

# *tsg101*: A Novel Tumor Susceptibility Gene Isolated by Controlled Homozygous Functional Knockout of Allelic Loci in Mammalian Cells

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## Summary

Using a novel strategy that enables the isolation of previously unknown genes encoding selectable recessive phenotypes, we identified a gene (*tsg101*) whose homozygous functional disruption produces cell transformation. Antisense RNA from a transactivated promoter introduced randomly into transcribed genes throughout the genome of mouse 3T3 fibroblasts was used to knock out alleles of chromosomal genes adjacent to promoter inserts, generating clones that grew in 0.5% agar and formed metastatic tumors in nude mice. Removal of the transactivator restored normal growth. The protein encoded by *tsg101* cDNA encodes a coiled-coil domain that interacts with stathmin, a cytosolic phosphoprotein implicated previously in tumorigenesis. Overexpression of *tsg101* antisense transcripts in naive 3T3 cells resulted in cell transformation and increased stathmin-specific mRNA.

## Introduction

It is now well recognized that conversion of a normal cell to a malignant one can result from recessive mutations in both alleles of genes that negatively regulate cell growth (for reviews see Klein, 1987; Weinberg, 1991; Knudson, 1993; Levine, 1993). The identification of genes whose homozygous disruption leads to tumorigenesis has been aided by the discovery of cancer-prone families and individuals who inherit a mutation in one allele of a tumor suppressor gene and therefore need only mutate the other allele for malignancy to occur (e.g., Cavenee et al., 1983; Friend et al., 1986). However, in the cells of normal individuals, an unmutated copy of a tumor suppressor gene ordinarily precludes phenotypic detection of recessive mutations in the other allele; both copies of the locus must be inactivated to produce phenotypic effects. Thus, it has not been practical to identify new genes that negatively regulate mammalian cell tumorigenesis by screening cell populations for mutations that cause malignant transformation.

For previously cloned genes, homozygous inactivation of chromosomally located alleles has been accomplished in cultured cells by homologous recombination and innovative selection techniques (e.g., Detloff et al., 1994; te Riele et al., 1990; Wu et al., 1994) and in transgenic animals by the mating of heterozygous individuals (Capecchi, 1989). Additionally, antisense RNA and oligonucleotides can disrupt the function of multiple alleles of a gene by, for example, interfering with translation or stability of the mRNA it encodes (e.g., Izant and Weintraub, 1984; Wagner, 1994). While such antisense

approaches have been useful in analyzing the biological functions of mammalian genes, they also have required prior cloning of, or knowledge of the DNA sequence of, the gene of interest.

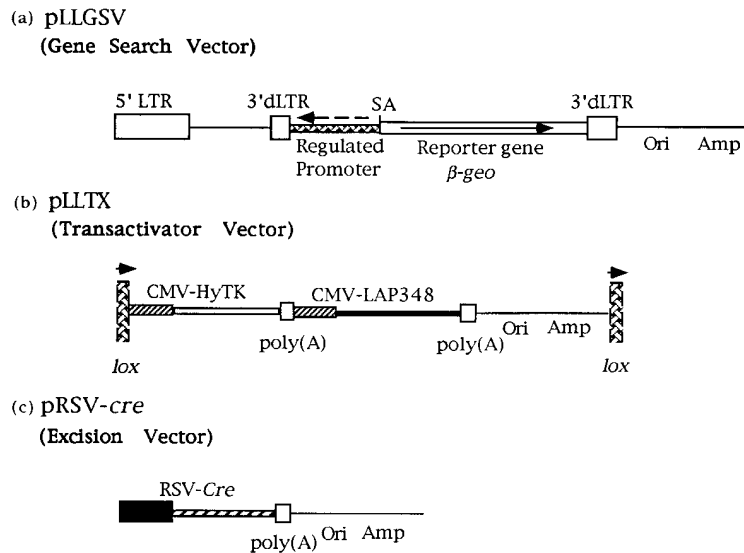
We report here a novel strategy that uses regulated antisense RNA initiated within a retrovirus-based gene search vector to identify previously unknown mammalian autosomal genes whose homozygous inactivation is associated with a defined phenotype, in this case, cellular transformation. By producing RNA complementary to transcripts from chromosomal genes that contain the retroviral vector, and consequently also complementary to mRNA encoded by other copies of these chromosomal genes, we generated cells able to grow in soft agar and form metastatic tumors in nude mice. The gene inactivated homozygously in a clone we analyzed contains a domain that interacts with stathmin (Sobel, 1991; Marklund et al., 1993), a highly conserved cytosolic phosphoprotein implicated in the coordination and relay of diverse signals controlling cell growth and differentiation.

## Results

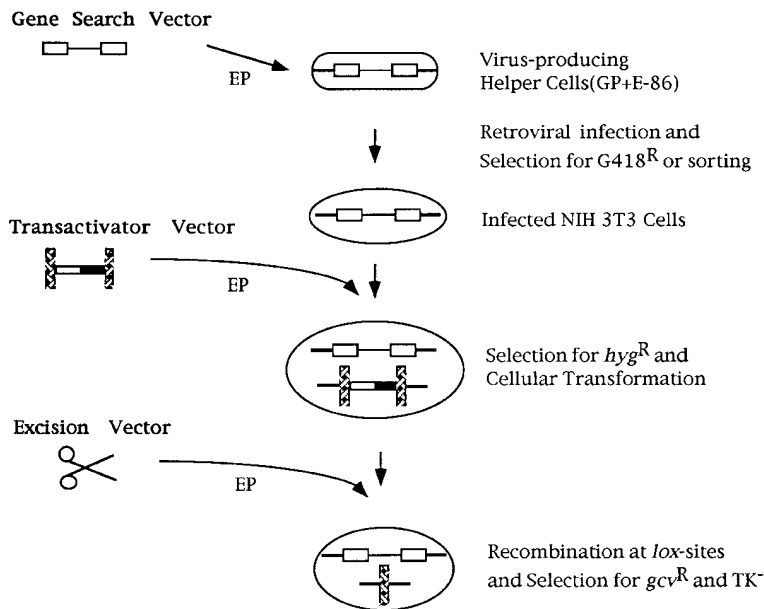
### Experimental Approach and Construction of Gene Search Vectors

The experimental strategy we used is shown in Figure 1. pLLGSV (Figure 1Aa), a Moloney murine leukemia virus-derived retroviral gene search vector containing the  $\beta$ -*geo* (Friedrich and Soriano, 1991) reporter gene, was introduced into NIH 3T3 cells, where it integrated at multiple chromosomal sites. As the proviral state of the retrovirus contained in pLLGSV lacks an enhancer and promoter in its long terminal repeats (LTR) (Hawley et al., 1987; Brenner et al., 1989), expression of  $\beta$ -*geo* in cells containing the provirus is dependent on transcription from the adjacent chromosomal DNA. Such expression yields resistance to the antibiotic G418 and production of  $\beta$ -galactosidase. An adenovirus-derived splice acceptor site located 5' to  $\beta$ -*geo* fuses  $\beta$ -*geo* mRNA to exons of chromosomally encoded transcripts. The SV40 T antigen minimal early promoter and 14 tandemly repeated copies of the *Escherichia coli lacZ* operator (Labow et al., 1990) are 5' to the splice acceptor site and in reverse (antisense) orientation to  $\beta$ -*geo*; transcription from this promoter can be activated in *trans* by LAP348 (Labow et al., 1990), which contains the operator-binding domain of the *E. coli* LacI repressor protein and the herpes simplex virus transactivation domain, VP16. The system was designed so that antisense RNA from the regulated SV40 promoter will inactivate  $\beta$ -*geo* fusion transcripts initiated in chromosomal genes that contain the pLLGSV-derived provirus and concomitantly will inactivate transcripts from other copies of these chromosomal genes. Clones in which such homozygous gene inactivation leads to identifiable phenotypes can be isolated from a heterogeneous cell population.

