to complementary strands of DNA, and will prime towards each other.

The TSG101 genes are isolated and obtained in substantial purity, generally as other than an intact mammalian chromosome. Usually, the DNA will be obtained
substantially free of other nucleic acid sequences that do not include a TSG101 sequence or fragment thereof, generally being at least about 50%, usually at least about 90% pure and are typically "recombinant", i.e. flanked by one or more nucleotides with which it is not normally associated on a naturally occurring chromosome.

The DNA sequences are used in a variety of ways. They may be used as probes for identifying other tsg101 genes. Mammalian homologs have substantial sequence similarity to the subject sequences, i.e. at least 75%, usually at least 90%, more usually at least 95% sequence identity with the nucleotide sequence of the subject DNA sequence. Sequence similarity is calculated based on a reference sequence, which may be a subset of a larger sequence, such as a conserved motif, coding region, flanking region, etc. A reference sequence will usually be at least about 18 nt long, more usually at least about 30 nt long, and may extend to the complete sequence that is being compared. Algorithms for sequence analysis are known in the art, such as BLAST, described in Altschul et al. (1990) J Mol Biol 215:403-10.

Nucleic acids having sequence similarity are detected by hybridization under low stringency conditions, for example, at 50°C and 10XSSC (0.9 M saline/0.09 M sodium citrate) and remain bound when subjected to washing at 55°C in 1XSSC. By using probes, particularly labeled probes of DNA sequences, one can isolate homologous or related genes. The source of homologous genes may be any mammalian species, e.g. primate species; murines, such as rats and mice; canines; felines; bovines; ovines; equines; etc.

The DNA may also be used to identify expression of the gene in a biological specimen. The manner in which one probes cells for the presence of particular nucleotide sequences, as genomic DNA or RNA, is well-established in the literature and does not require elaboration here.
Conveniently, a biological specimen is used as a source of mRNA. The mRNA may be amplified by RT-PCR, using reverse transcriptase to form a complementary DNA strand, followed by polymerase chain reaction amplification using primers specific for the subject DNA sequences. Alternatively, the mRNA sample is separated by gel electrophoresis, transferred to a suitable support, e.g. nitrocellulose, and then probed with a fragment of the subject DNA as a probe. Other techniques may also find use. Detection of mRNA having the subject sequence is indicative of TSG101 gene expression in the sample.

The subject nucleic acid sequences may be modified for a number of purposes, particularly where they will be used intracellularly, for example, by being joined to a nucleic acid cleaving agent, e.g. a chelated metal ion, such as iron or chromium for cleavage of the gene; as an antisense sequence; or the like. Modifications may include replacing oxygen of the phosphate esters with sulfur or nitrogen, replacing the phosphate with phosphoramidine, etc.

A number of methods are available for analyzing genomic DNA sequences for the presence of mutations. Where large amounts of DNA are available, the genomic DNA is used directly. Alternatively, the region of interest is cloned into a suitable vector and grown in sufficient quantity for analysis, or amplified by conventional techniques, such as the polymerase chain reaction (PCR). The use of the polymerase chain reaction is described in Saiki et al. (1985) Science 239:487, and a review of current techniques may be found in Sambrook, et al. Molecular Cloning: A Laboratory Manual, CSH Press 1989, pp.14.2-14.33.

PCR is particularly useful for detection of oncogenic mutations. In many cases such mutations involve a deletion at the TSG101 locus. For example, primers specific for TSG101 are used to amplify all or part of the gene. The amplification products are then analyzed for
size, where a deletion will result in a smaller than expected product. Where the deletion is very large, there may be a complete absence of the specific amplification product. Alternatively, analysis may be performed on mRNA from a cell sample, where the RNA is converted to cDNA, and then amplified (RT-PCR).

A detectable label may be included in the amplification reaction. Suitable labels include fluorochromes, e.g. fluorescein isothiocyanate (FITC), rhodamine, Texas Red, phycoerythrin, allophycocyanin, 6-carboxyfluorescein (6-FAM), 2',7'-dimethoxy-4',5'-dichloro-6-carboxyfluorescein (JOE), 6-carboxy-X-rhodamine (ROX), 6-carboxy-2',4',7',4,7-hexachlorofluorescein (HEX), 5-carboxyfluorescein (S-FAM) or N,N,N',N'-tetramethyl-6-carboxyrhodamine (TAMRA), radioactive labels, e.g. $^{32}$P, $^{35}$S, $^3$H; etc. The label may be a two stage system, where the amplified DNA is conjugated to biotin, haptens, etc. having a high affinity binding partner, e.g. avidin, specific antibodies, etc., where the binding partner is conjugated to a detectable label. The label may be conjugated to one or both of the primers. Alternatively, the pool of nucleotides used in the amplification is labeled, so as to incorporate the label into the amplification product.

The amplified or cloned fragment may be sequenced by dideoxy or other methods, and the sequence of bases compared to the normal TSG101 sequence. Hybridization with the variant, oncogenic sequence may also be used to determine its presence, by Southern blots, dot blots, etc. Single strand conformational polymorphism (SSCP) analysis, denaturing gradient gel electrophoresis (DGGE), and heteroduplex analysis in gel matrices are used to detect conformational changes created by DNA sequence variation as alterations in electrophoretic mobility. The hybridization pattern of a control and variant sequence to an array of oligonucleotide probes immobilised on a solid support, as
described in WO 95/11995, may also be used as a means of detecting the presence of variant sequences. Alternatively, where an oncogenic mutation creates or destroys a recognition site for a restriction endonuclease, the fragment is digested with that endonuclease, and the products size fractionated to determine whether the fragment was digested. Fractionation is performed by gel electrophoresis, particularly acrylamide or agarose gels.

The subject nucleic acids can be used to generate transgenic animals or site specific gene modifications in cell lines. The modified cells or animals are useful in the study of TSG101 function and regulation. For example, a series of small deletions and/or substitutions may be made in the TSG101 gene to determine the role of different exons in oncogenesis, signal transduction, etc. One may also provide for expression of the TSG101 gene or variants thereof in cells or tissues where it is not normally expressed or at abnormal times of development. In addition, by providing expression of TSG101 protein in cells in which it is otherwise not normally produced, one can induce changes in cell behavior.

DNA constructs for homologous recombination will comprise at least a portion of the TSG101 gene with the desired genetic modification, and will include regions of homology to the target locus. Alternatively, constructs may that do not target to the native locus, but integrate at random sites in the genome. Conveniently, markers for positive and negative selection are included. Methods for generating cells having targeted gene modifications through recombination are known in the art. For various techniques for transfecting mammalian cells, see Keown et al. (1990) Methods in Enzymology 185:527-537.

For embryonic stem (ES) cells, an ES cell line may be employed, or ES cells may be obtained freshly from a host, e.g. mouse, rat, guinea pig, etc. Such cells are
grown on an appropriate fibroblast-feeder layer or grown in the presence of leukemia inhibiting factor (LIF). When ES cells have been transformed, they may be used to produce transgenic animals. After transformation, the cells are plated onto a feeder layer in an appropriate medium. Cells containing the construct may be detected by employing a selective medium. After sufficient time for colonies to grow, they are picked and analyzed for the occurrence of homologous recombination. Those colonies that show homologous recombination may then be used for embryo manipulation and blastocyst injection. Blastocysts are obtained from 4 to 6 week old superovulated females. The ES cells are trypsinized, and the modified cells are injected into the blastocoel of the blastocyst. After injection, the blastocysts are returned to each uterine horn of pseudopregnant females. Females are then allowed to go to term and the resulting litters screened for mutant cells having the construct. By providing for a different phenotype of the blastocyst and the ES cells, chimeric progeny can be readily detected.

The chimeric animals are screened for the presence of the modified gene and males and females having the modification are mated to produce homozygous progeny. If the gene alterations cause lethality at some point in development, tissues or organs can be maintained as allogeneic or congenic grafts or transplants, or in in vitro culture. The transgenic animals may be any non-human mammal, such as laboratory animals, domestic animals, etc. The transgenic animals may be used in functional studies, drug screening, etc., e.g. to determine the effect of a candidate drug on tumor cells.

The subject gene may be employed for producing all or portions of the TSG101 protein. Peptides of interest include the coiled-coil domain (aa 231-302) and the proline-rich domain (aa 130-205). For expression, an
expression cassette may be employed, providing for a transcriptional and translational initiation region, which may be inducible or constitutive, the coding region under the transcriptional control of the transcriptional initiation region, and a transcriptional and translational termination region. Various transcriptional initiation regions may be employed which are functional in the expression host.

The peptide may be expressed in prokaryotes or eukaryotes in accordance with conventional ways, depending upon the purpose for expression. For large scale production of the protein, a unicellular organism or cells of a higher organism, e.g. eukaryotes such as vertebrates, particularly mammals, may be used as the expression host, such as E. coli, B. subtilis, S. cerevisiae, and the like. In many situations, it may be desirable to express the TSG101 gene in a mammalian host, whereby the TSG101 protein will be glycosylated.

With the availability of the protein in large amounts by employing an expression host, the protein may be isolated and purified in accordance with conventional ways. A lysate may be prepared of the expression host and the lysate purified using HPLC, exclusion chromatography, gel electrophoresis, affinity chromatography, or other purification technique. The purified protein will generally be at least about 80% pure, preferably at least about 90% pure, and may be up to and including 100% pure. By pure is intended free of other proteins, as well as cellular debris.

TSG101 polypeptides are useful in the investigation of the stathmin signaling pathway, which is involved in the regulation and relay of diverse signals associated with cell growth and differentiation. The coiled coil domain of TSG101 interacts with stathmin. The structure of TSG101 indicates that it is a transcription factor, which may act
as a downstream effector of stathmin signaling. The normal and mutated forms of TSG101 polypeptides may be used for binding assays with other proteins, to detect changes in phosphorylation, etc. that may affect this pathway. Yeast has been shown to be a powerful tool for studying protein-protein interactions through the two hybrid system described in Chien et al. (1991) P.N.A.S. 88:9578-9582.

Binding assays of TSG101 to DNA may be performed in accordance with conventional techniques for DNA footprinting, to determine the sequence motifs that are recognized by TSG101. In vitro transcription assays may be used, to determine how complexes comprising polymerase and transcriptional activation factors are affected by the presence of TSG101.

The polypeptide is used for the production of antibodies, where short fragments provide for antibodies specific for the particular polypeptide, whereas larger fragments or the entire gene allow for the production of antibodies over the surface of the polypeptide or protein. Antibodies may be raised to the normal or mutated forms of TSG101. The coiled coil, leucine zipper and proline rich domains of the protein are of interest as epitopes, particularly to raise antibodies that recognize common changes found in oncogenic TSG101. Antibodies may be raised to isolated peptides corresponding to these domains, or to the native protein. Antibodies that recognize TSG101 are useful in diagnosis, typing and staging of human tumors, e.g. breast carcinomas.

Antibodies are prepared in accordance with conventional ways, where the expressed polypeptide or protein may be used as an immunogen, by itself or conjugated to known immunogenic carriers, e.g. KLH, pre-S HBsAg, other viral or eukaryotic proteins, or the like. Various adjuvants may be employed, with a series of injections, as appropriate. For monoclonal antibodies,
after one or more booster injections, the spleen may be isolated, the splenocytes immortalized, and then screened for high affinity antibody binding. The immortalized cells, e.g. hybridomas, producing the desired antibodies may then be expanded. For further description, see Monoclonal Antibodies: A Laboratory Manual, Harlow and Lane eds., Cold Spring Harbor Laboratories, Cold Spring Harbor, New York, 1988. If desired, the mRNA encoding the heavy and light chains may be isolated and mutagenized by cloning in E. coli, and the heavy and light chains may be mixed to further enhance the affinity of the antibody.

The antibodies find particular use in diagnostic assays for carcinomas and other tumors associated with mutations in TSG101. Staging, detection and typing of tumors may utilize a quantitative immunoassay for the presence or absence of normal TSG101. Alternatively, the presence of mutated forms of TSG101 may be determined. A reduction in normal TSG101 and/or presence of abnormal TSG101 is indicative that the tumor is TSG101-associated.

A sample is taken from a patient suspected of having a TSG101-associated tumor. Samples, as used herein, include biological fluids such as blood, cerebrospinal fluid, tears, saliva, lymph, dialysis fluid and the like; organ or tissue culture derived fluids; and fluids extracted from physiological tissues. Also included in the term are derivatives and fractions of such fluids. Biopsy samples are of particular interest, e.g. carcinoma samples, organ tissue fragments, etc. Where metastasis is suspected, blood samples may be preferred. The number of cells in a sample will generally be at least about $10^3$, usually at least $10^4$ more usually at least about $10^5$. Usually a lysate of the cells is prepared.

Diagnosis may be performed by a number of methods. The different methods all determine the absence or presence of normal or abnormal TSG101 in patient cells suspected of
having a mutation in TSG101. For example, detection may utilize staining of histological sections, performed in accordance with conventional methods. The antibodies of interest are added to the cell sample, and incubated for a period of time sufficient to allow binding to the epitope, usually at least about 10 minutes. The antibody may be labeled with radioisotopes, enzymes, fluorescers, chemiluminescers, or other labels for direct detection. Alternatively, a second stage antibody or reagent is used to amplify the signal. Such reagents are well-known in the art. For example, the primary antibody may be conjugated to biotin, with horseradish peroxidase-conjugated avidin added as a second stage reagent. Final detection uses a substrate that undergoes a color change in the presence of the peroxidase. The absence or presence of antibody binding may be determined by various methods, including microscopy, spectrophotometry, scintillation counting, etc.

An alternative method for diagnosis depends on the in vitro detection of binding between antibodies and TSG101 in a lysate. Measuring the concentration of TSG101 binding in a sample or fraction thereof may be accomplished by a variety of specific assays. A conventional sandwich type assay may be used. For example, a sandwich assay may first attach TSG101-specific antibodies to an insoluble surface or support. The particular manner of binding is not crucial so long as it is compatible with the reagents and overall methods of the invention. They may be bound to the plates covalently or non-covalently, preferably non-covalently.

The insoluble supports may be any compositions to which polypeptides can be bound, which is readily separated from soluble material, and which is otherwise compatible with the overall method. The surface of such supports may be solid or porous and of any convenient shape. Examples of suitable insoluble supports to which the receptor is
bound include beads, e.g. magnetic beads, membranes and microtiter plates. These are typically made of glass, plastic (e.g. polystyrene), polysaccharides, nylon or nitrocellulose. Microtiter plates are especially convenient because a large number of assays can be carried out simultaneously, using small amounts of reagents and samples.

