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Gene Trapping Identifies Inhibitors of Oncogenic Transformation

THE TISSUE INHIBITOR OF METALLOPROTEINASES-3 (TIMP3) AND COLLAGEN TYPE I α2 (COL1A2) ARE EPIDERMAL GROWTH FACTOR-REGULATED GROWTH REPRESSORS*

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A gene trap strategy has been used to identify genes that are repressed in cells transformed by an activated epidermal growth factor (EGF)/EGF receptor signal transduction pathway. EGF receptor-expressing NIH3T3 cells (HER1 cells) were infected with a retrovirus containing coding sequences for the human CD2 antigen and for secreted alkaline phosphatase in the U3 region. By selecting for and against CD2 expression, we obtained clones in which the gene trap had integrated into genes selectively repressed by EGF. Two of these clones encoded for the secreted extracellular matrix proteins TIMP3 and COL1A2. We show here that both genes are downstream targets of RAS and are specifically repressed by EGF-induced transformation. Moreover, this strategy tags tumor suppressor genes in their normal chromosomal location, thereby improving target-specific screens for antineoplastic drugs.

Gene fusions between promoterless and/or enhancerless genes encoding easily assayable gene products with the controlling elements of cellular genes have provided valuable tools for studying gene function and regulation in prokaryotic and eukaryotic cells. Vectors developed for use in mammalian cells, referred to as "gene traps," insert a reporter gene into mostly random chromosomal sites, including transcriptionally active regions. By selecting for gene expression, recombinants are obtained in which the reporter gene is fused to the regulatory elements of endogenous genes. Transcripts generated by these fusions faithfully reflect the activity of the tagged cellular gene and thus provide an effective means to study the expression of genes in their normal chromosomal location. Moreover, gene traps are highly mutagenic both *in vitro* and *in vivo* and provide molecular tags to clone any gene whose function is linked to an observable phenotype (1–9).

Gene entrapment strategies have been successfully used to induce mutations in both cultured cells and transgenic mice. By using appropriate reporter systems, we and others (10–13) have identified genes that are either induced or repressed during important biological processes such as cellular differentiation or programmed cell death. To extend these observations, we sought to determine whether gene trapping would enable the recovery of genes with growth inhibitory and/or tumor suppressing functions. The basic assumption was that fusion transcripts induced between the selectable marker gene of a gene trap and the cellular sequences of a putative tumor suppressor gene would be repressed by an oncogenic signal. Such events can then be selected for by using a marker gene which allows selection for and against expression. Moreover, reporter genes expressed from the promoters of putative tumor suppressor genes should provide natural molecular targets for drug screening assays, thereby improving discovery of new anti-neoplastic drugs.

To test the suitability of this approach, a conditional transformation system was chosen that is based on a reversible activation of the epidermal growth factor receptor (EGFR[1]/HER1) signal transduction pathway. EGFR belongs to the HER family of tyrosine kinase receptors that have been implicated in the development and progression of several types of human cancer (14, 15). When overexpressed in NIH3T3 cells, EGFR transforms in a ligand-dependent manner and thus functions as an oncogene.

A gene trap retrovirus vector carrying coding sequences for the human CD2 cell surface receptor and for secreted human placental alkaline phosphatase (SEAP) in its U3 region was used to infect a subclone (HER1KA1) of an EGFR-expressing NIH3T3 cell line (16, 17). This subclone is highly and reversibly transformable by EGF. Selection for and against CD2 expression yielded clones in which the cell-provirus fusion transcripts were repressed by EGF-mediated transformation. Analysis of upstream proviral flanking sequences revealed that in two instances the gene trap integration had disrupted known genes. These genes code for the tissue inhibitor of metalloproteinases 3 (*Timp3*) (18) and for procollagen type α2 (*Col1A2*) (19).

We show here that *Timp3* and *Col1A2* are downstream targets of c-RAS within the EGF/EGFR signal transduction pathway. Both genes inhibit oncogenic transformation and are specifically repressed in EGF-transformed cells.