**A**



**B**



**C**

Formation of Fusion Transcript for cDNA Cloning

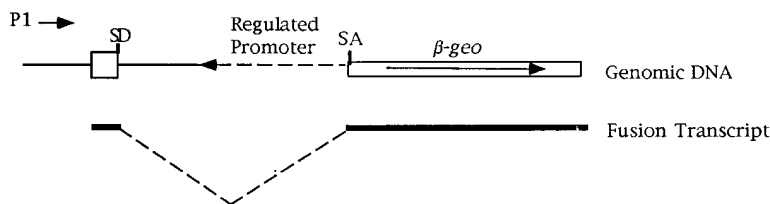


Figure 1. Vectors and Experimental Strategy

(A) The relevant segment of the pLLGSV gene search vector (a), the pLLTX transactivator vector (b), and the Cre-producing excision vector pRSV-cre are shown diagrammatically. Designations are: LTR (open boxes), retroviral long terminal repeat; 3' dLTR, defective 3' LTR lacking sequences required for production of virions (Brenner et al., 1989; Hawley et al., 1987); SA, splice acceptor site; Ori, plasmid replication origin; Amp, *bla* gene encoding resistance to ampicillin; regulated promoter, SV40 T antigen minimal early promoter linked to tandemly repeated copies of *E. coli lac* operator sequence (wavy line in closed box; arrow indicates direction of transcription);  $\beta$ -*geo*, reporter gene fusion of *E. coli lacZ* and *neo* (*aph*) genes (arrow indicates sense direction of fusion gene); CMV, cytomegalovirus promoter (diagonal lines); HyTK, hygromycin and thymidine kinase fusion gene (parallel horizontal lines); LAP348, transactivator gene (thick line); poly(A), signals determining the site of mRNA polyadenylation (small open boxes in [b] and [c]); RSV, Rous sarcoma virus promoter (closed box); *lox* (arrowheads indicate the orientation of *lox* sites shown by vertical helical lines), recognition site for Cre recombinase encoded by segment depicted in (c) by diagonal lines.

(B) Experimental strategy. Vectors were introduced into cells by electroporation (EP) where indicated. Fluorescence-activated cell sorting was done as described previously (Brenner et al., 1989); *hyg*, hygromycin; *gcv*, gancyclovir; P1, chromosomal promoter. Open boxes of gene search vector represent terminal LTRs. Open and shaded regions of transactivation vector are HyTK and LAP318 genes, respectively; hatched boxes are *lox* sites (see [A]). Other designations and experimental details are described in text.

(C) Diagrammatic representation of chromosomal DNA region containing the gene search vector and of fusion transcripts generated from chromosomal promoter, P1. Open boxes indicate chromosomal and  $\beta$ -*geo* genes. Thick lines represent fusion transcript sequences present in mRNA. Splice donor (SD) and acceptor (SA) sites are indicated. Broken lines correspond to chromosomal DNA sequences not represented in the spliced fusion transcript.

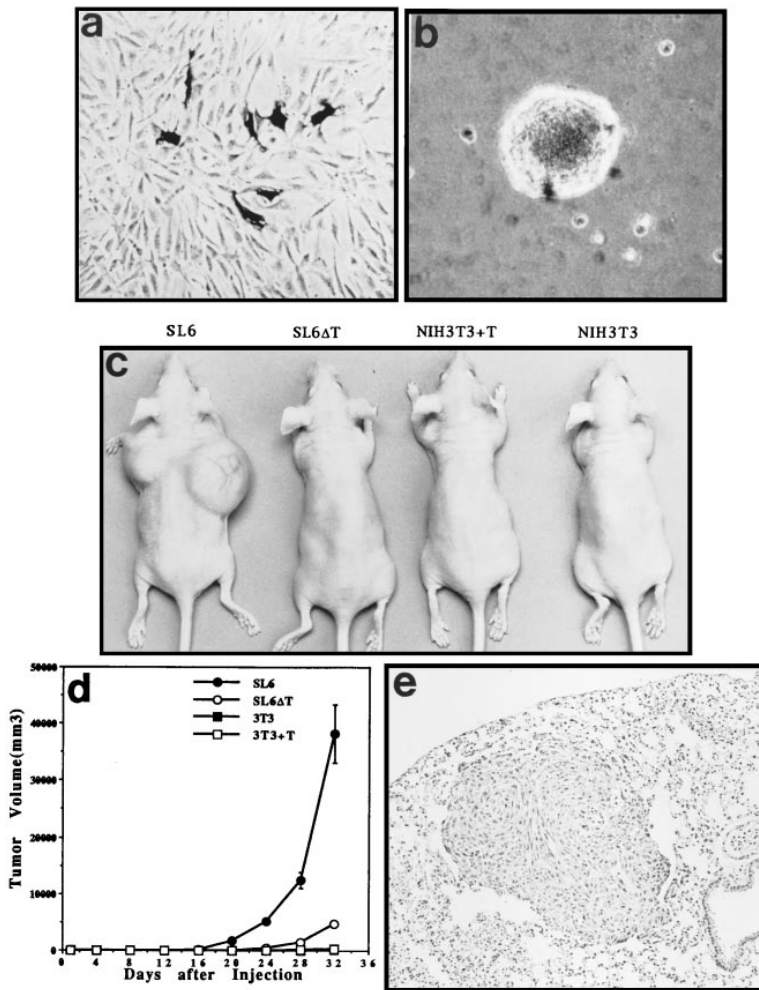


Figure 2. Isolation of Transformed Clone SL6 (a) NIH 3T3 cells stained with X-Gal 48 hr after retrovirus infection. Magnification is  $\times 100$ . (b) SL6 colony growing in 0.5% agar. Magnification is  $\times 100$ . (c) Tumorigenicity of SL6 cells. SL6-derived cells in which the transactivator has been deleted (i.e., SL6 $\Delta$ T), NIH 3T3 cells containing transactivator only (NIH 3T3 + T), and naive NIH 3T3 cells.  $10^5$  cells of each line were injected subcutaneously (see Table 1); a representative mouse receiving each line is shown 32 days after injection. (d) Growth curves in nude mice for the four cell lines described in (c). (e) Metastatic SL6 cells in lung 32 days after injection subcutaneously. Hematoxylin/eosin stain. Magnification is  $\times 40$ .