Patient sample lysates are then added to separately assayable supports (for example, separate wells of a microtiter plate) containing antibodies. Preferably, a series of standards, containing known concentrations of normal and/or abnormal TSG101 is assayed in parallel with the samples or aliquots thereof to serve as controls. Preferably, each sample and standard will be added to multiple wells so that mean values can be obtained for each. The incubation time should be sufficient for binding, generally, from about 0.1 to 3 hr is sufficient. After incubation, the insoluble support is generally washed of non-bound components. Generally, a dilute non-ionic detergent medium at an appropriate pH, generally 7-8, is used as a wash medium. From one to six washes may be employed, with sufficient volume to thoroughly wash non-specifically bound proteins present in the sample.

After washing, a solution containing a second antibody is applied. The antibody will bind TSG101 with sufficient specificity such that it can be distinguished from other components present. The second antibodies may be labeled to facilitate direct, or indirect quantification of binding. Examples of labels that permit direct measurement of second receptor binding include radiolabels, such as $^3$H or $^{125}$I, fluorescers, dyes, beads, chemiluminescers, colloidal particles, and the like. Examples of labels which permit indirect measurement of binding include enzymes where the substrate may provide for a colored or fluorescent product. In a preferred
embodiment, the antibodies are labeled with a covalently bound enzyme capable of providing a detectable product signal after addition of suitable substrate. Examples of suitable enzymes for use in conjugates include horseradish peroxidase, alkaline phosphatase, malate dehydrogenase and the like. Where not commercially available, such antibody-enzyme conjugates are readily produced by techniques known to those skilled in the art. The incubation time should be sufficient for the labeled ligand to bind available molecules. Generally, from about 0.1 to 3 hr is sufficient, usually 1 hr sufficing.

After the second binding step, the insoluble support is again washed free of non-specifically bound material. The signal produced by the bound conjugate is detected by conventional means. Where an enzyme conjugate is used, an appropriate enzyme substrate is provided so a detectable product is formed.

Other immunoassays are known in the art and may find use as diagnostics. Ouchterlony plates provide a simple determination of antibody binding. Western blots may be performed on protein gels or protein spots on filters, using a detection system specific for TSG101 as desired, conveniently using a labeling method as described for the sandwich assay.

By providing for the production of large amounts of TSG101 protein, one can identify ligands or substrates that bind to, modulate or mimic the action of TSG101. Areas of investigation include the development of cancer treatments. Drug screening identifies agents that provide a replacement for TSG101 function in abnormal cells. The role of TSG101 as a tumor suppressor indicates that agents which mimic its function will inhibit the process of oncogenesis. Of particular interest are screening assays for agents that have a low toxicity for human cells. A wide variety of assays may be used for this purpose, including labeled in
vitro protein-protein binding assays, electrophoretic mobility shift assays, immunoassays for protein binding, and the like. The purified protein may also be used for determination of three-dimensional crystal structure, which can be used for modeling intermolecular interactions, transcriptional regulation function, etc.

The term "agent" as used herein describes any molecule, protein, or pharmaceutical with the capability of altering or mimicking the physiological function of TSG101. Generally a plurality of assay mixtures are run in parallel with different agent concentrations to obtain a differential response to the various concentrations. Typically, one of these concentrations serves as a negative control, i.e. at zero concentration or below the level of detection.

Candidate agents encompass numerous chemical classes, though typically they are organic molecules, preferably small organic compounds having a molecular weight of more than 50 and less than about 2,500 daltons. Candidate agents comprise functional groups necessary for structural interaction with proteins, particularly hydrogen bonding, and typically include at least an amine, carbonyl, hydroxyl or carboxyl group, preferably at least two of the functional chemical groups. The candidate agents often comprise cyclical carbon or heterocyclic structures and/or aromatic or polyaromatic structures substituted with one or more of the above functional groups. Candidate agents are also found among biomolecules including peptides, saccharides, fatty acids, steroids, purines, pyrimidines, derivatives, structural analogs or combinations thereof.

Candidate agents are obtained from a wide variety of sources including libraries of synthetic or natural compounds. For example, numerous means are available for random and directed synthesis of a wide variety of organic compounds and biomolecules, including expression of
randomized oligonucleotides and oligopeptides. Alternatively, libraries of natural compounds in the form of bacterial, fungal, plant and animal extracts are available or readily produced. Additionally, natural or synthetically produced libraries and compounds are readily modified through conventional chemical, physical and biochemical means, and may be used to produce combinatorial libraries. Known pharmacological agents may be subjected to directed or random chemical modifications, such as acylation, alkylation, esterification, amidification, etc. to produce structural analogs.

Where the screening assay is a binding assay, one or more of the molecules may be joined to a label, where the label can directly or indirectly provide a detectable signal. Various labels include radioisotopes, fluorescers, chemiluminescers, enzymes, specific binding molecules, particles, e.g. magnetic particles, and the like. Specific binding molecules include pairs, such as biotin and streptavidin, digoxin and antidigoxin etc. For the specific binding members, the complementary member would normally be labeled with a molecule that provides for detection, in accordance with known procedures.

A variety of other reagents may be included in the screening assay. These include reagents like salts, neutral proteins, e.g. albumin, detergents, etc that are used to facilitate optimal protein-protein binding and/or reduce non-specific or background interactions. Reagents that improve the efficiency of the assay, such as protease inhibitors, nuclease inhibitors, anti-microbial agents, etc. may be used. The mixture of components are added in any order that provides for the requisite binding. Incubations are performed at any suitable temperature, typically between 4 and 40°C. Incubation periods are selected for optimum activity, but may also be optimized to
facilitate rapid high-throughput screening. Typically between 0.1 and 1 hours will be sufficient.

Other assays of interest detect agents that mimic TSG101 function. For example, candidate agents are added to a cell that lacks functional TSG101, and screened for the ability to reproduce TSG101 function, e.g. prevent growth of 3T3 cells in soft agar.

The compounds having the desired pharmacological activity may be administered in a physiologically acceptable carrier to a host for treatment of cancer attributable to a defect in tsg101 function. The inhibitory agents may be administered in a variety of ways, orally, topically, parenterally e.g. subcutaneously, intraperitoneally, intravascularly, etc. Topical treatments are of particular interest. Depending upon the manner of introduction, the compounds may be formulated in a variety of ways. The concentration of therapeutically active compound in the formulation may vary from about 0.1-100 wt.%.

The pharmaceutical compositions can be prepared in various forms, such as granules, tablets, pills, suppositories, capsules, suspensions, salves, lotions and the like. Pharmaceutical grade organic or inorganic carriers and/or diluents suitable for oral and topical use can be used to make up compositions containing the therapeutically-active compounds. Diluents known to the art include aqueous media, vegetable and animal oils and fats. Stabilizing agents, wetting and emulsifying agents, salts for varying the osmotic pressure or buffers for securing an adequate pH value, and skin penetration enhancers can be used as auxiliary agents.

The gene may also be used for gene therapy. Vectors useful for introduction of the gene include plasmids and viral vectors. Of particular interest are retroviral-based vectors, e.g. moloney murine leukemia virus and modified
human immunodeficiency virus; adenovirus vectors, etc. Gene therapy may be used to treat cancerous lesions, an affected fetus, etc., by transfection of the normal gene into suitable cells. A wide variety of viral vectors can be employed for transfection and stable integration of the gene into the genome of the cells. Alternatively, micro-injection may be employed, fusion, or the like for introduction of genes into a host cell. See, for example, Dhawan et al. (1991) Science 254:1509-1512 and Smith et al. (1990) Molecular and Cellular Biology 3268-3271.

Concurrent Disruption of Genes

The subject invention also provides a method for identifying a gene at a random chromosomal locus of a mammalian cell on the basis of a phenotype produced by homozygous inactivation of gene function. The method includes the concurrent inactivation of other copies of the genes or alleles of the gene that are substantially similar in their DNA sequence, followed by a determination of whether inactivation of the gene and its alleles has resulted in a cell phenotype distinguishable from the wild-type phenotype. Additionally, the chromosomal locus containing the inactivated gene may be identified.

One copy of the gene is inactivated by the integration of a DNA construct at a random or unselected chromosomal locus, or one that is selected for its proximity to an expressed gene. The construct integrated at the random chromosomal locus contains a transcription initiation region sequence responsive to a transactivation factor. Transcription occurs in the opposite direction to a coding region for a promoterless positive selection marker gene. Hereinafter, the transactivation factor responsive transcriptional initiation region will be referred to as the "TF promoter." The positive selection marker gene carried by the construct is expressed only when
the construct is integrated 3' in relation to an endogenous gene promoter, with the endogenous promoter directing transcription toward the positive selection marker gene. Expression of the positive selection marker gene may also require that it be in the correct reading frame to express an active positive selection marker, either fused or non-fused to the expression product of a portion of the endogenous gene at the locus of integration.

Additionally, either the TF promoter is activated by a factor which can be added to the medium or by a transactivation factor encoded and expressed by a second construct, which second construct is introduced into the host. This factor serves to activate antisense RNA transcription from the TF promoter. Antisense RNA transcription from the TF promoter extends in the 3' direction relative to the orientation of the TF promoter, and into the flanking chromosomal locus 5' to the insertion of the marker gene. Binding or hybridization of the antisense RNA transcription product initiated from the TF promoter inhibits expression of the other alleles which have substantially similar sequence to the chromosomal DNA sequence transcribed from the TF promoter, and sequences complementary to the antisense RNA. (By "similar sequence" is intended sufficient similarity to bind to a messenger RNA sequence to provide an observable change in the function of the messenger RNA sequence). In this way, the production of all of the same or allelic proteins, including enzyme isoforms, may be prevented.

By first introducing the construct with the promoterless marker gene, one can select for cells in which the marker gene is being expressed, so that there is a high likelihood that the construct is positioned in an actively transcribed gene. By expanding these cells, one may then use these cells for introduction of the expression construct containing the transactivation factor gene. This
expression construct will have its own marker gene, so that one can select for those cells which have the transactivation factor expression construct. The resulting cells should, for the most part, be cells which transcribe a transcription product antisense to the construct encoded by the gene containing the inserted construct, which results in inhibition of expression of proteins by all alleles of the locus at which the promoterless marker gene integrated.

The cells which have been selected for the markers associated with the two constructs may now be screened to determine whether gene inactivation has resulted in a specifically desired cell phenotype distinguishable from the wild-type phenotype, or alternatively selection of cells expressing the desired phenotype can be carried out. Additionally, the chromosomal locus flanking the 5' end of the integrated construct may be identified.

As a further check on whether the observed change in phenotype is as a result of the transcription of an antisense strand, the transcription of the antisense strand can be reversed by introducing a third construct into cells with a modified phenotype. This construct comprises an expression construct expressing a recombinase gene sequence that would serve to excise the transactivation gene or the TF promoter, where flanking consensus sequences recognized by the recombinase gene are present in the transactivation and/or TF promoter constructs, resulting in the excision of these elements, and such other portions of the construct as are deemed appropriate. Alternatively, turn off of the antisense promoter can be accomplished by using a promoter that is regulated by hormones, chemical agents, temperature, or other agents.

The method includes the preparation and introduction of a gene search construct that includes a TF promoter and a construct that expresses a transactivator protein,
normally the introduction being sequential, into a mammalian cell. (By "gene search construct" is intended a promoterless reporter gene and 5' sequences that may generate fusion transcripts originating in DNA 5' to the reporter gene, so that the fusion construct will include any portion of the coding region of the endogenous gene between the endogenous promoter and the 5' sequence of the construct, the 5' sequence of the construct and also all or functional part of the reporter gene. By "transactivator construct" is intended a DNA molecule which comprises a transcriptional initiation region, a translational initiation sequence, a coding sequence encoding a protein that activates the TF promoter, and a translational and transcriptional termination region. All of the regions and sequences will be functional in the host of interest). Preferably, a first construct ("gene search construct") comprising a promoterless marker gene and a transactivation factor responsive promoter ("TF promoter"), directing transcription in the opposite direction of the coding region of the promoterless marker gene, is introduced first into the cells and the cells expanded and selected for cells which express the promoterless marker gene. At this time, the TF promoter is inactive and will not interfere with the transcription of the promoterless marker gene.

A second construct may also be introduced which expresses a marker gene and a transactivation factor which acts on the TF promoter to activate the promoter, resulting in transcription of an antisense strand. As transcription of the antisense strand extends into the chromosomal DNA flanking the gene search construct, it serves to inactivate other genes having substantially similar sequences to the antisense strand of flanking DNA. Those cells having the gene search construct, with the first construct at a locus which provides for expression of the promoterless marker
gene, will under conditions of transactivation be screened or selected for change in phenotype.

Rather than activate the TF promoter with a transactivation factor from expression of the expression construct, one may employ a TF promoter which is responsive to agents, e.g., compounds or other stimuli, which may be added to the medium or provided as a change in environment, e.g. heat. There are many promoters which have responsive elements, e.g., tetracycline or hormonal, such as steroid, responsive elements, where compounds can be added to the medium which will turn on the TF promoter, e.g., tetracycline derivatives or hormones, such as glucocorticoids.

To further establish whether the change in phenotype is as a result of the production of an antisense sequence, one may provide for reversal of the transcription of the antisense sequence, by providing at least one of the constructs with sequences that result in excision of the DNA region between the excision sequences. One introduces a third construct, a recombinase expression construct, into the host comprising a marker gene and a gene encoding a recombinase which acts on the excision sequences for excision. The cells may then be screened for reversal of the phenotype, indicating that the phenotype was the result of the production of the antisense sequence. Alternatively, expression of the antisense promoter or of a transactivator protein that turns on this promoter can be regulated using one or more of the agents indicated above and below.

The transcriptional initiation region of the TF promoter or of the transactivator gene employed in this invention may be varied widely, as required by the particular application. The transcriptional initiation region of the TF promoter or transactivator gene may be constitutive or inducible, as appropriate, may include
enhancers, repressors, or other regulatory sequences, which may be regulated in cis or trans. Regulation may also be as a result of additives to the media, e.g., tetracycline or hormones, e.g., glucocorticoids. The initiation region may be from any source, where the initiation region is functional in the host. Thus, the initiation region may be from structural genes of the host, from viral genes functional in the host, or combinations of such promoter regions, or synthetic promoter regions, as appropriate.