MATERIALS AND METHODS

Plasmids and Viruses—The pGgU3CD2SEAPen(−) plasmid was derived from pGgU3HisTkNeoen(−) (2) by replacing the *His* coding se-

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[1] The abbreviations used are: EGFR, epidermal growth factor receptor; EGF, epidermal growth factor; PBS, phosphate-buffered saline; SEAP, secreted alkaline phosphatase; TIMP, tissue inhibitor of metalloproteinases; COL1A2, collagen type I α2; DMEM, Dulbecco's modified Eagle's medium; RACE, rapid amplification of cDNA ends; IRES, internal ribosomal entry site; LTR, long terminal repeat; kb, kiolobase pair(s); SRBC, sheep red blood cells; Atc, anhydrotetracycline.

quences in the U3 region of the 3′-LTR with CD2-IRES-SEAP. *CD2* sequences were obtained from πPH3CD2 (20). *IRES-SEAP* sequences were recovered from pSBC2-SEAP (21). pGgU3CD2SEAPen(−) was transfected into GP + E86 producer cells as described previously (2). A clonal cell line producing 1×10^5 Neo^R colony-forming units/ml was isolated and used to infect HER1KA1 cells at a multiplicity of infection = 3.

Timp3 sequences obtained from BSTIMP3™ (NMI, San Diego, CA) were ligated as *Eco*RI fragments into the corresponding sites of the pBabepuro polylinker. pBabeTIMP3 was transfected into BOSC23 cells and virus containing supernatants were used to infect HER1KA1 and HER1KA1/6.1 cells as described previously (22).

The human Ha-RAS^{Leu-61} coding sequences were recovered from pZIPNeoSVH-ras-leu61 (23) and cloned into the *Eco*RI/*Hin*dIII sites of the expression vector pTBC1 downstream of a tet07/CMV promoter (24) to obtain pTBC-Ha-RAS^{Leu-61}SEAP.

Cell Cultures and Transfections—NIH3T3-HER1 cells (17) were grown in agar cultures containing 25 ng/ml EGF (Sigma), and subclones were isolated after 14 days of incubation. Transformation efficiency of each subclone was assessed by estimating anchorage-independent growth in agar cultures. One clone (HER1KA1) with a stable transformation efficiency of 40% colony-forming units was chosen for further analysis. For agar cultures, 10^5 cells suspended in 5 ml of 0.25% (w/v) agar/DMEM were plated into 100-mm bacterial dishes on top of a gelified 0.5% (w/v) agar/DMEM bottom layer. Both layers contained 10% (v/v) newborn calf serum. Developing colonies were counted after 14 days incubation.

Inducible Ha-RAS expressing cell lines were obtained by co-transfecting pTBC-Ha-RAS^{Leu-61}SEAP, pUHD15.1 and pTBC1-Hygro into NIH3T3 cells and selecting in 125 μg/ml hygromycin as described previously (25). Isolated clones were tested for anhydrotetracycline (Acros Chimica, Nidderau, Germany)-regulated p21-Ha-RAS expression by Western blotting, using the polyclonal anti-Ha-RAS antibody C20 (Santa Cruz Biotechnology, Santa Cruz, CA), and by reversible tumorigenicity *in vitro* and *in vivo*.[2] The cell line N59.4/15, with tightly regulated Ha-ras expression, was selected for further experiments.

HER1-v-*src* cells were obtained by electroporating the expression plasmid pMSsrc (26) into 3T3-HER1 cells and selecting for transformation using standard focus forming assays (25).

Panning and Antibody Treatment—For panning, 100-mm bacterial plates were first treated with 10 ml of a 20 μg/ml solution of anti-human IgG antibody (Cappel/Organon-Teknika, Durham, NC). After incubating for 1.5 h, the plates were washed with 0.15 M NaCl and overlaid with 10 ml of 1% (w/v) bovine serum albumin in PBS. After incubating overnight at 4 °C, the bovine serum albumin was removed, and plates were treated with 3 ml of a LFA3-IgG_1 containing supernatant from COS 6 cells transfected with pCDM8IgG_1-LFA3 as described (27). Cells (1×10^7) were placed onto the antibody-treated plates and allowed to bind for 2 h at room temperature. Unbound cells were removed by gently washing the plates with PBS. Bound cells were recovered in DMEM supplemented with 10% newborn calf serum (BioWhittaker, Walkersville, MD) after trypsinizing for 5 min at 37 °C. Typically, over 95% of the recovered cells expressed CD2 as estimated by SRBC rosetting and fluorescent antibody staining.