### Isolation of Clones Showing Transformed Phenotype

We chose in these experiments to identify clones in which random homozygous knockout results in cellular transformation (Figure 2b). NIH 3T3 cells were infected with retrovirus particles that were derived from pLLGSV and thus carry  $\beta$ -*geo* and the inactive SV40 antisense promoter. Cells expressing  $\beta$ -*geo*, which contain the provirus integrated at a transcriptionally active chromosomal site, were selected for G418 resistance or collected by fluorescence-activated cell sorter for production of  $\beta$ -galactosidase (Brenner et al., 1989); cells obtained by either method showed deep blue staining by X-Gal (Figure 2a). To activate the SV40 antisense promoter contained in the provirus, cells pooled from  $5 \times 10^6$  G418-resistant clones were transfected with pLLTX (see Figure 1A), which encodes both LAP348 and HyTK, a fusion of a hygromycin resistance (*hyg*) gene and the herpes simplex virus thymidine kinase (TK) gene (Lupton et al., 1991). Transfectants expressing HyTK resist treatment with *hyg* but do not grow in cultures containing gancyclovir (*gcv*); in the absence of HyTK expression, cells are *hyg*-sensitive and *gcv*-resistant.

*hyg*-resistant colonies (i.e., those containing the pLLTX transactivator vector) were pooled, and their cells

were plated in media containing 0.5% agar, which stringently selects for growth of transformed clones (Freedman and Shin, 1974; Fisher et al., 1979; Cifone and Fidler, 1980; Li et al., 1989) (Figure 2b). Whereas  $4 \times 10^6$  cells containing the pLLGSV-derived provirus yielded more than 20 large colonies in soft agar upon transactivation, a similarly sized control population of uninfected NIH 3T3 cells showed no colonies (Figure 3A). Southern blot analysis (Figures 4A and 4B) indicated that the provirus had integrated normally at a single chromosomal site in several clones; one of these was expanded into a cell line designated SL6.

The SV40-derived antisense promoter contained in the pLLGSV-derived provirus was turned off by deleting the LAP348 transactivator gene expressed by pLLTX. This was done by transfecting SL6 cells with pRSV-*cre* (see Figure 1A), which encodes Cre, a site-specific recombinase (Sauer and Henderson, 1989) that acts on the lox sites of pLLTX (Figure 1A). Cells losing the LAP348/HyTK segment located between these lox sites were identified by their resistance to *gcv*, and loss of the LAP348/HyTK segment was further confirmed by Southern blotting (data not shown). A population of  $10^6$  cells derived from a pool of six randomly chosen *gcv*-resistant clones was designated SL6 $\Delta$ T. Coincident with

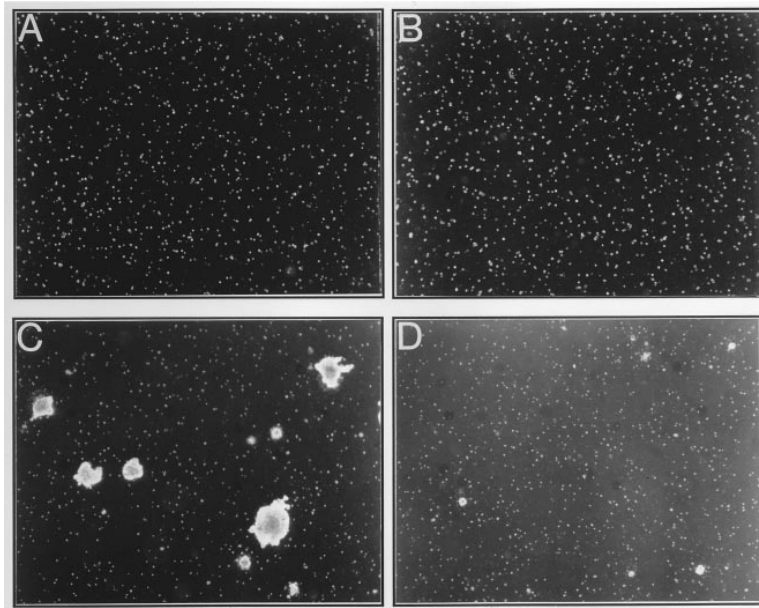


Figure 3. Effect of Transactivation of Antisense Promoter on Cell Growth

(A) NIH 3T3 cells.  
(B) NIH 3T3 cells containing the transactivator vector only.  
(C) SL6 cells.  
(D) Cells from which the transactivator has been excised (SL6ΔT). All cells were incubated for 3 weeks in 0.5% agar. Magnification is  $\times 40$ .

removal of the transactivator and consequent shutoff of the antisense promoter,  $\beta$ -galactosidase production resumed (Table 1), leading to dark blue staining of cells by X-Gal. In contrast to the parental SL6 line (see Figure 3C), SL6ΔT cells formed only occasional colonies in 0.5% agar, and these remained small despite prolonged culture (see Figure 3D). Control cell populations showed no colony formation (see Figures 3A and 3B).

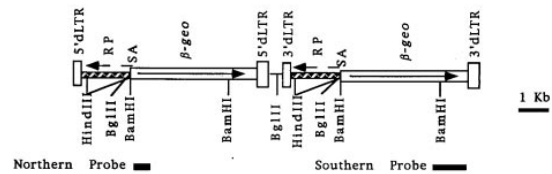
Hybridization of poly(A)<sup>+</sup> RNA from SL6ΔT cells with a  $\beta$ -*geo* DNA fragment (Figure 4C) showed an expected 7 kb band plus two additional bands that may have resulted from aberrant splicing events (Friedrich and Soriano, 1991). Northern blot analysis using as probe the cDNA sequence adjoining the pLLGSV gene search vector in SL6 (see below) confirmed that the 7 kb and 6.5 kb bands are fusion transcripts of  $\beta$ -*geo* with a chromosomally encoded exon. The cellular concentration of these chromosomally initiated fusion transcripts was not altered by activation of the SV40 antisense promoter (data not shown), suggesting that interference with  $\beta$ -*geo* gene function by antisense RNA did not result from inhibition of transcription or accelerated RNA degradation (see Discussion).

Injection of SL6 cells subcutaneously into nude mice produced large tumors at the sites of injection in all ten animals, and these tumors metastasized spontaneously to the lungs in eight of the ten mice (see Figure 2C and Table 1). While no tumors were produced by control cell populations, a single small nonmetastasizing tumor occurred in one animal at the site of injection of SL6ΔT cells, some of which had retained the ability to produce at least occasional small colonies on soft agar following turnoff of the antisense promoter, as noted above.