The promoter region may be a single promoter region (associated with a single gene) or a combination of 5' regions associated with different genes. The promoter region will usually be chosen to provide the desired level of transcription for the particular coding region or gene. Promoters which may find use in mammalian cells include SV40 promoters, glucocorticoid inducible promoters, CMV promoters, β-actin promoters, etc. The coding region or gene will be under the transcriptional and translational regulation of the initiation region. There will also be a translational and transcriptional termination region downstream in the direction of transcription from the gene. Since, for the most part, the termination region is not important to the functioning of expression, a wide variety of termination regions may be used from a wide variety of host genes, viral genes functional in the host, or the like.

For producing the antisense RNA, a promoter is employed which will not be active in the cell, except in conjunction with a transactivation factor or other inducing agent or condition necessary to activate the promoter. (See the above discussion concerning inducible promoters). This factor will be necessary for transcription initiation and can be supplied by a second construct introduced into the host cell or by adding the appropriate agent or providing the appropriate condition(s) for activation of the TF
promoter. With the expression construct which will not be required if induction of transcription does not require a transactivator protein, one can ensure strong binding of the transactivation factor to the TF promoter, desirably using a chimeric protein which combines a DNA-binding domain and a transcription factor, which directly or indirectly binds to RNA Polymerase II. The DNA-binding domain binds to a DNA sequence which desirably is not found in mammalian cells, and therefore would not be expected to bind at endogenous mammalian promoters. The DNA-binding domain allows for potent binding of the transactivation factor to a unique DNA sequence while orienting the transcription factor close to an RNA polymerase II binding site for interaction of the transcription factor with the transcriptional machinery of the host mammalian cell.

The marker gene of the expression construct expressing the transactivator may be a single gene or a fused gene comprising two different markers which may be selected differentially. Single gene markers include neo, which can provide for G418 resistance. Combination genes may include lacZ and aminoglycoside phosphotransferase (aph) which provides G418 resistance, hygromycin resistance (hyg) and the herpes simplex thymidine kinase (TK) gene, which provides resistance to hygromycin and sensitivity to ganciclovir.

The DNA-binding domain of the transactivator protein will usually be from a host foreign to the target host, usually a unicellular microorganism, insect, plant or the like, so as to be unlikely to be recognized by DNA binding proteins in the host, while the transcriptional activation domains will be from the host or other source which provides a factor which binds to the target host RNA polymerase II. Conveniently, the DNA-binding domain is derived from a DNA-binding protein isolated from bacterial cells and the transcription activation domain is derived
from a transcription activation domain that binds to RNA polymerase II from a common genus, e.g., mammalian cells for a mammalian host.

In a preferred embodiment, the transactivation factor contains the DNA-binding domain of the lac repressor at its amino terminus and the transcription activation domain from the herpes simplex virus virion protein 16 (VP16) at its carboxyl terminus, or the like.

The first DNA construct comprising the promoterless marker gene may also be referred to as the "knockout" construct. This knockout construct includes the TF promoter which comprises the DNA sequence bound by the transactivation factor. The expression construct comprising the transactivation factor gene or second construct could be part of the first construct or be introduced first, but this would not allow cells to be selected for appropriate integration at a gene locus and then subsequent expansion, without the complication of the antisense RNA also being produced, which might interfere with expression of the marker. It is therefore desirable that the second construct be introduced after cells having integration of the first construct downstream from a promoter have been selected, unless the transactivation factor gene is inducible, so that transcription of the transactivation gene may be initiated after selection for integration of the knockout construct.

The TF promoter typically includes a region consisting of sequence repeats, two or more, usually at least about 5 and not more than 20, and in a preferred embodiment, 14, which are tightly bound by the DNA-binding domain of the transactivation factor. Additionally, the TF promoter includes a promoter sequence for binding the RNA polymerase II of the host cell to place RNA polymerase II in close relationship with the transcription initiation domain. In a preferred embodiment the promoter sequence
responsive to the transactivation factor consists of a minimal SV-40 promoter, which lacks the enhancer sequences and GC-rich sequences typically found in the SV-40 early transcription promoter, but which can still bind the RNA polymerase, and spaced sets of lac operators located upstream from the promoter.

The lac operator sequences cause strong binding of the lac repressor DNA-binding domain at the transcription initiation region. The minimal SV-40 promoter binds the RNA polymerase II, which is also bound by the transcription factor of the chimeric protein to enhance transcription from the TF promoter.

The transcription initiation region is oriented so that RNA transcription initiated from the transcription initiation region extends into the chromosomal locus flanking the knockout construct at its 5′ end to provide for the antisense transcript on the non-coding strand, so as to be complementary to the sense strand from which the mRNA is transcribed.

The knockout construct also includes a coding region sequence for a positive selection marker. The term "coding region sequence" ordinarily refers to the coding region for a polypeptide without a promoter. The coding region sequence is located so as to allow for fusion of the coding region sequence with an exon of the gene into which the gene search construct is integrated, usually upstream (5′ direction) in relationship to the direction of transcription of the TF promoter. The coding region sequence is conveniently downstream of the TF promoter, where a splice site may be employed which is positioned to remove the TF promoter when the coding region sequence is spliced to a chromosomal exon. Since the positive selection marker coding region sequence lacks a promoter and the transactivatable antisense promoter region sequence is oriented in the opposite direction, the selection marker
coding region sequence is only expressed if the knockout construct is integrated downstream from an endogenous gene promoter. Additionally, when integrated within a translated portion of the gene, the coding region needs to be in the correct reading frame to form a fused protein consisting of an active positive selection marker and the truncated polypeptide of the gene. This can be done by including 5′ to the positive selection marker a splice acceptor sequence in one or more translational reading frames.

Optionally, the knockout construct contains a splice acceptor sequence which is located usually about 20 or fewer base pairs upstream of the positive selection marker region, although it may be within or downstream from the TF promoter. The splice acceptor sequence is useful in case the knockout construct has integrated at an intron or 3′ UTR of a chromosomal gene and is employed for splicing the precursor RNA to incorporate the positive selection marker gene sequence in the mRNA. If the coding sequence is incorporated into the 5′ UTR, the coding sequence will include an initiation codon.

The knockout construct with the 3′-splice site may be organized with the TF promoter upstream or downstream from the 3′-splice site (if downstream, desirably the TF promoter sequence will lack a stop codon in phase with the coding region sequence); the 3′-splice site; and the coding region sequence.

To obtain integration one may introduce the bare DNA into the host cell. Preferably a construct will be used which enhances integration, such as an integrating virus, e.g., self-inactivating Moloney Murine Leukemia Virus, adenovirus, transposons and a transposase, etc. Alternatively integrating of DNA can be accomplished following introduction of naked DNA by electroporation.

Depending upon the particular vector employed for
integration, the integration may be more or less random, depending on whether the vector has sequence preferences. It should be noted that retroviral insertions have some preference for actively transcribed regions, so that there will be some enrichment for integration into genes with retroviral integration sequences.

Stable maintenance of the first and second constructs in the mammalian cell and integration of the knockout construct at a random chromosomal locus as described above, where the promoterless marker gene is placed under the transcriptional construct of an endogenous promoter, results in (i) expression of the positive selection marker coding region sequence, and (ii) in conjunction with transactivation factor expression or availability, binding of the factor to the transcription initiation region sequence activating antisense RNA transcription extending into the 5’ region of the gene locus in relation to the TF promoter.

One copy of the gene is inactivated by the insertion of the knockout construct in its sequence. Expression of other copies of genes similar, particularly complementary, to the antisense transcript is inhibited. The antisense RNA forms duplexes with cellular RNAs that are similar to the antisense RNA transcript. Duplex formation inhibits the function of such cellular RNAs. Such inactivation of the related genes may cause a change in the phenotype of the cells.

In order to investigate whether a change in phenotype is associated with the antisense RNA production, one provides for reversal of the production of the RNA antisense. Optionally, the first and/or second constructs may contain two site-specific recombination sites delimiting the regions associated with the production of the antisense sequence. That is, they may delimit the TF promoter and/or the transactivation gene sequence.
Preferably, these sites delimit the transactivation gene sequence or functional portion thereof, e.g., promoter or coding region. In order to provide for excision of the sequence(s) associated with the production of the antisense RNA, a third DNA construct (recombinase expression construct) containing a recombinase gene may be prepared and integrated into the host comprising the first and second constructs. A marker gene may also be provided as part of the construct, so as to select for cells into which the recombinase construct has integrated. These selected cells may be expanded and screened for change of phenotype. Alternatively, where the transactivation factor is provided extrinsic to the cells, the medium or environment may be changed to stop transcription of the antisense sequence. Another alternative is to regulate the antisense (TF) promoter by use of hormones, Tc, etc.

If regulation by removal of the transactivator is desired, the sequence between the consensus sequences for deletion comprises a negative selection marker, particularly in conjunction with the gene expressing the transactivation factor. Expression of the recombinase gene typically causes the excision of a fragment of the transactivation factor construct’s DNA which contains the gene for the transactivation factor and the gene for the negative selection marker. The cells may then be selected for lack of expression of the negative selection marker.

The recombinase is an enzyme which causes the excision of any DNA sequence that is delimited by site-specific recombination sites. These site-specific recombination sites are sequences typically between 20 to 100 base pairs, usually about 30 to 50 base pairs and are exogenous to the host cell. Site-specific recombination sites contain two recombinase recognition sequences in inverted orientation at an overlap region. The recombination sites are oriented as repeats to cause
segment excision. The recombinase may be a member of the integrase protein family which includes cre protein, int protein and FLP protein.

Expression of the recombinase in a mammalian cell causes excision of the DNA fragment which is delimited by the two site-specific recombination sites and which will desirably include the transactivation factor coding region sequence. The transactivation factor is therefore no longer expressed, and antisense RNA transcription is no longer initiated from the knockout construct. Typically, the cell phenotype, where there are two or more copies of the gene, should revert to the wild-type phenotype, where the previous change in phenotype was as a result of the production of the antisense sequence.

All the DNA constructs contain selection marker gene sequences for monitoring insertion of the constructs in mammalian cells comprising the different construct genes and for allowing for selection of such cells substantially free of other cells not comprising the construct sequences.

The positive selection marker gene is a gene sequence that allows for selection of target cells in which the subject constructs have been introduced. Positive selection marker genes include the neo gene for resistance to G418, the hygromycin resistance gene, and the like. A negative selection marker gene is typically the herpes simplex virus thymidine kinase (tk) gene, whose expression can be detected by the use of nucleoside analogs, such as acyclovir or gancyclovir, for their cytotoxic effects on cells that contain a functional tk gene.

As discussed above, the knockout construct contains a coding region sequence for a positive selection marker to select for cells expressing the positive selection marker. The positive selection marker is only expressed if it is downstream in relationship to an endogenous promoter and,
when fused to a coding region, is fused in frame with the native protein.

The transactivation factor construct contains a positive selection marker gene to select for cells expressing the positive selection marker. The transactivation construct may also include a negative selection marker gene in the DNA fragment that is excised from the construct in the presence of a recombinase. In this manner the excision of the DNA fragment containing the transactivation factor gene sequence is monitored.

Additionally, the constructs may contain other sequences required for manipulation of the constructs. For example, restriction sites are necessary for manipulating the sequences in the constructs. Other sequences which may be present include primer initiation sequences for amplifying DNA, origins for cloning, markers for cloning hosts, sequences aiding in integration into the host chromosome, and the like.

The constructs may be included in vectors for introducing the constructs into mammalian cells. When the vectors are introduced into a cell by retroviral infection, these sequences include long terminal repeats and packaging signals. When the introduced vectors are to replicate episomally in a mammalian cell, the vectors include a viral origin of replication.

According to the subject invention, both knockout and transactivator constructs ordinarily are introduced into the target mammalian cells. Particularly the mammalian cells are mouse cells, rat cells, primate cells, e.g., sequentially human cells, rabbit cells or the like. Other eukaryotic hosts may also be used, such as plant cells, insect cells, fish cells, fungal cells, and the like. The mammalian cells may be normal cells, in a differentiated or undifferentiated state, e.g., stem cells. Alternatively, the cells may be transfected with naked DNA.
Desirably, the cells are maintainable in culture and allow for the introduction of new genetic material.

The constructs may be introduced into the target cell in accordance with known ways. For example, the constructs may be introduced by retroviral infection, electroporation, fusion, polybrene, lipofection, calcium phosphate precipitated DNA, or other conventional techniques. Particularly, the knockout construct is introduced by viral infection for largely random integration of the construct in the genome. The transactivation construct is introduced into cells by any of the methods described above. After introduction of each construct into target mammalian cells, the cells are grown in a selective medium to select for cells that express the appropriate selection markers, substantially free of cells that do not express the selection markers. For example, cells receiving a knockout construct containing the neomycin coding region sequence are grown in a medium containing G418, and cells receiving a transactivation construct containing the hygromycin resistance gene sequence are grown in a medium containing hygromycin.

Stable expression of the first positive selection marker coding sequence indicates that the knockout construct has been integrated into a chromosomal locus, downstream of an endogenous promoter. Stable expression of the second positive selection marker gene sequence indicates that the transactivation construct has been stably introduced in the cells.

The cells that have received the knockout construct and stably express both positive selection markers are assayed for a cell phenotype distinguishable from the wild-type phenotype. Different types of phenotypes may include changes in growth pattern and requirements, sensitivity or resistance to infectious agents or chemical substances, changes in the ability to differentiate or nature of the
differentiation, changes in morphology, changes in response to changes in the environment, e.g., physical changes or chemical changes, changes in response to genetic modifications, and the like.

For example, the change in cell phenotype may be the change from normal cell growth to uncontrolled cell growth. The cells may be screened by any convenient assay which provides for detection of uncontrolled cell growth. One assay which may be used is a methylcellulose assay with bromodeoxyuridine (BrdU). Another assay which is effective is the use of growth in agar (0.3 to 0.5% thickening agent). A test for tumorigenicity may also be used, where the cells may be introduced into a susceptible host, e.g. immunosuppressed, and the formation of tumors determined.