For complement lysis, Leu5b antibody (Becton-Dickinson, Heidelberg, Germany) was first allowed to bind the CD2 receptor by incubating 1–2×10^6 cells in 0.5 ml of PBS with 10 μl of Leu5b antibody for 30 min on ice. Cells were lysed by adding 1 ml of a 50% solution of rabbit complement (Cedarlane, Hornby, Ontario, Canada) as suggested by the manufacturer. Following incubation for 30 min at 37 °C, the complement was removed by several washings, and surviving cells were recovered in DMEM. In typical experiments, more than 90% of the cells were killed by antibody plus complement treatment.

CD2 Receptor Analysis—SRBC rosetting was performed by incubating aminoethylthioronium bromide-treated sheep red blood cells (SRBC) with CD2 expressing cells for 30 min at room temperature as described (28). CD2 receptor density was estimated as described by Lischke *et al.* (29). Briefly, 2×10^4 cells were treated in 100 μl of PBS containing 3% (w/v) bovine serum albumin and 10 μl of Leu5b antibody. Following incubation for 30 min on ice, cells were centrifuged at 400 × *g* and recovered in 100 μl of PBS containing 100 μg/ml horseradish peroxidase goat anti-mouse IgG (Dianova, Hamburg, Germany). After 30 min on ice, cells were washed 3 × in PBS and resuspended in 50 μl of 0.1 M Tris/HCl, pH 8.5. Fifty microliters of a solution containing the horseradish peroxidase-specific substrate were added to the cells, and light emission was measured for 1 s in a Micro-Lumat LB96 P luminometer (Berthold).

Estimation of SEAP Activity—SEAP activity was estimated in supernatants derived from 1×10^4 cells incubated in 0.5 ml of DMEM for 48 h at 37 °C. SEAP levels were quantified using the PhosphaLight kit (Tropix, Bedfort, MA) according to the manufacturer's instructions. Results are expressed in milliunits/ml calculated from a standard of human placental alkaline phosphatase (Calbiochem) as described (25).

Amplification of Upstream Sequences—5′-RACE was performed with 1 μg of total RNA using the 5′-RACE kit from Life Technologies, Inc., following the manufacturer's instructions. The specific CD2 reverse primers were as follows: 5′-CAAGTTGATGTCCTGACCCAAG-3′ and 5′-GGTTTCCAAGGCATTCGTAATCTC-3′ (nested). Amplification reactions were allowed to proceed for 20 cycles at 94 °C for 1 min, 55 °C for 1 min and 72 °C for 1 min, and 30 cycles at 94 °C for 45 s, 60 °C for 1 min, and 72 °C for 1 min. The RACE products were cloned into pCR2.1 vectors (Invitrogen, San Diego, CA) and sequenced as described previously (8).

RESULTS

Construction of a Gene Trap Retrovirus Expressing Two Selectable Genes from Dicistronic Transcripts (U3CD2SEAP)—A U3CD2SEAP gene trap vector was derived from the Moloney murine leukemia virus-based pGgtkNeoU3His(en−) (2) by replacing the *His* gene with coding sequences for the human cell surface receptor (CD2) and for the secreted human placental alkaline phosphatase (SEAP) (20, 21). To enable translation of both genes from dicistronic transcripts, the *SEAP* gene was separated from *CD2* by an internal ribosomal entry site (IRES) (21). This gene trap allows positive and negative selection as well as a quantitative monitoring of transcriptional activity. To enable selection for high virus titer producer cells, the viral construct also contained an independent selectable marker gene (*neo*) controlled by an herpes simplex virus thymidine kinase promoter between the LTRs (Fig. 1*A*) (2).

The plasmid was transfected into GP + E86 helper cells, and supernatants from neomycin-resistant lines producing recombinant virus were used to infect HER1KA1 cells. As has been shown in previous studies, virus replication and long terminal repeat (LTR)-mediated duplication places the sequences inserted into U3 just 30 nucleotides downstream from the flanking cellular DNA. This enables their expression from integrations into transcribed genes (Fig. 1*A*) (2, 30).