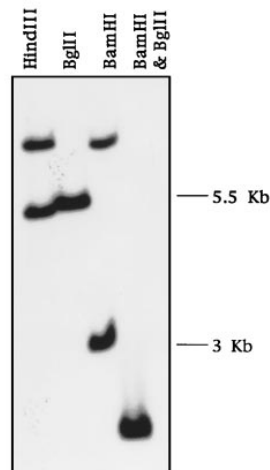
#### cDNA Cloning and Sequence Analysis

Chromosomally initiated  $\beta$ -*geo* fusion transcripts from SL6ΔT cells were cloned as described in Experimental Procedures. The 120 bp cDNA segment found in multiple clones contained 70 bp of a nonvector sequence that was fused in frame to the splice acceptor site 5' to

#### A. Map of Integrated Provirus



#### B. Southern Blot



#### C. Northern Blot

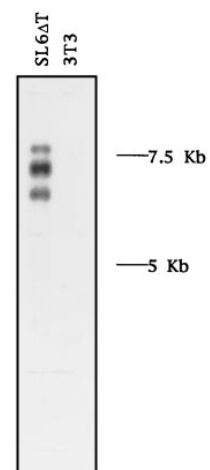


Figure 4. Structure of Integrated Provirus in SL6 Cells and Results of Southern and Northern Blot Analyses

(A) Map of integrated provirus. RP, regulated antisense promoter. Other designations are as in Figure 1. Relevant restriction sites are shown, along with the regions used as probes for Southern and Northern blots.

(B) 20  $\mu$ g of genomic DNA used for each restriction digestion was probed with an 1.3 kb *neo* gene single-strand DNA probe.

(C) 2  $\mu$ g of polyadenylated mRNA was probed with a 320 bp fragment containing 5' *lacZ* gene sequences, generated as described above. Positions of size markers are indicated.

*β-geo*. A database search using the BLAST program (Altschul et al., 1990) showed 97% identity between this sequence and a partial transcript of unknown function expressed during differentiation of F9 mouse embryonal carcinoma cells (Nishiguchi et al., 1994).

A cDNA library from mouse NIH 3T3 cells was screened with the 70 bp cDNA probe to obtain the full-length gene. Plasmids from four hybridizing colonies contained an identical insert having a 1143 bp open translational reading frame predicted to encode a 381 amino acid protein of 43,108 Da (Figure 5A). The gene specifying this protein was designated as tumor susceptibility gene 101 (*tsg101*); 4 amino acids downstream of its ATG putative translation start codon and 72 nt from the start of the cDNA was a splice donor consensus sequence (AG).

Analysis of *tsg101* cDNA and protein sequences by a BLAST Program search of databases of the National Center for Biotechnology Information showed identity, with two mismatches, of amino acids 231–301 of *tsg101* to cc2, an  $\alpha$ -helix domain (Figure 5C) encoded by a short cDNA sequence whose product interacts with stathmin (Maucuer et al., 1995), an evolutionarily conserved phosphoprotein implicated in the integration and relay of diverse signals regulating cell growth (Sobel, 1991). The algorithm of Stock and colleagues (Lupas et al., 1991) predicts with a probability of greater than 99.8% that the helical domain of Tsg101 will form a coiled-coil structure (Figure 5B).

Protein pattern and motif searches of Tsg101 (Prints) identified three separate DNA-binding motifs characteristic of transcription factors: a leucine-zipper motif (Landschulz et al., 1988; O'Shea et al., 1989a) within the cc2 domain (Figure 5C); a segment (amino acids 37–46) resembling the helix–turn–helix domain of the bacteriophage  $\lambda$  repressor (i.e., HTHLAMBDA) (Brennan and Matthews, 1989); and a Zn-cys zinc-finger binuclear cluster signature (FUNGALZCYS) domain (amino acids 73–83) (Pan and Coleman, 1990). Preceding the leucine zipper is a proline-rich region (amino acids 129–200; 31% proline) typical of the activation domains of transcription factors (Mitchell and Tjian, 1989). Also present in the Tsg101 protein are seven potential protein kinase C phosphorylation sites (amino acids 11, 38, 85, 88, 215, 225, 357), five potential casein kinase II phosphorylation sites (amino acids 38, 210, 249, 265, 290), two potential N-myristoylation sites (amino acids 55, 156), and three potential N-glycosylation sites (amino acids 44, 150, 297) (Bairoch and Bucher, 1994).

### Overexpression of *tsg101* Antisense RNA Causes Transformation of Naive 3T3 Cells and Elevation of Stathmin mRNA

Synthesis of *tsg101* antisense RNA under control of the cytomegalovirus promoter in naive NIH 3T3 cells resulted in cellular transformation, as indicated by the ability to form foci in monolayer culture and show anchorage-independent growth in soft agar (Figures 6 and 7, Table 2). These colonies grew somewhat more slowly than the ones produced by SL6 cells, which contain a retroviral insertion in one copy of *tsg101* and thus require antisense knockout of only the other gene copy for cell transformation to occur. Additionally, as seen in Figures 6 and 7 and Table 2, overexpression of the *tsg101* cDNA isolate we cloned in the sense direction also resulted in focus formation and colony growth in soft agar, although at considerably lower frequency than in the antisense direction, implying that excessive amounts of Tsg101 protein from this cDNA can also accomplish cellular transformation. In control experiments, cells transfected with vector DNA lacking the insert mock-transfected cells, and cells expressing *tsg101* sense transcripts carrying deletions or insertions that cause frameshift mutations and premature termination of the protein showed no focal transformation or colony growth on soft agar. Consistent with previous data showing that the intracellular concentration of stathmin is increased in tumor cells (Hanash et al., 1988; Roos et al., 1993; Brattsand et al., 1993), the steady-state level of stathmin mRNA was elevated in cells containing constructs expressing full-length *tsg101* transcripts in both the sense and antisense directions (Figure 8), but not in cells receiving mutated sense direction constructs.

### Discussion

The work reported here demonstrates the utility of a novel strategy for isolating mammalian cell autosomal genes associated with defined phenotypes that are recessive. Using antisense RNA originating at a chromosomally integrated promoter functionally to inactivate alleles of chromosomal genes containing the integrated promoter, we identified a gene whose antisense-RNA-dependent knockout reversibly converted mouse 3T3 fibroblasts to metastasizing tumor cells. The approach used is notable in that it allows the identification or selection or both of cells showing a phenotypic trait requiring homozygous knockout of gene function, in the

Table 1. Characterization of SL6

	3T3 –	3T3 +	SL6 $\Delta$ T –	SL6 +
Transactivator	–	+	–	+
$\beta$ -galactosidase activity (U/ $\mu$ g) <sup>a</sup>	9.3	10.1	1225.8	19.9
Growth in 0.5% agar <sup>b</sup>	0/10 <sup>5</sup>	0/10 <sup>5</sup>	20/10 <sup>5</sup>	>1000/10 <sup>5</sup>
Tumorigenicity in nude mice	0/10	0/10	1/10	10/10
Spontaneous lung metastasis <sup>c</sup>	0/10	0/10	0/10	8/10

<sup>a</sup> Mean of triplicates. Units/ $\mu$ g cell protein.

<sup>b</sup> Colonies/cells plated. The colonies formed by SL $\Delta$ T cells, which lack the LAP348/HyTK segment, were much smaller than those formed by SL6 cells, which contain this segment (see Figure 3).