Alternatively, the change in cell phenotype may be the change from a normal metabolic state to an abnormal metabolic state. In this case, cells are assayed for their metabolite requirement, such as amino acids, sugars, cofactors, or the like, for growth. Initially, about 10 different metabolites may be screened at a time to assay for utilization of the different metabolites. Once a group of metabolites has been identified that allows for cell growth, where in the absence of such metabolites the cells do not grow, the metabolites are screened individually to identify which metabolite is assimilable or essential.

Alternatively, the change in cell phenotype may be a change in the structure of the cell. In such a case, cells might be visually inspected under a light or electron microscope.

The change in cell phenotype may be a change in the differentiation program of a cell. For example, the differentiation of myoblasts to adult muscle fibers can be investigated. The differentiation of myoblasts can be induced by an appropriate change in the growth medium and can be monitored by determining the expression of specific
polypeptides, such as myosin and troponin, which are
expressed at high levels in adult muscle fibers.

The change in cell phenotype may be a change in the
commitment of a cell to a specific differentiation program.
For example, cells derived from the neural crest, if
exposed to glucocorticoids, commit to becoming adrenal
chromaffin cells. However, if the cells are exposed instead
to fibroblast growth factor or nerve growth factor, the
cells eventually become sympathetic adrenergic neuronal
cells. If the adrenergic neuronal cells are further
exposed to ciliary neurotrophic factor or to leukemia
inhibitory factor, the cells become cholinergic neuronal
cells. Cells transfected by the method of the subject
invention can therefore be exposed to either
glucocorticoids or any of the factors, and changes in the
commitment of the cells to the different differentiation
pathways can be monitored by assaying for the expression of
polypeptides associated with the various cell types.

After establishing a change in phenotype, the
chromosomal region flanking the knockout construct DNA may
be identified using PCR with the construct sequence as a
primer for unidirectional PCR, or in conjunction with a
degenerate primer, for bidirectional PCR. The sequence may
then be used to probe a cDNA or chromosomal library for the
locus, so that the region may be isolated and sequenced.
Alternatively, the region knocked out by antisense RNA may
be sequenced and, if a large enough portion is identified,
the coding region may be used in the sense direction and a
polypeptide sequence obtained. The resulting peptide may
then be used for the production of antibodies to isolate
the particular protein. Also, the peptide may be sequenced
and the peptide sequence compared with known peptide
sequences to determine any homologies with other known
polypeptides. Various techniques may be used for
identification of the gene at the locus and the protein
expressed by the gene, since the subject methodology provides for a marker at the locus, obtaining a sequence which can be used as a probe and, in some instances, for expression of a protein fragment for production of antibodies. If desired the protein may be prepared and purified for further characterization.

The subject method, in another aspect, is employed to identify the function of a gene when at least part of the sequence of the gene is known. The method includes the inactivation of both gene copies to determine a change in cell phenotype, or a loss of function, associated with the inactivation of specific alleles of the gene.

The method includes the preparation of a knockout construct including both the promoter region sequence and the positive selection marker coding region sequence. Additionally, the construct contains two homologous recombination sites delimiting the promoter region sequence and the positive selection marker sequence. These homologous recombination sites are homologous to sequences of the known gene and allow for insertion of the knockout construct sequence flanked by the two recombination sites into the known gene.

These homologous recombination sites will typically be not more that about 2 kbp, usually not more than 1 kbp. The sites will typically be not less than 0.05 kbp, usually not less that 0.1 kbp. The regions of homology between these recombination site sequences and the target sequences will typically be at least about 90%, usually greater than 95%. The regions of homology are preferably within coding regions, such as exons, of the gene.

Additionally, the knockout construct may contain the transactivation factor gene sequence, so that no other construct is required for performing the subject invention.

Using this approach, one may inhibit expression of the alleles of a gene, where only a partial sequence is
known and determine whether the expression product has an
effect on phenotype, since all of the copies of the gene
and related alleles may be inhibited from expression. In
this manner, without knowing what the gene is, one may
conveniently determine whether the function of the gene is
of interest.

The following examples are offered by way of
illustration and not by way of limitation.

**Experimental**

**Example 1**

The method described below allows for the
identification and isolation of new genes involved in the
regulation of cell growth and differentiation. Preparation
of constructs, methods for mammalian cell transformation,
assays for uncontrolled cell growth, and methods for
identifying the new gene are provided.

**Results**

Experimental Approach and Construction of Gene
Search Vectors. pLLGSV, a retroviral gene search vector
derived from self-inactivating Moloney murine leukemia
virus (MLV) (Hawley et al., *PNAS USA* (1987) 84:2406-2410;
Brenner et al., *PNAS USA* (1989) 86:5517-5521) carries the
β-geo (Friedrich and Soriano, *Genes & Develop.* (1991)
5:1513-1523) reporter gene. This reporter, a fusion of the
*E. coli lacZ* and aminoglycoside phosphotransferase (*aph* or
"neo") genes, encodes resistance to the antibiotic G418,
which was used to select and identify cells containing
virus integrated into transcriptionally active chromosomal
DNA regions behind chromosomal promoters. An adenovirus-
derived splice acceptor (Friedrich and Soriano, 1991 *supra*)
was inserted at the 5' end of β-geo to enhance the fusion
of β-geo mRNA to upstream transcripts encoded by
chromosomally-encoded exons. 5' to, and in reverse
orientation to β-geo, is a regulated promoter formed by fusion of the SV40 early T antigen minimal promoter sequence to 14 E.coli lacZ operators (Labow et al., Mol. Cell. Biol. (1990) 10:3343-3356); this promoter has no transcription activity, but can be highly activated in trans by a transactivator, Lap348 (Labow et al., 1990, supra), containing the operator-binding domain of the E. coli lacI repressor and the herpes simplex virus transactivation domain VP16. The system was designed to generate large amounts of antisense RNA, which interact not only with the sense RNA encoded by the allele with the integrated gene search vector, but also with the sense RNA encoded by other allele(s) of the same gene.

pLLGAV was first transfected into helper cells (GP+E-86) to generate infectious viruses to infect NIH3T3 cells. A population of G418 resistant NIH3T3 cells, containing the pLLGSV vector integrated at transcriptionally active sites behind chromosomal promoters throughout the 3T3 cell genome, were transfected with transactivator vector pLLTX. pLLTX encodes both the Lap348 and HyTK, a fusion of a hygromycin resistance (hyg) gene and the herpes simplex virus thymidine kinase (TK) gene (Lupton et al., Mol. Cell. Biol. (1991) 11:3374-3378). Transfectants expressing HyTK are resistant to hyg but sensitive to gancyclovir (gcv), which specifically kills cells expressing herpes TK. In contrast, in the absence of HyTK expression, cells are hyg-sensitive and gcv-resistant. Two lox sites from bacteriophage P1 flanking the transactivator and HyTK genes allow excision of the Lap348/HyTK segment from chromosomes of cells by Cre, a lox-specific recombinase (Sauer and Henderson, Nature (1989) 298:447-451) expressed from pRSV-cre introduced into hyg resistant cells by electroporation. Cells in which the Lap348/HyTK segment has been excised, and in which the
regulated promoter consequently has been turned off, are detected by their resistance to gcv.

hyg resistant NIH3T3 cells were plated in 0.5% agarose to select for transformation phenotype, i.e., to select genes whose inactivation may contribute to cellular transformation. Excision of LAP348 from transformed cells by Cre generated transactivator deleted clones. Comparing the phenotypes of the cells with transactivator present and cells with transactivator deleted, further confirms that cellular transformation results from transactivator generated antisense RNA. Cells with transactivator deleted can be used for cloning of the gene containing the gene search vector.

Isolation of Clones Showing Transformed Phenotype.

2.5 x 10^8 NIH 3T3 cells were infected with viral supernatant from a culture of a pLLGSV-transfected helper cell clone selected for its ability to produce a high titer of infectious virus. Infected cells containing chromosomally integrated pLLGSV were either selected on plates for G418 resistance or collected by fluorescence-activated cell sorting (Brenner et al., 1989, supra) for β-galactosidase activity; the cell population obtained by either method showed variable degrees of deep blue staining by X-gal. A pool of more than 5 x 10^6 clones containing retroviral integrations selected for G418 resistance was transfected with the transactivator vector pLLTX by electroporation; colonies selected for hyg resistance were pooled and plated in 0.5% agarose. Whereas no cells in a similarly-sized uninfected NIH 3T3 population formed colonies on this concentration of agarose, the pLLGSV infected population produced 20 colonies. One of these clones, SL6 was expanded into cell line, which was transfected with pRSV-cre to generate cells with deleted transactivator (SL6ΔT cells. Both SL6 and SL6ΔT cells were injected into nude mice subcutaneously, where only SL6 cells were highly
tumorigenic. Although SL6ΔT cells produced a small tumor in one mouse, neither control NIH3T3 cells nor NIH3T3 cells transfected with pLLTX alone produced any tumor. Only SL6 cells produced spontaneous metastases to the lung. Replating of SL6, SL6ΔT and control cells into 0.5% agarose showed that only SL6 cells formed large colonies. To examine the regulation of reporter gene expression by transactivator, SL6 and SL6ΔT cells were assayed for β-galactosidase activity (Table 1). When transactivator was present in SL6 cells, expression of reporter gene was almost complete by shut off, compared to background control cells; when transactivator was removed by cre-lox recombination in SL6ΔT cells, the reporter gene was highly expressed. These results indicate that transactivator generated antisense RNA can effectively inactivate gene expression.

<table>
<thead>
<tr>
<th>Table 1. Characterization of SL6</th>
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<tr>
<td>Transactivator</td>
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<tr>
<td>β-Galactosidase Activity (U/µg)</td>
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<tr>
<td>Growth in 0.5% Agarose</td>
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<tr>
<td>Tumorigenicity in Nude Mice</td>
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<tr>
<td>Spontaneous Lung Metastasis</td>
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^aMeans of triplicates.
^bThe colonies formed by SL6 without transactivator were significantly smaller than those formed by SL6 with transactivator.
^cMice were sacrificed at day 32 with lung metastases were confirmed by histology.

A genomic southern blot of SL6 cells using an 1.3 kb neo fragment probe showed a single chromosomal integration of pLLGSV; both the reporter gene and the regulated promoter were faithfully duplicated in accordance with the retroviral life cycle. Northern blotting of poly(A) RNA
isolated from SL6AT using a 550 bp fragment of 5' β-geo as a probe, showed a major transcript of 7 Kb in length, and two transcripts of 7.5 Kb and 6.5 Kb in smaller amount. Hybridization with the cloned gene confirmed that the 7 Kb and 6.5 Kb transcripts were fusion transcripts of the reporter gene and mRNA initiated at a chromosomally-located promoter external to the vector. During cDNA cloning (see below), we also isolated many alternatively spliced cDNA products, in which the splice acceptor site of the second copy of the reporter gene in the provirus had been spliced to several cryptic splice donors of the first reporter gene, and such aberrant splicing may result in multiple transcripts in Northern blots, as has been observed previously (Friedrich and Soriano, 1991, supra).

cDNA Cloning and Sequence Analysis. A biotin labeled oligodeoxyribonucleotide that corresponds to the 5' end of β-geo was used to select β-geo fusion mRNA from SL6AT cells by hybridization; the hybridized mRNAs were purified using streptavidin-coated paramagnetic particles, reverse transcribed, converted to double strand cDNA, cloned into the E. coli plasmid pAmp1, and sequenced by standard methods. The cloned 120 bp cDNA segment contained 70 bp of a novel sequence fused in frame to the splice acceptor site 5' to β-geo. A data base search using the BLAST program (Altschul et al., J. Mol. Biol. (1990) 215:403-410) showed 97% identity to a mouse partial cDNA sequence of unknown function identified by its expression during differentiation of F9 mouse embryonal carcinoma cells (Nishiguchi et al., (1994) J. Bio. Chem. 116:128-139.

A mouse NIH 3T3 cell cDNA library was screened with the 70 bp cDNA probe to obtain a full length gene. Four positive clones were isolated, and all contained a 1148 bp open translational reading frame (ORF) encoding a predicted 381 amino acid protein of 43,108 kDa. The gene defined by this sequence was designated as tumor susceptibility gene
A potential consensus sequence for initiation of translation, followed by an adenosine residue three bases upstream of a putative ATG translation start codon, was located near the 5' end of the \textit{tsg101}. A splice donor consensus sequence (AG) was observed 72 nucleotides into the cDNA sequence analyzed and four codons downstream of the ATG.

The sequence of full length \textit{tsg101} cDNA and the predicted amino acid sequence of the Tsg101 protein were used to search the non-redundant DNA and protein sequence databases of the National Center for Biotechnology Information using the BLAST program. This analysis indicated that amino acids 231 to 301 of \textit{tsg101} are identical, except for two mismatches to cc2, an \textalpha{}-helix domain encoded by a partial cDNA clone identified by its ability to express a protein that interacts with stathmin (Maucuer et al., \textit{PNAS USA} (1995) 92:3100-3104); an evolutionarily-conserved phosphoprotein implicated in the integration and relay of diverse signals regulating cell growth (Sobel, \textit{Trends Biochem. Sci.} (1991) 16:301-305). The algorithm of Stock and colleagues (Lupas et al., \textit{Science} (1991) 252:1162-1164) predicts with a probability of \textasciitilde{}99.8\% that the helical domain of Tsg101 will form a coiled-coil structure. A protein pattern search of full length Tsg101 identified a leucine zipper domain within the coiled-coil domain of Tsg101, consistent with the observed ability of the cc2 domain to interact with stathmin. Additionally, seven potential protein kinase C phosphorylation sites (aa11, 38, 85, 88, 215, 225, 357), five potential Casein kinase II phosphorylation sites (aa38, 210, 249, 265, 290), two potential N-myristoylation sites (aa55, 156), and three potential N-glycosylation sites (aa44, 150, 297) were present in Tsg101 (Bairoch and Bucher, \textit{Nucleic Acids Res.} (1994) 22:3583-9).

A protein motif search (Prints, Leeds University, UK)
showed that aa37-46 of Tsg101 resembles the helix-turn-helix signature domain of the bacteriophage λ repressor (i.e., HTHLAMBDA) (Brennan and Matthews, J. Biol. Chem. (1989) 264:1903-1906), and that aa73-83 resembles a fungal Zn-cys bi-nuclear cluster signature (FUNGALZCYS) (Pan and Coleman, PNAS USA (1990) 87:2077-2081).