Selection for and against CD2 Expression Identifies Clones with U3CD2SEAP Provirus Integrations in EGF-responsive Genes—HER1KA1 cells infected with U3CD2SEAP retrovirus were selected for CD2 expression by "panning" using a fusion protein that contained the natural ligand of CD2 (LFA3) and human IgG1 (LFA3-IgG_1) (20, 31). The recovered cells, comprising at least 2×10^4 transcribed integrations, were exposed to transforming concentrations of EGF (25 ng/ml) (16) for 72 h and then treated with monoclonal anti-CD2-antibody and complement. This was expected to eliminate integrations into constitutively expressed genes and thus enrich for integrations into genes repressed by transformation. The surviving cells were subdivided into 48 pools which were assayed for EGF-regulated integrations by estimating at least two of the following parameters: (i) binding of aminoethylisothioronium bromide-treated SRBC to the CD2 receptor (SRBC rosetting) (28); (ii) CD2-receptor density using a recently described enzyme-linked immunosorbent assay for cell-surface receptors (29); and (iii) measuring SEAP levels in culture supernatants using a chemoluminometric assay (25). Two pools were selected and plated into semi-solid cultures to isolate clones. Twelve clones of each pool were expanded and analyzed by Southern blotting. The clones HER1KA1/6.1, HER1KA1/11, and HER1KA1/3 were unique and had 5, 1, and 1 proviral integrations, respectively (data not shown). Since HER1KA1/6.1 and HER1KA1/11 were particularly sensitive to EGF, they were chosen for fur-

[2] T. Beckers, E. Thoenes, and P. Hilgard, manuscript in preparation.