<sup>c</sup> Mice were sacrificed at day 32, and the lung metastases were confirmed by histological examination. No metastases to other organs were observed. No evidence of metastasis was seen in the lungs of control animals by either gross or histological examination.

**A**

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1 CCCCTCTGCCTGTGGGACGGAGGAGCGGCCATGGCTGTCGGAGAGTCAAGTGAAGAAG
61 ATGATGTCACAGTACAAATATAGAGATCTAACCGTCCGTCAAACTGTCATGTCATCGCT
MetMetSerLysTyrLysTyrArgAspLeuThrValArgGlnThrValAsnValIleAla 20
121 ATGTACAAAGATCTCAAACCTGATTGGATTTCATATGTTTTAATGATGGCAGTCCAGG
MetTyrLysAspLeuLysProValLeuAspSerTyrValPheAsnAspGlySerSerArg 40
181 GAGCTGGTGAACCTCACTGGTACAAATCCAGTGCCTATCGAGGTAATATATATAATATT
GluLeuValAsnLeuThrGlyThrIleProValArgTyrArgGlyAsnIleTyrAsnIle 60
241 CCAATATGCCTGTGGCTGGACACATACCCATATAACCCCTATCTGTTTTGTTAAG
ProIleCysLeuTrpLeuLeuAspThrTyrProTyrAsnProProIleCysPheValLys 80
301 CCTACTAGTTCAATGACTATTAACAGGAAAGCATGGATGCAAATGGGAAAACTAC
ProThrSerSerMetThrIleLysThrGlyLysHisValAspAlaAsnGlyLysIleTyr 100
361 CTACCTTATCTACATGACTGGAACATCCACGGTCAGAGTTGCTGGAGCTTATCAAAATC
LeuProTyrLeuHisAspTrpLysHisProArgSerGluLeuLeuGluLeuIleGlnIle 120
421 ATGATTGTGATATTGGAGAGGAGCCTCCAGTCTTCTCCCGCCTACTGTTTCTGCATCC
MetIleValIlePheGlyGluGluProProValPheSerArgProThrValSerAlaSer 140
481 TACCCACCACACAGCAACAGGGCCCAAAATACCTCTACATGCCAGGCATGCCAAGT
TyrProTyrTyrThrAlaThrGlyProProAsnThrSerTyrMetProGlyMetProSer 160
541 GGAATCTCGCATATCCATCTGGATACCTCCCAACCCAGTGGTTATCTGGCTGTCTT
GlyIleSerAlaTyrProSerGlyTyrProProAsnProSerGlyTyrProGlyCysPro 180
601 TACCCACCTGTGGCCATACCTCCACACAAGCTCACAGTACCTTCCAGCCTCCT
TyrProProAlaGlyProTyrProAlaThrThrSerSerGlnTyrProSerGlnProPro 200
661 GTGACCACCTGTGGTCCAGCAGAGATGGCACAATCAGTGAGGACACTATCCGTGCATCT
ValThrThrValGlyProSerArgAspGlyThrIleSerGluAspThrIleArgAlaSer 220
721 CTCATCTCAGCAGTCACTGACAACTGAGATGGCGGATGAAGGAGAAATGGATGGTGCC
LeuIleSerAlaValSerAspLysLeuArgTrpArgMetLysGluGluMetAspGlyAla 240
781 CAGGCAGAGCTTAATGCCTTGAACAGCAACAGAGGAAGATCTGAAAAAGGCCACGAAA
GlnAlaGluLeuAsnAlaLeuLysArgThrGluGluAspLeuLysLysGlyHisGlnLys 260
841 CTGGAAGAGATGGTCCACCCGCTTAGATCAAGAAGTGTGAGTGAATGATAAAAAATAGAA
LeuGluGluMetValThrArgLeuAspGlnGluValAlaGluValAspLysAsnIleGlu 280
901 CTTTTGAAAAAGAGGATGAAGAAGTAACTAAGTTCTGCTCTGGAGAAAATGGAATCAATCT
LeuLeuLysLysLysAspGluGluLeuSerSerAlaLeuGluLysMetGluAsnGlnSer 300
961 GAAAAATATGATATTGATGAAGTTATCATTCACAGCCCACTGTATAACAGATTCTA
GluAsnAsnAspIleAspGluValIleIleProThrAlaProLeuTyrLysGlnIleLeu 320
1021 AATCTGTATGCAGAGAAAATGCTATTGAAGACACTATCTTTACCTTGGAGAAGCTTTG
AsnLeuTyrAlaGluGluAsnAlaIleGluAspThrIlePheTyrLeuGlyGluAlaLeu 340
1081 CGCGGGGAGTCATAGACCTGGATGTGTTCTGAAACAGTCCGCTCCTGTCCCGTAAA
ArgArgGlyValIleAspLeuAspValPheLeuLysHisValArgLeuLeuSerArgLys 360
1141 CAGTTCACGCTAAGGGCACTAATGCAAAAGGCAAGGAAGACTCGGGCCTTAGTGACCTC
GlnPheGlnLeuArgAlaLeuMetGlnLysAlaArgLysThrAlaGlyLeuSerAspLeu 380
1201 TACTGACATGTGCTGCTCAGTGGAGACCGACTCTCCGTAAGCATTCTTTCTTCTTCT
Tyr
1261 TTTTCTCATCAGTAGAACCCACAATAAGTTATTGTCAGTTTATCATTCAAGTGTAAATAT
1321 TTTGAATCAATAATATATTTTCTGTTTCTTGGGTAAAACCTGGCTTTTATTAATGCAC
1381 TTTCTACCCTCTGTAAGCGTCTGTGCTGTGCTGGGACTGACTGGGCTAATAAAAATTTGT
1441 TGCATAAA

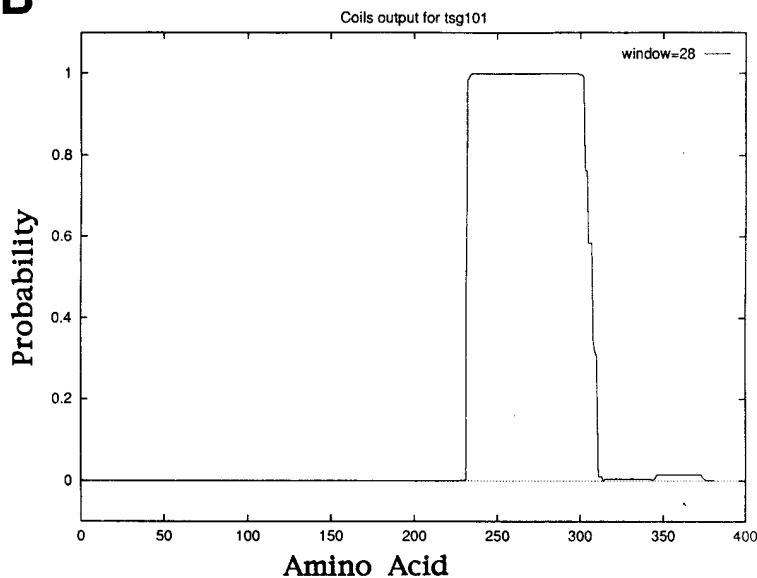
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Figure 5. Sequences and Structural Features of *tsg101*

(A) Sequence of *tsg101* cDNA and predicted amino acid sequence of the Tsg101 protein. The site of translation initiation is underlined. The splice junction 5' to  $\beta$ -*geo* is boxed, and the poly(A) signal is shown in bold type. The predicted coiled-coil domain is shaded.