Expression of tsg101 Sense and Antisense RNA Cause Transformation of Naive NIH3T3 Cells. To confirm the role of tsg101 in cell growth, we investigated the effects of overexpression of tsg101 in sense and antisense orientations in naive NIH 3T3 cells. In both instances, the tsg101 sequence was expressed in stably transfected cells under control of the cytomegalovirus (CMV) promoter. Expression of tsg101 in either the sense or antisense orientation resulted in transformation of naive NIH3T3 cells, as indicated by the ability to form colonies on 0.5% agarose. Whereas no colonies were observed in cells transfected with the vector lacking the insert or in mock transfected cells.

Experimental Procedures

Construction of Vectors. To construct the self-inactivated retroviral gene search vector pLLGSV, a 4.3 kb XhoI-XhoI fragment from pSAβ-geo (Friedrich and Soriano, Genes & Develop. (1991) 5:1513-1523), containing β-geo reporter gene and a splice acceptor sequence 5' to the reporter, was ligated into a XhoI linker site of pACYC184 plasmid (Chang and Cohen, J. Bacteriol. (1978) 134:1141-1156) that had been digested with Tth1111 and XbaI. The NheI site of pACYC was then deleted and the XhoI site 5' to the β-geo reporter gene was converted into an NheI site by linker insertion; a 1.45 kb PvuII-StuI fragment containing 14 lac operator repeats and a SV40 minimal promoter sequence from pL14CAT (Labow et al., 1990, supra) was introduced into an SpeI 5' to the splice acceptor site and
β-geo in the opposite orientation to β-geo. The polyadenylation signal of β-geo was deleted by XbaI digestion and replaced with a NheI linker. This 5.4 kb NheI-NheI fragment was then ligated in the same orientation as retroviral transcription, into a NheI site at the deleted 3' LTR of pHHAM (Hawley et al., PNAS USA (1987) 84:2406-2410) after NheI partial digestion.

The transactivator vector pLLTX was derived from pHCMVLP348 (Labow et al., Mol. Cell, Biol. (1990) 10:3343-3356). The HindIII site at the 3' end of the transactivator was first deleted and a 1952 bp SfiI fragment containing a HyTK gene expression cassette (Lupton et al., Mol. Cell. Biol. (1991) 11:3374-3378), was ligated into the HindIII site upstream of transactivator to yield pLAPHyTK. A 200 bp DNA fragment containing two directly repeated loxP sites derived from pBS30 (Sauer and Henderson, Nucleic Acids Res. (1989) 17:147-161) was introduced into a ClaI site of pLAPHyTK to give pLLTX. pBS30 was first digested with Sall and BamHI, and ligated with a HindIII linker; then the vector was digested with AatII and XhoI to generate this 200 bp fragment with two directly repeated loxP sites. This 200 bp fragment was ligated into a ClaI site of pLAPHyTK to give pLLTX.

To construct the expression vector pLLEXP I, a 1410 bp fragment [containing a human β-actin promoter, the puromycin resistance gene pac, and an SV40 poly(A) site] was first cloned into the BamHI site of pBR332 to generate pBR-β-pac. The SfiI fragment containing the HyTK gene expression cassette (Lupton et al., 1991, supra) was then inserted into a BamHI site of pBR-β-pac, after BamHI partial digestion to give pBR-β-pac-HyTK. The expression vector pLLEXP I was generated by NheI and BglII digestion of pBR-β-pac-HyTK to remove the HyTK gene and replaced by cDNA inserts.
Cell Culture and Transfection. NIH 3T3 cells (ATCC) and GP+E-86 cells (Markowitz et al., J. Virol. (1988) 62:1120-1124) were cultured in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% calf serum (3T3) or 10% newborn calf serum (GP+E-86), 100U/ml penicillin, and 100 mg/ml streptomycin. DNA transfection was carried out by electroporation (Potter et al., PNAS USA (1984) 81:7161-7165) using Cell-Porator Electroporation systems I (Life Technologies, Inc.) and Lipofectamin (Life Technologies, Inc.) according to the protocol of the manufacturer.

Retroviral Infection of Mouse Fibroblast NIH3T3 Cells. To generate infectious retrovirus, pLLGSV was linearized by treatment with ScaI and transfected into helper cell line GP+E-86 by electroporation. The transfected GP+E-86 cells were replated on day 3 and selected with 800 µg/ml G418 for 2-3 weeks. All G418 resistant clones were isolated and expanded in 24-well plates. Culture supernatant from each clone was incubated with NIH 3T3 cells in the presence of polybrene (8 µg/ml) for 8 hr, and the frequency of integration behind the chromosomal promoter was subsequently determined by X-gal staining of the infected NIH 3T3 cells. The helper cell clones giving the highest frequency of integrations behind chromosomal promoters were expanded and culture supernatant was collected for large scale infection of NIH 3T3 cells.

Isolation of Transformed Clones and Tumorigenicity Assay. Cultures of G418 resistant NIH 3T3 cells were trypsinized and transfected with HindIII linearized pLLTX DNA by electroporation. The transfected cells were selected with 500 µg/ml of hygromycin for 12-18 days. All hygromycin resistant clones were plated into 0.5% agarose (Li et al., J. Natl. Cancer Inst. (1989) 81:1406-1412), 4 to 6 weeks later, the colonies formed in 0.5% agarose were isolated and expanded to cell lines. To assay the tumorigenicity of the transfected cells, 10⁵ cells were
injected into nude mice (NIH nu/nu, female and 6 weeks of age) subcutaneously over the lateral thorax. The animals were examined twice weekly and sacrificed five weeks later. The neoplastic nature of local tumors and lung metastases were confirmed by histologic examination (Fidler, Cancer Metastasis Rev. (1986) 5:29-49).

**cDNA Cloning and Screening of cDNA Library.** A biotin labeled oligodeoxynucleotide (27 mer) that corresponds to the 5' end of the β-geo reporter gene was hybridized with polyadenylated mRNA from SL6AT cells, and captured with Streptavidin paramagnetic particles (Promega). The oligo-hybridized mRNA was eluted and reverse transcribed with a gene specific primer corresponding to a sequence located upstream of the biotin labeled oligo into first strands of cDNA. A uracil DNA glycosylase (UDG) cloning site (Booth et al., Gene (1994) 146:303-308) was incorporated into the gene specific primer to facilitate cDNA cloning. The first strand cDNA was then 3' tailed with (dG)n by terminal transferase, and converted into ds cDNA using a UDG-oligo d(c)20 primer and DNA polymerase. The ds cDNAs were cloned into the UDG-cloning vector pAMP1 (Life Technologies, Inc.) and screened for fusion to β-geo. A 70 bp cDNA segment of novel sequence fused in frame to the splice acceptor site 5' to β-geo was used as a probe to screen a mouse NIH 3T3 cDNA library (Stratagene). Positive clones were sequenced with Sequenase 2.0 (USB) for both strands.

**Southern and Northern Blot Analysis.** Genomic DNA was isolated by standard procedure. Total RNA was isolated with RNA STAT-60 (TEL-TEST), and poly(A) mRNA was isolated with PolyATtract (Promega). Both DNA and RNA blots were probed with PCR generated single-stranded DNA probes.
Example 2.

Chromosomal mapping studies assigned TSG101 to human chromosome 11 band p15, a region showing loss of heterozygosity primarily in breast cancer but also in other human malignancies, and proposed previously to contain tumor suppressor gene(s). Intragenic deletions in TSG101 were identified in six of fourteen metastatic breast cancer cell lines, and six of fourteen primary human breast carcinomas had mutations in the gene. All of these mutations directly removed all or part of the cc2 domain. These findings support the conclusion that TSG101 is a suppressor of abnormal cell growth and additionally demonstrate that this gene has an important role in human breast cancer.

Results

Cloning and Characterization of Human TSG101 cDNA. tsg101 was initially identified in mouse cells by a novel gene discovery approach that enables regulated functional inactivation of multiple copies of previously unknown genes and selection for cells that show a phenotype resulting from such inactivation. To obtain TSG101, the human homolog of mouse tsg101, the 1448 bp mouse cDNA sequence was used to query dbEST of the National Cancer for Biotechnology Information (NCBI) by the BLAST program. Ten human partial cDNA sequences (Expressed Sequences Tags, EST) included in the database showed 85% to 95% identity to mouse tsg101 cDNA. A 27 bp sequence contained within a region of 100% identity between ESTs H53754 and 230135 was used to design the UDG primers Pa-UDG and Pd-UDG; these primers plus two other UDG primers (Pb-UDG and Pc-UDG) corresponding to sequences bracketing the vector cloning site of a lgt10-based human cDNA library were used to amplify by PCR the 5’ (Pc-UDG and Pd-UDG) and 3’ (Pa-UDG and Pb-UDG) segments of human TSG101 cDNA, employing total DNA
isolated from the human cDNA library as template. The longest 5' and 3' PCR products were then joined in the UDG cloning vector pAMPl.

A 1494 bp cloned human cDNA insert was termed full length TSG101 cDNA. Sequence analysis of this cDNA identified a 1140 bp open reading frame predicted to encode a 380 amino acid protein with a molecular mass of 42.841 kDa and a pI of 5.87. The human and mouse cDNAs are 86% identical at the nucleotide level. The predicted proteins are 94% identical and are distinguished by 20 amino acid mismatches and one gap. A coiled-coil domain (human TSG101 aa 231-302) and a proline-rich domain (human TSG101 aa 130-205, 32% proline) typical of the activation domains of transcription factors are highly conserved between the human and mouse proteins, with only one amino acid mismatch in each of the two domains. The leucine zipper motif in the coiled-coil domain of the human TSG101 protein is identical to the one in the mouse protein. Other conserved features identified in human TSG101 include seven putative protein kinase C phosphorylation sites (aa 11, 38, 68, 89, 215, 225, 357), five potential case in kinase II phosphorylation sites (aa 38, 210, 249, 265, 290) and three potential N-glycosylation sites (aa 44, 150, 297). Analysis of the human TSG101 cDNA and protein sequences by the BLAST program search of NCBI database did not reveal any significant homology with the sequences for any other human genes.

Expression of TSG101 in human tissues was examined on a multiple-tissue Northern blot probed with full length tsg101 cDNA. A single 1.5 kb transcript was observed in all eight human tissues tested and was slightly more prominent in RNA isolated from liver and pancreas. The size of this transcript indicates that the 1494 bp cDNA corresponds to full length native TSG101 mRNA.
Chromosomal localization of human and mouse TSG101 genes. By using PCR primers that specifically amplify a human TSG101 sequence from the 3'-untranslated region, genomic DNA from a panel of 18 human x Chinese hamster hybrid cell lines was analyzed. The expected 210 bp PCR product was obtained only from hybrid cell lines that had retained human chromosome 11 and from total human genomic DNA, but not from hamster DNA. The human-specific PCR product was also generated from a cell line (31-2A HAT) that retained only the short arm of chromosome 11 (11p), whereas no PCR amplification was observed using the same primers in a cell line that had only the long arm of chromosome 11 (11q). By concordant segregation and by excluding all other chromosomes, the human TSG101 gene is assigned to chromosome arm 11p.

To obtain a human TSG101 genomic DNA probe suitable for mapping by fluorescence in situ hybridization (FISH), the same set of PCR primers employed for the analysis of hybrid cell lines was used to screen a PAC library containing human genomic DNA inserts. Two overlapping clones, PAC1 and PAC2, each containing ~150 kb inserts, were isolated and confirmed to contain TSG101 human genomic DNA by Southern blotting using a 5' human TSG101 cDNA fragment as probe. Fluorescence in situ hybridization of the two PAC clones to human chromosome spreads gave identical results, which confirmed the localization of TSG101 on chromosome arm 11p by our somatic cell hybrid analysis. A fluorescence signal on both chromatids of both copies of chromosome 11 was seen in 20 metaphase cells analyzed. Based on the chromosomal R-banding pattern, TSG101 is assigned to chromosome 11 bands p15.1-p15.2.

Radiation hybrid (RH) mapping provides another independent approach to map human genes and to position them relative to polymorphic markers on the linkage map. PCR typing for human TSG101 of the Stanford G3 human RH
mapping panel revealed a positive result in 11 of the 83 RH cell lines (retention frequency 13.25%). By two point linkage analysis TSG101 was found to be closely linked to Sequence Tagged Site (STS) markers D11S921, D11S899, and D11S1308. Both D11S921 and D11S1308 are on the Whitehead Institute integrated map and radiation hybrid map and their physical positions approximately correspond to 11p15.

To map tsg101 in the mouse, a mapping panel of 22 mouse x rodent hybrid cell lines was analyzed by PCR using mouse gene-specific primers. The presence or absence of mouse chromosome 7 in hybrid cell lines was in complete concordance with the 202 bp mouse tsg101 PCR product. All other mouse chromosomes were excluded by at least 3 discordant hybrids. An attempt to place the gene on the mouse linkage map by typing an interspecies backcross panel was not successful, as no difference between C57BL/6 and M. spretus patterns were detectable by single strand conformational analysis (SSCA) of PCR products. Given the known conserved syntenic regions on human chromosome 11p and mouse chromosome 7, the mapping of the mouse gene provides further evidence that the human and mouse sequences we have cloned are true TSG101 gene homologs.

Analysis of TSG101 Mutations in Human Breast Cancers. Extensive studies have shown deletion or loss of heterozygosity of markers at or near the 11p15 band in a variety of human malignancies, primarily breast cancers, but also Wilms’ tumor, and ovarian and testicular malignancies, suggesting that this region contains one or more tumor suppressor genes. Moreover, a region mapping between 11p15.4 and 11pcen was deleted in approximately 30% of 171 sporadic breast tumors analyzed. The notion that chromosome 11 contains a tumor suppressor gene specifically implicated in the pathogenesis of human breast cancer is supported by evidence that introducing a normal chromosome 11 or segments of this chromosome into breast cancer cells
reverses their metastatic potential, as well as other properties associated with oncogenesis. The finding that homozygous inactivation of tsg101 converts mouse fibroblasts into metastasizing cancer cells suggests that this gene functions as a suppressor of malignant cell growth. To investigate the role for TSG101 in human breast cancer, cDNA isolated from ten breast cancer cell lines and fourteen primary human breast tumors was examined specifically for mutations in TSG101, comparing these cDNAs with cDNA obtained from three normal fibroblast cell lines, eleven tumor cell lines derived from other types of cancers, and matched normal breast tissue from the individuals that were the source of the primary breast cancers.