(B) Coiled-coil structure prediction (Lupas et al., 1991) of *tsg101* structure.

(C) Sequence alignment of *tsg101* and cc2. Amino acid positions defining the leucine-zipper motif are boxed. Sites of apparent sequence difference are indicated by open letters.

**B****C**

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tsg101 231 RMKEEMDGAQAEINAKRTEEDLKKGHQKLEEMVTRLDQE
RMKEEMDGAQAEINAKRTEEDLKKGHQKLEEMVTRLDQE
cc2 1 RMKEEMDGAQAEINAKRTEEDLKKGHQKLEEMVTRLDQE

tsg101 VAEVDKNIELLKKKDEELSSALEKMENQSENNDIDEVIPTA 313
VAEV KNIELLKKKDEELSSALEKMENQSENNDIDEVIPTA
cc2 VAEV@KNIELLKKKDEELSSALEKMENQSENNDIDEVIPTA 83

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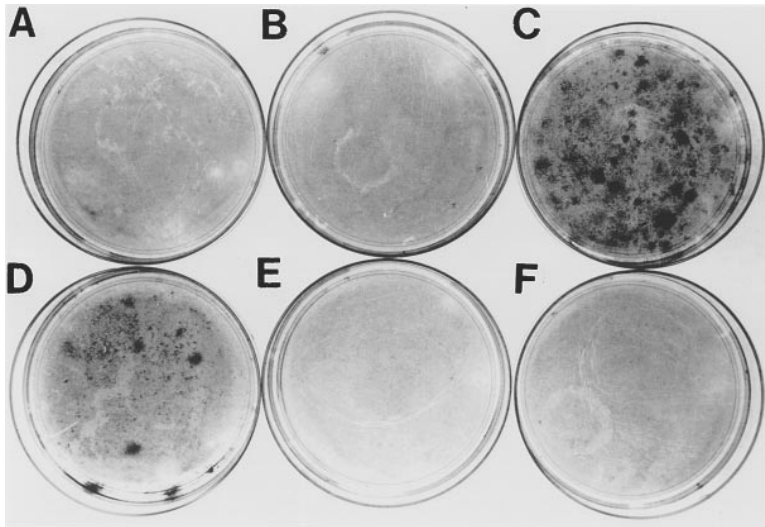


Figure 6. Focus Formation of NIH 3T3 Cells Transfected by *tsg101* cDNA and Control Constructs

(A) Mock transfection (no DNA).  
(B) Vector control lacking *tsg101* cDNA insert.  
(C and D) Vector containing the full-length *tsg101* cDNA insert in the antisense and sense orientation, respectively.  
(E) Vector containing *tsg101* $\Delta_{10-23}$  insert (sense mutant 1).  
(F) Vector containing *tsg101* $_{1254}$  insert (sense mutant 2).  
The frequency of focus formation in multiple assays is shown in Table 2.

absence of prior isolation of the gene or information about its sequence. To isolate genes that negatively regulate tumorigenesis, we identified NIH 3T3 clones that formed colonies in 0.5% agar, which previously has been used stringently to select transformed cells showing high metastatic potential (Cifone and Fidler, 1980; Li et al., 1989), and then confirmed that this phenotype was dependent on expression of antisense RNA from the gene-search vector. The strategy we used is potentially applicable for isolating cells in which homozygous knockout of unknown genes produces other selectable phenotypes such as resistance to infectious agents or chemical substances.

Whereas retrovirus integrations are largely random events, preference for integration at the 5' ends of functional genes has been observed (Rohdewohld et al., 1987). Use of the  $\beta$ -*geo* reporter gene allows cells containing integrations in transcriptionally active genes to

be selected and also allows monitoring of the effects of antisense RNA on transcripts initiated in the flanking chromosomal DNA sequence. Reversibility of antisense inhibition and the tumorigenic phenotype was accomplished in these experiments by Cre/lox-mediated deletion of a gene encoding a transactivator protein. Promoters controlled by tetracycline (Gossen et al., 1995), heavy metal ions (Mayo et al., 1982), or hormones (Lee et al., 1981) may provide possible alternatives for regulating the production of antisense RNA from gene search vectors.

Hybridization of antisense RNA with sense RNA can lead to degradation of both strands of the RNA duplex (Izant and Weintraub, 1984, 1985), inhibit translocation of the sense RNA from nucleus to cytoplasm (Kim and Wold, 1985), interfere with mRNA splicing (Temsamani et al., 1991; Volloch et al., 1991a, 1991b), inhibit the initiation of translation (Melton, 1985; Stephenson and

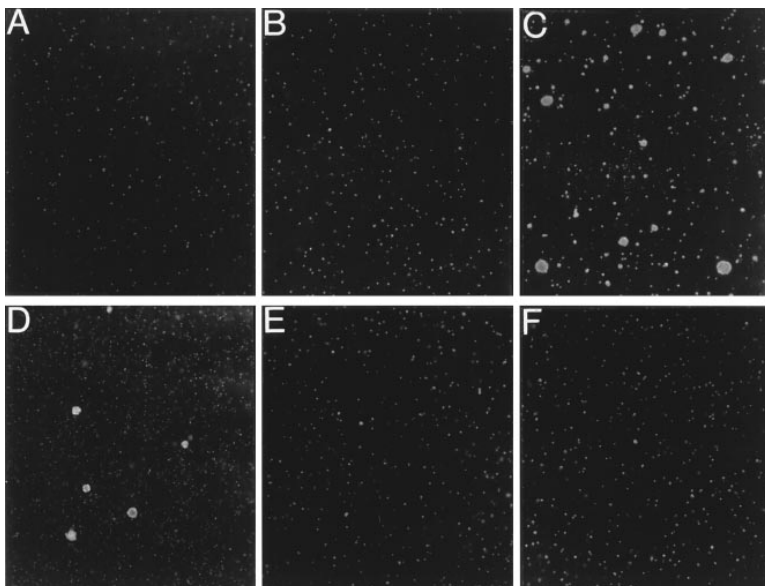


Figure 7. Colony Formation by NIH 3T3 Cells Transfected by *tsg101* cDNA and Control Constructs

Vector DNA (5  $\mu$ g) was introduced into NIH 3T3 cells by LipofectAMINE. Transfected cells were selected for growth in 1  $\mu$ g/ml puromycin for 14 days, and resistant cells were plated in 0.35% agar and incubated for 3 weeks. Magnification is  $\times 40$ . The efficiency of colony formation is shown in Table 2. Panel designations are as in Figure 6.

Table 2. Focus and Colony Formation by NIH 3T3 Cells Transfected with *tsg101* cDNA Constructs

Cell Line <sup>a</sup>	Transfection Efficiency <sup>b</sup> (Mean ± SE)	Focus Formation <sup>c</sup> (Mean ± SE)	Growth in Soft Agar <sup>d</sup> (Mean ± SE)	Efficiency of Colony Formation in Soft Agar (%)
No DNA	0	0	0	0
Vector only	167 ± 17	0	0	0
Antisense	154 ± 16	116 ± 15	473 ± 42	0.473
Sense	164 ± 20	22 ± 4	39 ± 7	0.039
Sense Mutant 1	143 ± 21	0	0	0
Sense Mutant 2	155 ± 22	0	0	0

<sup>a</sup> 10<sup>5</sup> NIH 3T3 cells were transfected with 5 µg of *tsg101* DNA construct or control construct as indicated (see Experimental Procedures). For focus assays, half of the transfected cell population was cultured in 1 µg/ml of puromycin to determine transfection efficiency, and the other half was plated in the absence of antibiotic for focus formation. For soft agar assays, transfected cells were selected by growth in 1 µg/ml of puromycin for 14 days, and 10<sup>5</sup> puromycin-resistant cells were then plated in 0.35% agar in 100 mm plates in the absence of antibiotics.

<sup>b</sup> Measured as the number of puromycin-resistant colonies selected in medium containing 1 µg/ml puromycin per µg pf plasmid DNA.

<sup>c</sup> Number of foci formed per µg of plasmid DNA in medium containing 10% calf serum. Foci were counted 14 days after plating of cells. The data represent averages from three independent experiments, each done in triplicate.

<sup>d</sup> Number of colonies formed by 10<sup>5</sup> puromycin-resistant cells in 0.35% agar. All bottom layers were 0.6% agar. Colonies were counted 21 days after plating. The data represent averages of three independent experiments, each done in triplicate.

Zamecnik, 1978), or all of the above. As the cellular concentration of chromosomally encoded *tsg101* message did not change significantly in the presence of antisense transcripts, their effects on *tsg101* gene function seem likely to result from a mechanism other than RNA degradation.

*tsg101* was chosen for study because its functional disruption led both to cellular transformation and to tumors that metastasized spontaneously in nude mice. Deletion of the transactivator gene required for the production of antisense transcripts complementary to *tsg101* mRNA reversed these phenotypes, although rare cells in the population retained an ability, after removal of the transactivator, to generate very small colonies in soft agar and a small nonmetastasizing tumor. These observations suggest that some of the growth-related

changes resulting from knockout of *tsg101* gene function are not fully reversible.

*tsg101* cDNA encodes a region virtually identical to cc2, a coiled-coil domain of a mouse protein shown to interact with stathmin in the yeast two-hybrid system (Maucuer et al., 1995). The human homolog of *tsg101* encodes a highly similar domain (L. L. and S. N. C., unpublished data). Stathmin (Sobel, 1991), also known as oncoprotein 18 (Marklund et al., 1993), is phosphorylated in vivo in response to growth and differentiation factors (Doye et al., 1990) and also during T cell activation (Gratiot-Deans et al., 1992), cell cycle transitions (Brattsand et al., 1994; Strahler et al., 1992), embryonic development (Doye et al., 1992), and tissue regeneration (Koppel et al., 1993). Expression of stathmin is increased in acute leukemia and in highly malignant lymphoma

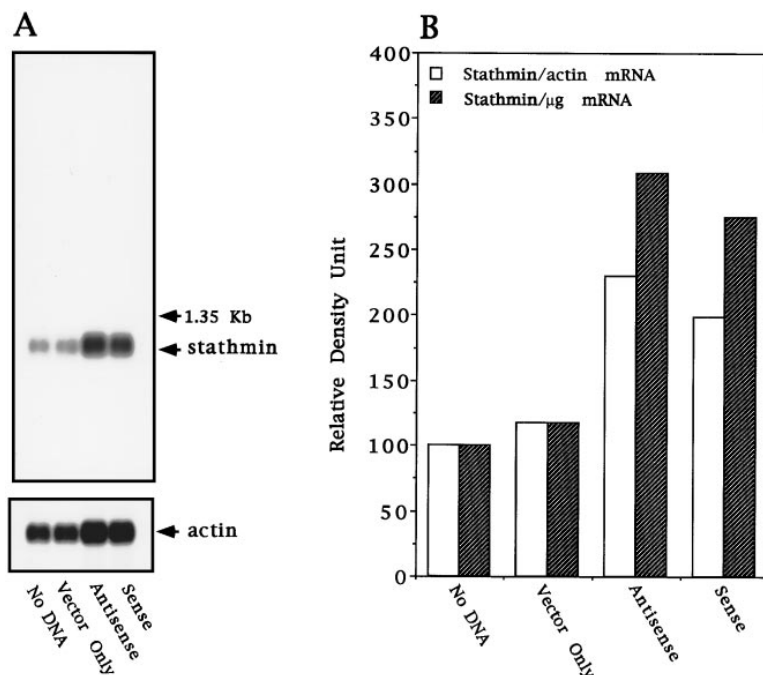


Figure 8. Effect of *tsg101* on Expression of Stathmin mRNA

(A) Northern blot analysis of stathmin mRNA. Polyadenylated mRNA was isolated from each transfected cell line, and 1 µg mRNA was loaded in each lane for Northern blotting. A 450 bp stathmin DNA fragment generated by polymerase chain reaction from a mouse cDNA library was used as probe; blots were then stripped and reprobbed with a 1.5 kb β-actin fragment for the internal control.

(B) Quantitation of stathmin mRNA, expressed as density units relative to β-actin mRNA. NIH 3T3 cells were transfected as indicated in Figures 6 and 7, which also describe the constructs used.

and neuroblastoma cells (Hanash et al., 1988; Brattsand et al., 1993; Roos et al., 1993). We observed increased stathmin mRNA in cells overexpressing *tsg101* antisense RNA or mRNA encoding the full-length protein, both of which conditions resulted in cell transformation.

The presence of a multiple DNA-binding domain in Tsg101 and the occurrence of a proline-rich region near the leucine-zipper DNA-binding motif in its coiled-coil region suggest that Tsg101 may function as a transcription factor (Mitchell and Tjian, 1989). The overall structure of Tsg101 is analogous to the structure of proteins encoded by nuclear oncogenes such as *fos* and *jun*, which are known to form heterodimers that bind to DNA and modulate transcription (Halazonetis et al., 1988; O'Shea et al., 1989b). Our finding of typical phosphorylation sites in multiple locations on Tsg101 suggests that the function of this protein may be regulated by phosphorylation, as has been shown for stathmin (Sobel, 1991).