RT-PCR using primers that bracketed or were within the TSG101 protein-coding region showed deletions in TSG101 transcripts in five of the ten breast cancer cell lines examined. These were localized by PCR amplification using sets of primers for different segments of the gene, and the fragments generated by these primers were cloned in the pCNTR plasmid vector for sequencing. The sequence of cloned TSG101 cDNA from the independently isolated and maintained cell lines revealed a 85 bp deletion predicted to remove 28 aa (codons 5-32) and to generate, after codon 32, a frameshift predicted to lead to premature termination of the TSG101 protein 10 codons later. Sequence analysis of RT-PCR fragments amplified from cell lines L7 and L8 showed deletions that remove most or all of the coiled coil domain. While a deletion of ~250 bp was observed in RT-PCR cDNA generated from a fifth cell line (MDA-MB-436), using primers that amplify a 490 bp TSG101 region located 5' to the coiled coil domain, the highly heterogeneous nature of this cell line complicated further analysis of transcripts. Three human fibroblast lines, three melanoma cell lines,
two Wilms' tumor cell lines, two neuroblastoma cell lines, two sarcoma cell lines, a bladder cancer cell line, and one tumor cell line from a Beckwith-Wiedemann syndrome patient showed only normal length TSG101 transcripts by RT-PCR analysis. Normal length TSG101 transcripts were also present in cultures of breast cancer cell lines that produced truncated TSG101 transcripts, and variability in the ratio of normal-length and truncated TSG101 transcripts in different experiments suggested heterogeneity in these tumor cell lines.

RT-PCR generated 1389 bp fragments amplified from each of the four breast cancer cell lines that contained specifically mapped TSG101 deletions were cloned, and several of the clones generated from each cell were analyzed by sequencing. Comparison of the sequences from these cloned cDNAs with the sequence of TSG101 cDNA from two normal human lines and from the melanoma cell lines revealed a C to T transition in codon 107 of transcript from L8, indicating the existence of two separate TSG101 alleles in this breast cancer cell line.

The sequences obtained were compared with the sequences of RT-PCR products from transcripts of normal human fibroblasts (cell lines 0 and 1) and human melanoma lines (cell line 2 and 3). A point mutation in TSG101 was identified in breast cancer cell line 8. This C to T transition results in change codon 107 from Trp to Arg. No point mutations in TSG101 were found in an initial analysis of other tumor cell lines or in the TSG101 sequence of melanoma cells or normal fibroblasts.

To search for mutation(s) in other TSG101 alleles within the cell lines containing deletions in one allele of TSG101, the cloned 1389 bp full length RT-PCR fragments from the four breast cancers carrying TSG101 deletions (cell lines 4, 6, 7, and 8) were sequenced. PCR amplification of the corresponding regions of TSG101
genomic DNA using primers derived from intron and exon sequences showed deletions of the expected size in the genomic DNA of all of the four breast cancer cell lines (L4, L6, L7 and L8) whose cDNA had been characterized by sequencing: a 322 bp genomic PCR fragment was amplified from cell line L8, a 192 bp fragment from cell line L7 and a genomic fragment containing a deletion of about 85 bp was amplified from cell lines L4 and L6. Southern blotting of the amplified genomic fragments using a TSG101-specific DNA probe confirmed that the cDNA deletions characterized had resulted from the deletion of TSG101 genomic DNA. No deletions were observed in TSG101 genomic DNA amplified from normal fibroblast cell controls.

*Primary breast cancer cells produce deleted TSG101 transcripts.* The above experiments indicate that TSG101 is mutated in a significant fraction of the breast cancer cell lines that were studies. The association of TSG101 mutations with human breast cancer implied by these cell line data was further demonstrated by analysis of fourteen primary breast tumors, along with matched normal breast tissue obtained from the individuals that were the source of these tumors: six of fourteen primary breast cancers (P1, P2, P3, P4, P5 and P6) produced TSG101 transcripts containing a deletion, whereas corresponding deletions were not observed in transcripts from matched normal breast tissue. Two of the five primary breast cancers that contained deleted TSG101 transcripts produced two different truncated transcript species, implying the existence of mutations in two TSG101 alleles in these cancer cells. Non-deleted TSG101 transcripts were also detected in varying amounts in primary cancer specimens that produced truncated TSG101 transcripts; while some of these transcripts may result from the concurrent presence of non-tumor cells in the tissue specimens analyzed, as has been observed previously, other non-deleted transcripts may be
derived from tumor cells and contain point mutations, as
was found for breast cancer cell line L8.

Sequence analysis of truncated cDNA fragments
amplified from primary breast cancers identified the TSG101
5 sequences that were deleted in these primary cancers.
Three primary tumors, (P1, P3 and P6) had deletions in the
same cDNA region. Transcripts of one primary tumor deleted
in TSG101 (P4) also contained a 63 bp insertion at the
deletion junction. All of the deletions that were
10 sequenced affected the TSG101 protein-coding sequence and
removed all or part of the stathmin-interacting cc2 domain.

The extraordinary conservation observed between the
mouse and human TSG101 proteins is consistent with its
important biological role. Both the coiled-coil and
15 proline-rich domains are nearly identical, and the
potential phosphorylation and N-glycosylation sites are
completely conserved between the human and mouse protein.
Chromosomal mapping of TSG101 to human chromosome 11 and
mouse chromosome 7, which share conserved syntenic regions,
20 demonstrate that the human gene and mouse genes are
homologs.

Both the mouse and human TSG101 proteins contain a
coiled coil domain nearly identical to one previously shown
to interact with stathmin, a phosphoprotein proposed to
25 function in the coordination and relay of diverse signals
regulating cell proliferation and differentiation. The
presence of multiple DNA-binding domains in the TSG101
protein and a proline-rich domain near the leucine zipper
DNA binding motif of this protein indicates that the TSG101
30 gene product is a transcription factor, and therefore a
downstream effector of stathmin action.

Deletions in TSG101 were detected in 40% of the
metastatic breast cancer cell lines analysed and in the
same percentage of primary tumors. A point mutation was
35 also identified in a non-deleted TSG101 allele in one of
the primary tumors (MDA-MB-468; L8) and deletions in two different TSG101 transcript species were demonstrated for two primary breast cancers by gel analysis and sequencing. The data indicate that this may represent sporadic-type breast carcinomas.

All of the deletions, both in breast cancer cell lines and in primary breast tumors affected the stathmin-interacting coiled-coil domain of the TSG101 protein. The deletions in cancer cell lines MDA-MB-435 (L7) and MDA-MB-468 (L8) included part or all of the coiled coil domain, whereas the deletions observed near the N-terminal end of the protein in cell lines MDA-MB-231 (L4) and MDA-MB-453 (L6) were predicted to generate a frame shift from the point of deletion and terminate the protein by a stop codon 10 amino acids later - prior to the coiled-coil domain. All of the mutations resulted in total or partial deletion of the cc2-coding sequence.

Interestingly, a mutation of the p53 gene was also reported in MDA-MB-468 (L8), which contains mutations in two TSG101 alleles, suggesting the possibility that p53 and TSG101 have additive roles in the step-wise progression of breast-cancer to the fully malignant state.

It is noteworthy that the breast cancer cell lines having a DNA deletion that contains the TSG101 gene have also been shown to have high metastatic potential in nude mice. Line MDA-MB-435 (L7) was reported previously to contain a single copy of 11 p in many cells of the population, and FISH analysis has indicated that the majority of MDA-MB-435 cells contain only a single copy of TSG101. This cell line was found to have a high metastatic potential and is negative for both estrogen and progesterone receptors. Introduction of a copy of normal chromosome 11 significantly suppressed this metastatic potential. These observations are consistent with the finding that LOH at 11p15 in primary human breast tumors is
associated with poor survival after metastasis and the suggestion that LOH at 11p15 is involved in late stage tumor progression.

The TSG101 gene and the protein it encodes are useful for not only the diagnosis of human breast cancer and other human cancers as well, but also for gaining an increased understanding of mechanisms of tumorigenesis.

Experimental Procedures

cDNA and Genomic DNA Cloning. The two UDG-primers derived from ESTs H53754 and Z30135 were [SEQ ID NO:5] Pa-UDG (5'AGGUCAUGAUUGUGUAUUUGGAGAUG3') and [SEQ ID NO:6] Pd-UDG (5'CAUCUCCAAAACCACCAUCAUGACCU 3'). Two UDG-primers derived from the lgt10 cloning site are [SEQ ID NO:7] Pb-UDG (5'CAUCAUCAUGAGGTTGGCTATGAGTATTTCCTTCAG3') and [SEQ ID NO:8] Pc-UDG(5'CUACUACUACUACACCTTTTGAGCAAGTCAGCCTGGTT3'). 5'(Pc-UDG and Pd-UDG) and 3'(Pa-UDG and Pb-UDG) segments were amplified by PCR as following condition: 100 μl final volume of 20 mM Tris-HCl pH 8.55, 3.3 mM MgCl₂, 16 mM (NH₄)₂SO₄, 150 μg/ml BSA, 300 μM each dNTP, 1 μl human placenta lgt10 cDNA library (titer 10⁶/μl, ATCC), 0.2 μl of KlenTaqLA (Barnes (1994) P.N.A.S. 91:2216-2220), in a Perkin-Elmer Cetus thermal cycler for 40 cycles of: 95°C for 45 s (for denaturation), annealing and extending at 72°C for 1 min. The PCR products were visualized in ethidium bromide-stained low melting agarose gels, purified and cloned into pAMP1 cloning vector (Life Technologies, Inc.). Multiple clones were isolated and both strands of the cDNA inserts were sequenced using Sequenase 2.0 (USB).

The PCR product made using primers, [SEQ ID NO:9] 5' CTGATAACCAAGCTGGAGGTAGCTCTTTC3' (forward primer) and [SEQ ID NO:10] 5'ATTTAGCAGTCCCCAACATTCAGCAAA3' (reverse primer) were used to screen a PAC library containing human genomic DNA insert (Genome Systems, Inc.), yielding two overlapping clones, PAC1 and PAC2, each containing inserts about 150 kb
long. The presence of TSG101-specific sequences within these inserts was confirmed by Southern blotting, using a 5' fragment of human TSG101 cDNA as probe.

Cell Lines and Cell Culture. Human breast cancer cell lines (MDA-MB-231, MDA-MB-436, MDA-MB-435, MDA-MB-468, MDA-MB-157, MDA-MB-175VII, MDA-MB361, BT-483, and MCF-7), Wilms tumor cell lines (G401 and SK-NEP-1), and primary cultures of human normal fibroblast (CCD-19Lu and MRC-9) were obtained from American Type Culture Collection. Two melanoma cell lines (A375P and A375SM) were provided by I. J. Fidler. Two pleomorphic sarcoma cell lines (FB 309 and FB 310), a normal fibroblast line (FB316), a bladder carcinoma (FB241), two neuroblastoma cell lines (FB616 and FB617) and a Beckman-Wiedemann syndrome tumor cell line (FB583) were obtained from the collection of Ute Francke. All cell lines were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 100 U/ml penicillin, and 100 μg/ml streptomycin, except for breast cancer BT-483 cells, which were cultured in RPMI-1640 medium with 20% fetal bovine serum and two Wilms tumor cell lines (G401 and SK-NEP-1), which were cultured in McCoy's 5a medium with 10% fetal bovine serum. Cell lines from the Francke collection were cultured in Minimal Essential Medium a supplemented with 10% fetal bovine serum.

Fourteen primary breast tumors and matched normal breast tissues from the same patients were obtained from Cooperative Human Tissue Network and from Biochain Institute, Inc. (San Leandro, CA).

Northern Blot Analysis. A Northern blot filter of multiple normal tissue mRNA was purchased (Clontech). Radioactively-labeled single anti-sense strand DNA probe generated from full length human TSG101 cDNA by 40 cycles of primer extension, using [³²P]dCTP, was hybridized to the filter using standard methods. The same blot was stripped
and hybridized with a human β-actin probe synthesized by random priming as an internal loading control.

Somatic cell hybrids, PCR amplifications, and SSCA. The human TSG101 gene was localized to a human chromosome using a panel of 18 human X Chinese hamster hybrid cell lines derived from several independent fusion experiments (summarized in Francke et al. (1986) Cold Spring Harb. Symp. Quant. Biol. 2:855-866). The mouse tsg101 gene was mapped by analyzing a mapping panel of 20 mouse X Chinese hamster and two mouse X rat somatic cell hybrid lines derived from four independent fusion experiments, as described previously in Li et al. (1993) Genomics 18:667-672. The PCR primers used to amplify human and murine TSG101 sequences were derived from the 3' -untranslated region; the human primers were those employed to clone TSG101 genomic DNA as described above. The murine primers were: [SEQ ID NO:11] 5'GAGACCGACCTCTCCGTAAGACTACTT3' (forward primer) and [SEQ ID NO:12] 5'TAGCCCAAGTCAGCCAGCAGCAG 3'(reverse primer). PCR conditions were 95°C, 2 min; then 35 cycles of 94°C, 30 seconds; 68°C, 30 seconds; 72°C, 1 min; followed by 72°C, 7 min. To distinguish the PCR products from human and hamster sequences in some of hybrid lines, single-strand conformation analysis (SSCA) was carried out as described previously in Li et al. (1995) Cytogenet Cell Genet 71:301-305.

Fluorescence in situ hybridization. The chromosomal localization of the human TSG101 gene was independently determined by fluorescence in situ hybridization (FISH). Two genomic PAC1 and PAC2 clones carrying ~150 kb inserts, each containing overlapping human TSG101 sequences, were labeled with biotin-1 1-dUTP by nick-translation using commercial reagents (Boehringer Mannheim). Each labeled probe was hybridized at a concentration of 200 ng/50μl per slide to pre-treated and denatured metaphase chromosomes.
from human Lymphocytes. Hybridization, signal detection and amplification, as well as microscopy analysis and digital imaging were performed as previously described in Li et al. (1995) Cyto genet. Cell Genet. 68:185-191.

Human radiation hybrid mapping panel. The Stanford G3 radiation hybrid (RH) mapping panel was purchased from Research Genetics, Inc. and was used to further define the localization of the human TSG101 gene on human chromosome 11. This panel consists of 83 RH clones of the whole human genome with a resolution of approximately 500 kb. All 83 RH cell lines were typed for the human TSG101 gene by using primers and PCR conditions as described above. The results were sent to Stanford Human Genome Center for analysis with a software package of two-point and multipoint maximum likelihood methods, described by Boehnke et al. 1991.

RT-PCR and Sequencing of cDNAs. Total RNA was isolated using RNA Stat-60 (TEL-TEST). 10 μg of total RNA was treated with 10 units of RNase-free DNase I (Boehringer Mannheim) for 10 min, extracted with phenol-chloroform twice, and precipitated with ethanol. First strand cDNAs were synthesized by SuperScript II™ RNase H- reverse transcriptase (Life Technologies) using the TSG101-specific primer [SEQ ID NO:13] P2 (5'ATTAGCACTCACAACATGAGCAAA3') and the human GAPDH antisense primer [SEQ ID NO:14] (5'GTCTTCTGGGTCAGTGGCAT3') as a control. 1-2 μl of each product was used for PCR amplification with primer sets indicated. Primers used were [SEQ ID NO:15] P1 (5'CGGGTGTCCAGAGCCACGTGTAAGAAA3'), [SEQ ID NO:16] P3 (5'CCTTACCAACTGTTGGTCCATATCCTG3'), [SEQ ID NO:17] P4 (5'CCTTACGCTGTTGCTGCTGTTAAT3') and [SEQ ID NO:18] P5 (5'CACAGTCTAGTGGTGGCGTTC3'). PCR amplifications were carried out in 50 μl final volume of 20 mM Tris-HCl pH 8.55, 3.3 mM MgCl₂, 16 mM (NH₄)₂SO₄, 150 μg/ml BSA, 300 μM each dNTP, 0.2 μl of KlentaqLA (Barnes, supra.), in a Perkin-Elmer/Cetus thermal cycler for 35 cycles of 95°C
for 45 s (for denaturation), 65°C for 30 s (for annealing) and extension at 72°C for 30 s to 1 min and 30 s. The PCR products were visualized in ethidium bromide-stained low melting agarose gels, gel fragments were purified (Qiagen) and cloned into pCNTR cloning vector (5 Prime - 3 Prime, Inc.) Multiple clones were isolated and sequenced using Sequenase 2.0 (USB).

For primary tumors and matched normal breast tissue, P1 and P4 primers were used for first PCR amplification for 25 cycles. The amplified products were diluted 50-fold, 1 ml of the diluted product was used for a second round PCR amplification for 30 cycles using two nested primers, P6 (SEQ ID NO:21) AGCCAGCTCAAGAAAAATGGTGTCACAG, and P7 (SEQ ID NO:22) TCACCTGAGACCGCGACGTCTCTTCTTGCTT. The PCR products were resolved in 1.5% agarose gel stained with ethidium bromide, DNA fragments were cut from gels, and purified with QIAquick gel extract kit (Qiagen). The sequence of cDNA derived from primary breast cancers and matched normal breast tissue was determined using an Applied Biosystems model 310 genetic analyzer.

**PCR amplification of genomic DNA.** Genomic DNA was isolated by standard methods. 50 ng of RNAse A treated genomic DNA was used as a template for PCR amplifications. The primers used were:

**Cell line 8, (SEQ ID NO:23) TTCTGAAGGTCTCTGTGAGACAATAG**;
and (SEQ ID NO:24) CCTCCAGCTGCTATCAGGAAAG;
**Cell line 7, (SEQ ID NO:25) CAGTAGGGATGGCACAAATCAGCGAGGA** and (SEQ ID NO:26) GGTCAGTGCCTCTACACAACCCAAAGTTAA; **cell lines 4 and 6, (SEQ ID NO 27) CCGGTGTGCGAGCCAGCCTCAAGAAA** and (SEQ ID NO:28) TTTATTTTTTTTCAAAGGTCTCTGTTCTC. PCR amplifications were carried out in 50 ml final volume of 20 mM Tris-HCl pH 8.55, 3.3 mM MgCl₂, 16 mM (NH₄)₂SO₄, 150 mg/ml BSA, 300 mM each dNTP, 0.2 ml of KlentaqLA, in a Perkin Elmer/Cetus thermal cycler for 40 cycles at 95°C for
45 s, 65°C for 30 s, and extension at 72°C for 30 s to 1 min.

All publications and patent applications cited in this specification are herein incorporated by reference as if each individual publication or patent application were specifically and individually indicated to be incorporated by reference.

Although the foregoing invention has been described in some detail by way of illustration and example for purposes of clarity of understanding, it will be readily apparent to those of ordinary skill in the art in light of the teachings of this invention that certain changes and modifications may be made thereto without departing from the spirit or scope of the appended claims.
SEQUENCE LISTING

(1) GENERAL INFORMATION:

5  (i) APPLICANT: THE BOARD OF TRUSTEES OF THE LEcLAND STANFORD JUNIOR UNIVERSITY

(ii) TITLE OF INVENTION: MAMMALIAN TUMOR SUSCEPTIBILITY GENES AND THEIR USES

(iii) NUMBER OF SEQUENCES: 28

10  (iv) CORRESPONDENCE ADDRESS:
    FISH AND RICHARDSON, P.C.
    2200 SAND HILL ROAD
    MENLO PARK, CA 94025

(v) COMPUTER READABLE FORM:
    (A) MEDIUM TYPE: Floppy disk
    (B) COMPUTER: IBM PC compatible
    (C) OPERATING SYSTEM: PC-DOS/MS-DOS
    (D) SOFTWARE: PatentIn Release #1.0, Version #1.30

(vi) CURRENT APPLICATION DATA:
    (A) APPLICATION NUMBER:
    (B) FILING DATE:
    (C) CLASSIFICATION:

(viii) ATTORNEY/AGENT INFORMATION:
    (A) NAME: SHERWOOD, Pamela J.
    (B) REGISTRATION NUMBER: 36,677

(ix) TELECOMMUNICATION INFORMATION:
    (A) TELEPHONE: 415-322-5070
    (B) TELEFAX: 415-854-0675

(2) INFORMATION FOR SEQ ID NO:1:

30  (i) SEQUENCE CHARACTERISTICS:
    (A) LENGTH: 1448 base pairs
    (B) TYPE: nucleic acid
    (C) STRANDEDNESS: single
    (D) TOPOLOGY: linear

35  (ii) MOLECULE TYPE: cDNA

(ix) FEATURE:
    (A) NAME/KEY: CDS
    (B) LOCATION: 61..1203

40  (xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

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Val Phe Asn Asp Gly Ser Ser Arg Glu Leu Val Asn Leu Thr Gly Thr
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ATC CCA GTG TGT TAT CGA GGT AAT ATA TAT AAT ATT CCA ATA TGC CTG
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CCT ACT AGT TCA ATG ACT ATT AAA ACA GGA AAG CAT GTG GAT GCA AAT
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Pro Thr Ser Ser Met Thr Ile Lys THR Lys His Val Asp Ala Asn
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115 120 125
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540
Thr Ala Thr Gly Pro Pro Asn Thr Ser Tyr Met Pro Gly Met Pro Ser
145 150 155 160
GGA ATC TCT GCA TAT CCA TCT GGA TAC CCT CCC AAC CCC AGT GGT TAT
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365
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(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 381 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

Met Met Ser Lys Tyr Lys Tyr Arg Asp Leu Thr Val Arg Gln Thr Val Lys Val Ile Ala Met Tyr Lys Asp Leu Lys Pro Val Leu Asp Ser Tyr Val Phe Asn Asp Gly Ser Ser Arg Glu Leu Val Asn Leu Thr Gly Thr Ile Pro Val Arg Tyr Arg Gly Asn Ile Tyr Asn Ile Pro Ile Cys Leu Trp Leu Leu Asp Thr Tyr Asn Pro Pro Ile Cys Phe Val Lys Pro Thr Ser Ser Met Thr Ile Lys Thr Gly Lys His Val Asp Ala Asn Gly Lys Ile Tyr Leu Pro Tyr Leu His Asp Trp Lys His Pro Arg Ser Glu Leu Leu Glu Leu Ile Gln Ile Met Ile Val Ile Phe Gly Gly Glu Pro Pro Val Phe Ser Arg Pro Thr Val Ser Ala Ser Tyr Pro Pro Tyr Thr Ala Thr Gly Pro Pro Asn Thr Ser Tyr Met Pro Gly Met Pro Ser Gly Ile Ser Ala Tyr Ser Gly Tyr Pro Pro Asn Pro Ser Gly Tyr Pro Gly Cys Pro Tyr Pro Pro Ala Gly Pro Tyr Pro Ala Thr Thr Ser Ser Gln Tyr Pro Ser Gln Pro Pro Val Thr Val Gly Pro Ser Arg Asp Gly Thr Ile Ser Glu Asp Thr Ile Arg Ala Ser Leu Ile Ser Ala Val Ser Asp Lys Leu Arg Trp Arg Met Lys Glu Glu Met Asp Gly Ala Gln Ala Glu Leu Asn Ala Leu Lys Arg Thr Glu Glu Asp Leu Lys
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Ala Glu Val Asp Lys Asn Ile Glu Leu Leu Lys Lys Asp Glu Glu
275 280 285
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290 295 300
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305 310 315 320
Asn Leu Tyr Ala Glu Gin Ala Ile Gin Gin Asp Thr Ile Phe Tyr Leu
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(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
    (A) LENGTH: 1494 base pairs
    (B) TYPE: nucleic acid
    (C) STRANDEDNESS: single
    (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE:
    (A) NAME/KEY: CDS
    (B) LOCATION: 120..1259

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

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240
AACTAATGGA CCTCACTGAA ACAATCCCCTG TGCTTTATAG AGGTAATACA TACATATTC
300
CAATATGCTT ATGGCTACTG GACACATACC CATATAATCC CCCATCTCTG TTTGTAAGC
360
CTACTAGTTC AATGACTATT AAAACAGGGA AGCATGTTGA TGCAATGGGC AAGATATATTC
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CACCTGGTTGG TCCATATCCT GCCACAACAA GTTCTCAGTA CCCCCTTCAG CCTCCTGTTGA
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CCACGTGTGG TCCCAGTAGG GATGACGACAA TCAGGAGAGG CACACATCGGA GCCCTCTCTCA
780
TCTCTGGCCTG CAGTGACAAA CTGAGATGGC GGAATGAAGGA GGAATGGCAG GTGCCCACG
840
CAGACCATCAA TGCCCTGAAA CGAAGAAGAG AAGACCTGAA AAAGGTCTAC CAGAAACTGG
900
AAGAGATGGT TACCCGTTTA GATCAAGAAG TAGCCGAGGT TGATAAAAAC ATAGAAACTTT
960
TGAAACAGAA GGATGAAGAA CTACGTTTCTG CTCTGGAAAA AATGGAAAAAT CAGCTGAAAA
1020
ACAATGATAT CGTGAAGATT ATCATCACCA CAGCTCCCTT ATACAAACAG ATCCCTGATTC
1080
TGGGATAGAG AGAAAACGCT ATGGAAGACAA CTATTTTTTA CTTGGAGGAA GCCCTGAGAA
1140
GGGGCGTGGAT AGACCTGGAT TCCTCTCTGA AGCAGTTAGG TCCTCTGTCG CGTAACAGTT
1200
TCCAGCTGAG GCCAATATGG CAAAAGGCAA GAAGAAGCTGG CGTGTCATGT GACCTCTACT
1260
GACTCTCTCG ATACACGCTG GAGGTTGAGC TCCTCTTAAA GATTCTCTTC CTCTTTTTTA
1320
TCAGTGGGTT CCCAGAATAA GTTATTCGAG TTATCATACG AAGTGTAAAATTATTTGAAT
1380
CAATTTATA TTTTCTGTTT TCTTTTGGTA AAGACTGGCT TTATTTAATG CACTTTCTAT
1440
CCCTCTGAAA CTTTTTGGTC TGAATGTGTTG GACTGCTAAA TAAAAWTTGT TTTT
1494

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:
   (A) LENGTH: 380 amino acids
   (B) TYPE: amino acid
   (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein
- 73 -

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

Met Val Ser Lys Tyr Lys Tyr Arg Asp Leu Thr Val Arg Glu Thr Val
1 5 10 15  
Asn Val Ile Thr Leu Tyr Lys Asp Leu Lys Pro Val Leu Asp Ser Tyr
20  25  30  
Val Phe Asn Asp Gly Ser Ser Arg Glu Leu Met Asn Leu Thr Gly Thr
35  40  45  
Ile Pro Val Pro Tyr Arg Gly Asn Thr Tyr Asn Ile Pro Ile Cys Leu
50  55  60  
Trp Leu Leu Asp Thr Tyr Pro Tyr Asn Pro Pro Ile Cys Phe Val Lys
65  70  75  80  
Pro Thr Ser Ser Met Thr Ile Lys Thr Gly Lys His Val Asp Ala Asn
85  90  95  
Gly Lys Ile Tyr Leu Pro Tyr Leu His Glu Trp Lys His Pro Glu Ser
100 105 110  
Asp Leu Leu Gly Leu Ile Gln Val Met Ile Val Val Phe Gly Asp Glu
115 120 125  
Pro Pro Val Phe Ser Arg Pro Ile Ser Ala Ser Tyr Pro Pro Tyr Gin
130 135 140  
Ala Thr Gly Pro Pro Thr Ser Tyr Met Pro Gly Met Pro Gly Gly
145 150 155 160  
Ile Ser Pro Tyr Pro Ser Gly Tyr Pro Pro Asn Pro Ser Gly Tyr Pro
165 170 175  
Gly Cys Pro Tyr Pro Pro Gly Gly Pro Tyr Pro Ala Thr Thr Ser Ser
180 185 190  
Gln Tyr Pro Ser Gln Pro Pro Val Thr Thr Val Gly Pro Ser Arg Asp
195 200 205  
Gly Thr Ile Ser Glu Asp Thr Ile Arg Ala Ser Leu Ile Ser Ala Val
210 215 220  
Ser Asp Lys Leu Arg Trp Arg Met Lys Glu Glu Met Asp Arg Ala Gin
225 230 235 240  
Ala Glu Leu Asn Ala Leu Lys Arg Thr Glu Glu Asp Leu Lys Gly Lys
245 250 255  
His Gln Lys Leu Glu Glu Met Val Thr Arg Leu Asp Gln Glu Val Ala
260 265 270  
Glu Val Asp Lys Asn Ile Glu Leu Leu Lys Lys Asp Glu Glu Leu
275 280 285  
Ser Ser Ala Leu Glu Lys Met Glu Asn Gln Ser Glu Asn Asp Ile
290 295 300  
Asp Glu Val Ile Ile Pro Thr Ala Pro Leu Tyr Lys Gln Ile Leu Asn
305 310 315 320  
Leu Tyr Ala Glu Glu Ala Ile Glu Asp Thr Ile Phe Tyr Leu Gly
325  330  335
Glu Ala Leu Arg Arg Gly Val Ile Asp Leu Asp Val Phe Leu Lys His
340  345  350
Val Arg Leu Leu Ser Arg Lys Gln Phe Gln Leu Arg Ala Leu Met Gln
355  360  365
Lys Ala Arg Lys Thr Ala Gly Leu Ser Asp Leu Tyr
370  375  380