While cells homozygously inactivated for *tsg101* gene expression by antisense RNA were selected by their transformed phenotype, we observed subsequently that overexpression of *tsg101* mRNA in the sense direction in naive NIH 3T3 cells also resulted in transformation, as assayed both by focus formation in monolayer cultures and by colony growth in soft agar. These foci and colonies, which were observed at a lower frequency than those generated by cells expressing *tsg101* antisense RNA, were abolished by frameshift mutations in the Tsg101 open reading frame. Our results suggest either that *tsg101* expression outside of a relatively narrow range can lead to cell transformation or, alternatively, that the *tsg101* clone we isolated and studied contains a dominant negative mutation that interferes with the function of coexisting wild-type genes. Such dominant negative effects were observed for early isolates of the p53 tumor suppressor gene, which, although ordinarily a down-regulator of cell growth, was identified initially by the ability of overproduced mutant p53 protein to cause cellular transformation (Finlay et al., 1988).

## Experimental Procedures

### Description of Vectors

pLLGSV is a pHHAM-derived (Hawley et al., 1987) retroviral vector lacking the 3' LTR promoter and enhancer and containing the  $\beta$ -*geo* reporter gene (Friedrich and Soriano, 1991), a fusion of the *E. coli lacZ* and aminoglycoside phosphotransferase (*aph* or "*neo*") genes, in the 3' LTR; duplication of this LTR during chromosomal integration places  $\beta$ -*geo* near the 5' end of the provirus. The SV40 T antigen minimal early promoter and 14 copies of the *E. coli lac* operator (Labow et al., 1990) are inserted 5' to an adenovirus-derived splice acceptor sequence placed 5' to  $\beta$ -*geo* and in an antisense direction to  $\beta$ -*geo*.

The transactivator vector pLLTX was derived from pHCMVLAP348 (Labow et al., 1990). As strong LAP348-inducible promoters are not effectively inhibited by isopropyl- $\beta$ -D-thiogalactopyranoside (Baims et al., 1991), pLLTX was engineered to allow ready excision of the transactivator by the Cre protein. It contains a HyTK gene expression cassette (Lupton et al., 1991) ligated into a HindIII site upstream of the transactivator gene; this is bracketed by *loxP* sites derived from pBS30 (Sauer and Henderson, 1989).

pLLEXP I, which was used to overexpress the *tsg101* gene in naive 3T3 cells, is a pBR322-derived construct that includes a puromycin resistance gene, *pac* (Morgenstern and Land, 1990), driven by a

human  $\beta$ -actin promoter, as a selection marker. 5' to *pac* is a cytomegalovirus promoter and SV40 poly(A) site. *tsg101* cDNAs were inserted between the cytomegalovirus promoter and polyadenylation site in either the sense or antisense direction, as indicated. The *tsg101* mutant, *tsg101*<sub>10-23</sub> (sense mutant 1), was generated by a BglII deletion of codons 10–23, which causes a frameshift that generates a translation stop signal 7 codons past the deletion terminus. *tsg101*<sub>254</sub> (sense mutant 2) was generated by a 4 bp insertion at codon 254, which produces a frameshift and a translation stop signal 20 codons later.

### Cell Culture and Transfection

NIH 3T3 cells (American Type Culture Collection) and GP+E-86 helper cells (Markowitz et al., 1988) were cultured in Dulbecco's modified Eagle's medium supplemented with 10% calf serum (3T3) or 10% newborn calf serum (GP+E-86), 100 U/ml penicillin, and 100  $\mu$ g/ml streptomycin. Transfection was carried out by electroporation (Potter et al., 1984), using the Cell-Porator Electroporation System I (Life Technologies, Inc.) or LipofectAMINE (Life Technologies, Inc.) as recommended by the manufacturer.

### Retroviral Infection of Mouse Fibroblast NIH 3T3 Cells

To generate infectious retrovirus, pLLGSV was linearized by treatment with *ScaI* and introduced into GP+E-86. Transfected cells were replated on day 3 and selected using 800  $\mu$ g/ml G418 for 2–3 weeks. G418-resistant clones were isolated and expanded individually in 24-well plates. Culture supernatant from each clone was incubated with NIH 3T3 cells in the presence of polybrene (8  $\mu$ g/ml) for 8 hr, and the frequency of integration behind an active chromosomal promoter was subsequently determined by X-Gal staining. Helper cell clones showing the highest frequency of integration were expanded, and the culture supernatant was collected for large-scale infection of NIH 3T3 cells.

### Isolation of Transformed Clones and Tumorigenicity Assays

Suspensions of G418-resistant NIH 3T3 cells prepared by trypsin treatment were transfected with pLLTX DNA by electroporation. Following selection in hygromycin (500  $\mu$ g/ml) for 12–18 days, hygromycin-resistant clones were plated onto 0.5% agar (Cifone and Fidler, 1980; Li et al., 1989) and the colonies that formed after 4–6 weeks were isolated and expanded to cell lines. Focus formation and soft agar assays of cellular transformation by antisense and sense constructs containing *tsg101* cDNA were done as described (Cartwright et al., 1987), except that cells were transfected using LipofectAMINE. Tumorigenicity was assayed by injection of  $10^5$  cells into nude mice (NIH nu/nu, female, and 6 weeks of age) subcutaneously over the lateral thorax. Animals were examined twice weekly and sacrificed 5 weeks later. Lung metastases and the neoplastic nature of local tumors were confirmed by histologic examination.

### cDNA Cloning and Screening of cDNA Library

For cDNA cloning, a biotin-labeled oligodeoxyribonucleotide (27 mer) that corresponds to the 5' end of the  $\beta$ -*geo* reporter gene was hybridized with polyadenylated mRNA from SL6 $\Delta$ T cells and captured with Streptavidin paramagnetic particles (Promega). Hybridizing mRNA was eluted and reverse-transcribed using a gene-specific primer that contained a uracil DNA glycosylase cloning site (Booth et al., 1994) and corresponded to a sequence located 5' to the biotin-labeled oligonucleotide. (dG)*n* was added to the 3' end of first-strand cDNA by terminal transferase, and double-strand cDNA was synthesized using a uracil DNA glycosylase-oligo d(C)<sub>20</sub> primer and DNA polymerase; this was inserted into the uracil DNA glycosylase-cloning vector pAMP1 (Life Technologies, Inc.), and the resulting clones were screened for fusion of upstream transcript sequences to  $\beta$ -*geo*. A 70 bp probe containing the sequence fused in-frame to the splice acceptor site 5' to  $\beta$ -*geo* was used to screen a mouse NIH 3T3 cDNA library (Stratagene). Both strands of positive clones were sequenced using Sequenase 2.0 (USB).

### Southern and Northern Blot Analysis

Genomic DNA was isolated by standard procedures. Total RNA was isolated with RNA STAT-60 (TEL-TEST), and poly(A)<sup>+</sup> mRNA was

isolated using PolyAtract (Promega). DNA and RNA blots were probed with single-strand DNA probes. Autoradiograms of Northern blots were scanned with Scanmaster 3™ (Howtek) and analyzed using the Quality One program (pdi).

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#### GenBank Accession Number

The accession number for the *tsg101* cDNA sequence is U52945.