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 9 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:
His Thr His Leu Ala Met Asx Asp Ala
1  5

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 10 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:
Phe Xaa Asn Gly Ala Leu Glx Cys Tyr Ser
1  5  10

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 27 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "Primer"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:
AGGUCAUGAU UUGGUAAUUU GGAGAUG
27
(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:
   (A) LENGTH: 27 base pairs
   (B) TYPE: nucleic acid
   (C) STRANDEDNESS: single
   (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
     (A) DESCRIPTION: /desc = "Primer"

(x) SEQUENCE DESCRIPTION: SEQ ID NO:8:

CAUCUCCAAA UACCACAAUC AUGACCU

(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:
   (A) LENGTH: 39 base pairs
   (B) TYPE: nucleic acid
   (C) STRANDEDNESS: single
   (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
     (A) DESCRIPTION: /desc = "Primer"

(x) SEQUENCE DESCRIPTION: SEQ ID NO:9:

CAUCAUCAUC AUGAGGTGCG TTATGAGTAT TTCTTCCAG

(2) INFORMATION FOR SEQ ID NO:10:

(i) SEQUENCE CHARACTERISTICS:
   (A) LENGTH: 39 base pairs
   (B) TYPE: nucleic acid
   (C) STRANDEDNESS: single
   (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
     (A) DESCRIPTION: /desc = "Primer"

(x) SEQUENCE DESCRIPTION: SEQ ID NO:10:

CUACUACUAC UACACCTTTT GACGAAGTTC AGCCTGGTT

(2) INFORMATION FOR SEQ ID NO:11:

(i) SEQUENCE CHARACTERISTICS:
   (A) LENGTH: 28 base pairs
   (B) TYPE: nucleic acid
   (C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "Primer"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:
CTGATAACAG CTGGAGGCTC AGCTCTTC
28

(2) INFORMATION FOR SEQ ID NO:12:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 28 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "Primer"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:
ATTAGCAGT CCAACATTC AGCACAAA
28

(2) INFORMATION FOR SEQ ID NO:13:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 28 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "Primer"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:
GAGACCGACC TCTCGTAAA GCATTCTT
28

(2) INFORMATION FOR SEQ ID NO:14:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 28 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "Primer"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:
TAGCCCCGTC AGTCCCCAGCA CAGCACAG
28
(2) INFORMATION FOR SEQ ID NO:15:

(i) SEQUENCE CHARACTERISTICS:
    (A) LENGTH: 28 base pairs
    (B) TYPE: nucleic acid
    (C) STRANDEDNESS: single
    (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
    (A) DESCRIPTION: /desc = "Primer"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

ATTAGACGT CCCAACATTG AGCACA

(2) INFORMATION FOR SEQ ID NO:16:

(i) SEQUENCE CHARACTERISTICS:
    (A) LENGTH: 25 base pairs
    (B) TYPE: nucleic acid
    (C) STRANDEDNESS: single
    (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
    (A) DESCRIPTION: /desc = "Primer"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:

GTCTTCTGGA TGGCAGTGAT GGCAT

(2) INFORMATION FOR SEQ ID NO:17:

(i) SEQUENCE CHARACTERISTICS:
    (A) LENGTH: 27 base pairs
    (B) TYPE: nucleic acid
    (C) STRANDEDNESS: single
    (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
    (A) DESCRIPTION: /desc = "Primer"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:17:

CGGGTGTCGG AGAGCCAGCT CAAGAAA

(2) INFORMATION FOR SEQ ID NO:18:

(i) SEQUENCE CHARACTERISTICS:
    (A) LENGTH: 28 base pairs
    (B) TYPE: nucleic acid
    (C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
    (A) DESCRIPTION: /desc = "Primer"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:18:
5 CTTACCCAC CTGGTGTCG ATATCGTG
28

(2) INFORMATION FOR SEQ ID NO:19:

(i) SEQUENCE CHARACTERISTICS:
    (A) LENGTH: 26 base pairs
    (B) TYPE: nucleic acid
    (C) STRANDEDNESS: single
    (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
    (A) DESCRIPTION: /desc = "Primer"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:19:
15 CCTCCAGCTG ATATCGAGA AGTCGT
26

(2) INFORMATION FOR SEQ ID NO:20:

(i) SEQUENCE CHARACTERISTICS:
    (A) LENGTH: 27 base pairs
    (B) TYPE: nucleic acid
    (C) STRANDEDNESS: single
    (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
    (A) DESCRIPTION: /desc = "Primer"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:20:
25 CACAGTCGAG AGTGGGAG CGTATTTC
27

(2) INFORMATION FOR SEQ ID NO:21:

(i) SEQUENCE CHARACTERISTICS:
    (A) LENGTH: 27 base pairs
    (B) TYPE: nucleic acid
    (C) STRANDEDNESS: single
    (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
    (A) DESCRIPTION: /desc = "Primer"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:21:
AGCCAGCTCA AGAAATGGT GTCCAAG

(2) INFORMATION FOR SEQ ID NO:22:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 28 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "Primer"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:22:
TCACTGAGAC CGGCAGCTTT TCTTGCTT

(2) INFORMATION FOR SEQ ID NO:23:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 27 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "Primer"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:23:
TTCTGAAGGT TCTGTGAGA CAAATAG

(2) INFORMATION FOR SEQ ID NO:24:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 22 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "Primer"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:24:
CCTCCAGCTG GTATCAGAGA

(2) INFORMATION FOR SEQ ID NO:25:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 27 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "Primer"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:25:
CAGTAGGGAT GCCACAATCA GCGAGGA

(2) INFORMATION FOR SEQ ID NO:26:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 27 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "Primer"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:26:
GGTCAGTGCC TCTACAAACC AAGTTAA

(2) INFORMATION FOR SEQ ID NO:27:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 27 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "Primer"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:27:
CGGGTGTCCG AGGCCAGCT CAAGAAA

(2) INFORMATION FOR SEQ ID NO:28:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 29 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "Primer"
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:28:
TTTATTTTTT TACAAAGGTT TCTGTTCTC
WHAT IS CLAIMED IS:

1. An isolated nucleic acid encoding a TSG101 protein, or fragment of at least about 100 nt in length thereof, as other than an intact chromosome.

2. An isolated nucleic acid according to Claim 1, wherein said TSG101 protein is a mammalian protein.

3. An isolated nucleic acid according to Claim 2, wherein said nucleic acid comprises an oncogenic mutation.

4. An isolated nucleic acid according to Claim 3, wherein said oncogenic mutation disrupts the coiled coil domain.

5. An expression cassette comprising a transcriptional initiation region functional in an expression host, a nucleic acid having a sequence of the isolated nucleic acid according to Claim 1 under the transcriptional regulation of said transcriptional initiation region, and a transcriptional termination region functional in said expression host.

6. A cell comprising an expression cassette according to Claim 5 as part of an extrachromosomal element or integrated into the genome of a host cell as a result of introduction of said expression cassette into said host cell and the cellular progeny of said host cell.

7. A method for producing TSG101 protein, said method comprising:
   growing a cell according to Claim 6, whereby said TSG101 protein is expressed; and
   isolating said TSG101 protein free of other proteins.
8. A purified polypeptide composition comprising at least 50 weight % of the protein present as a TSG101 protein or a fragment thereof.

9. A purified polypeptide composition according to Claim 8, wherein said TSG101 protein is a mammalian protein.

10. A monoclonal antibody binding specifically to a TSG101 protein.

11. A method for characterizing the phenotype of a tumor, the method comprising:
   detecting the presence of an oncogenic mutation in TSG101 in said tumor,
   wherein the presence of said oncogenic mutation indicates that said tumor has a TSG101-associated phenotype.

12. A method according to Claim 19, wherein said carcinoma is a breast carcinoma.

13. A method for inactivating multiple copies of a gene at an expressed unselected chromosomal locus of eukaryotic cells, comprising:
   introducing into said eukaryotic cells a knockout DNA construct to produce a genetically modified cell mixture, said knockout DNA construct comprising at least (i) an agent regulated promoter ("TF promoter") oriented for RNA transcription in the opposite direction to (ii) a first positive selection marker coding sequence located 5' of said TF promoter, wherein a transactivation factor is provided extrinsic to said eukaryotic cells or intrinsic to said cells by introducing a transactivation DNA construct, said transactivation DNA construct comprising at least (i)
a gene sequence for a second positive selection marker, and
(ii) a gene sequence for said transactivation factor which
binds to said transcription initiation region of said
knockout construct to initiate RNA transcription, whereby
an antisense RNA is produced of the sequence of integration
of knockout construct locus; and
growing said genetically modified cell mixture in a
selective medium to obtain selected genetically modified
cells, said genetically modified selected cell being
characterized by (i) expression of said first positive
selection marker coding sequence resulting from said
knockout DNA construct being integrated downstream of a
promoter for said gene at said random chromosomal locus and
under its transcriptional regulatory control, and, in the
presence of said agent or when present, (ii) expression of
said second positive selection marker gene sequence
resulting in production of transactivator factor, wherein
the first copy of said gene at said chromosomal locus is
inactivated by integration of said knockout construct
downstream of said promoter and any other similar genes are
inactivated by said antisense RNA.

14. The method of claim 13, further including the steps
of assaying said genetically modified for a change in cell
phenotype associated with inactivating multiple copies of
said gene, and determining the gene at said locus.

15. The method of claim 13, wherein said introducing
step includes introducing said knockout and transactivation
DNA constructs successively so that said knockout construct
is introduced first to produce first genetically modified
cells and said transactivation DNA construct is introduced
later to produce second genetically modified cells.
16. The method of claim 15, wherein said knockout construct and said transactivation DNA construct comprise first and second positive selection markers, respectively, and said growing step includes growing said genetically modified cells which comprise said knockout construct in a first selective medium to obtain first selected cells expressing said first selection marker and growing said second genetically modified cells in a second selective medium to obtain second selected cells expressing both positive selection marker sequences.

17. The method of claim 13, wherein said knockout DNA construct further includes a splice acceptor sequence which is 5' in relationship to said positive selection marker coding region sequence.

18. The method of claim 17, wherein said splice acceptor sequence is 3' in relationship to said TF promoter.

19. The method of claim 13, wherein said transactivation factor includes a transcription activation domain and a DNA-binding domain, and said TF promoter includes a promoter sequence linked to multiple copies of a sequence which binds said DNA-binding domain, said DNA binding domain being exogenous to said eukaryotic cells.

20. The method of claim 19, wherein said promoter sequence comprises a domain derived from a viral transcription regulatory protein gene and said DNA-binding domain is derived from the lac repressor protein, and said sequence which binds said DNA-binding domain includes multiple copies of the lac operator sequence.

21. A knockout DNA construct sequence comprising a promoterless positive selection marker coding sequence and
a TF promoter responsive to a transactivation factor located in the direction of transcription of said coding sequence 5' of said coding sequence and oriented for transcription in the direction opposite said coding sequence.

22. A transactivation DNA construct sequence comprising a gene sequence for a transactivation factor, a gene sequence for a positive selection marker, and a gene sequence for a negative selection marker, said gene sequence for a transactivation factor and said gene sequence for said negative selection marker being delimited by two site-specific recombination sites.
INTERNATIONAL SEARCH REPORT
International application No.
PCT/US96/18828

A. CLASSIFICATION OF SUBJECT MATTER
IPC(6) : C12Q 1/68; C12N 15/00; C07H 21/04
US CL : 435/6, 172.3, 320.1; 536/24.5
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED
Minimum documentation searched (classification system followed by classification symbols)
U.S. : 435/6, 172.3, 320.1; 536/24.5

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
APS, Medline, Biosis
search terms: antisense, promoter, upstream, readthrough, flanking, cre, lox, site specific recombination,

C. DOCUMENTS CONSIDERED TO BE RELEVANT

<table>
<thead>
<tr>
<th>Category*</th>
<th>Citation of document, with indication, where appropriate, of the relevant passages</th>
<th>Relevant to claim No.</th>
</tr>
</thead>
<tbody>
<tr>
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<td>--------</td>
</tr>
</tbody>
</table>

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:
  "A" document defining the general state of the art which is not considered to be of particular relevance
  "B" earlier document published on or after the international filing date
  "L" document which may throw doubt on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
  "O" document referring to an oral disclosure, use, exhibition or other means
  "P" document published prior to the international filing date but later than the priority date claimed
  "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principles or theory underlying the invention
  "X" document of particular relevance; the claimed invention cannot be considered novelty or cannot be considered to involve an inventive step when the document is taken alone
  "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
  "*" document number of the same patent family

Date of the actual completion of the international search: 11 FEBRUARY 1997

Date of mailing of the international search report: 19 MAR 1997

Name and mailing address of the ISA/US
Commissioner of Patents and Trademarks
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Authorized officer: JOHN S. BRUSCA
Telephone No. 308-0196

Form PCT/ISA/210 (second sheet) (July 1992)
INTERNATIONAL SEARCH REPORT

Box I  Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1.☐ Claims Nos.:
   because they relate to subject matter not required to be searched by this Authority, namely:

2.☐ Claims Nos.:
   because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3.☐ Claims Nos.:
   because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II  Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1.☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2.☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3.☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4.☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest ☐☐ The additional search fees were accompanied by the applicant's protest.
☒ No protest accompanied the payment of additional search fees.

Form PCT/ISA/210 (continuation of first sheet(1))(July 1992)