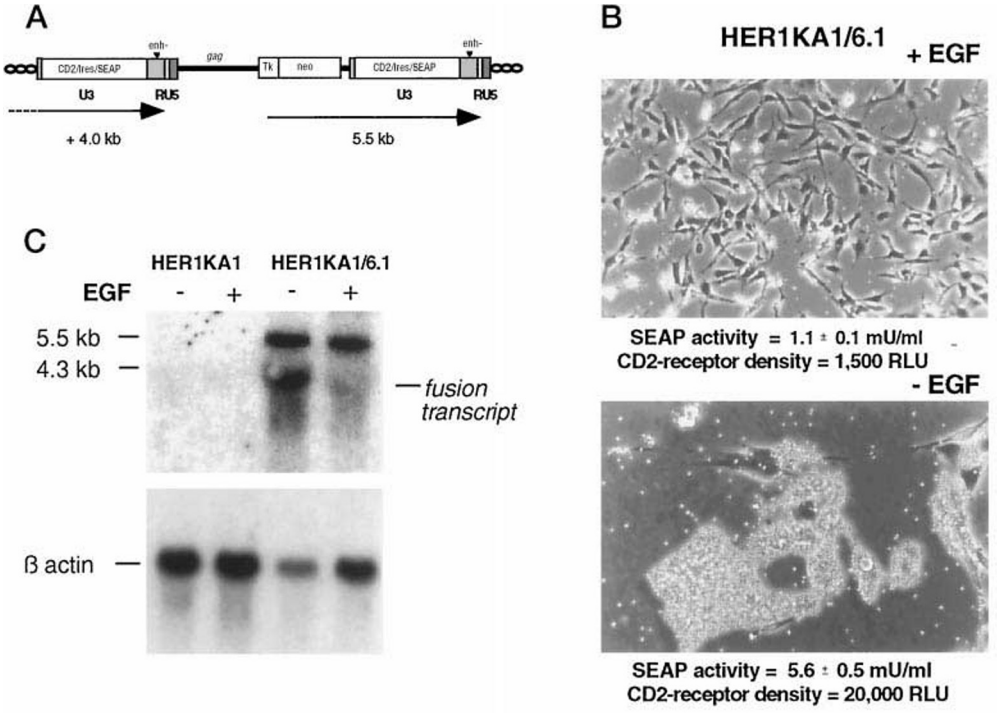

FIG. 1. **EGF-regulated gene trap expression in HER1KA1/6.1 cells.** *A,* structure of the U3CD2SEAP gene trap provirus and predicted transcripts. *B,* CD2 receptor and SEAP expression in HER1KA1/6.1 cells in presence (*top*) and absence (*bottom*) of EGF. Assays were performed with exponentially growing cells after incubating for 48 h with or without 25 ng/ml EGF. SRBC rosetting was examined using dark field microscopy at 200 × magnification. SEAP and CD2 receptor levels are shown below. Results are means ± S.D. from 2 to 3 independent experiments. *RLU*, relative light units. *C,* Northern blot analysis of proviral fusion transcripts. Total RNAs (10 μg/lane) were fractionated on formaldehyde-agarose gels, blotted onto nylon filters, and hybridized to ^{32}P-labeled internal ribosomal entry site (IRES)- and β-actin-specific probes.

ther analysis (Fig. 1*B* and data not shown).

To exclude the possibility that more than one provirus had disrupted an expressed gene in the HER1KA1/6.1 clone, mRNA recovered from both EGF-treated and untreated cells was analyzed by Northern blotting. When hybridized to an IRES-specific probe, two major transcripts were detected in absence of EGF, *e.g.* a 4.3-kb cell-provirus fusion transcript initiating in a nearby cellular promoter and terminating in the polyadenylation site of the 5′-proviral LTR, and a 5.5-kb transcript initiating at the internal thymidine kinase promoter and terminating in the polyadenylation site of the 3′-LTR (Fig. 1*A*). Although this suggests that CD2 might be translated from both transcripts, previous studies have clearly shown that only the reporter genes of the 5′-LTR are efficiently translated from cell-provirus fusion transcripts (1, 2, 32, 33). In contrast to the proviral transcripts that continued to be expressed in EGF-treated cells, fusion transcripts disappeared almost completely, suggesting that they were repressed by EGF (Fig. 1*C*). The results indicate that in HER1KA1/6.1 cells only one out of five proviral integrations is expressed from a nearby cellular promoter.

Proviruses in HER1KA1/6.1 and HER1KA1/11 Cells Disrupt Known Genes—To ascertain that the gene traps had disrupted expressed cellular genes in HER1KA1/6.1 and HER1KA1/11 cells, the cellular components of the respective cell-provirus fusion transcripts (Fig. 1*C*) were amplified by 5′-RACE by using CD2-specific primers (8). When the amplification product from HER1KA1/6.1 was hybridized to RNA from wild type HER1KA1 cells, it detected a major cellular transcript of 4.5-kb which was readily repressed by EGF (Fig. 2*A, lanes 1* and *2*). Similarly, sequences amplified from HER1KA1/11 cells hybridized to a cellular transcript of 5.2 and 5.7 kb (Fig. 2*B, lanes 1* and *2*).

Sequence analysis of the cloned HER1KA1/6.1 and

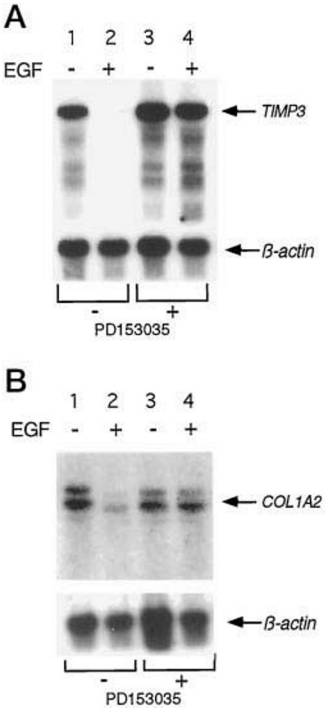

FIG. 2. **EGF-mediated repression of *Timp3* and *Col1A2* expression in wild type HER1KA1 cells.** *A*, Northern blot analysis of *Timp3* transcripts. Cells were incubated 48 h with or without EGF (25 ng/ml) (*lanes 1* and *2*) and with or without 1 μM EGFR tyrosine kinase antagonist PD153035 (*lanes 3* and *4*). RNAs (10 μg/lane) were hybridized on Northern blots to ^{32}P-labeled HER1KA1/6.1 (TIMP3) RACE product- or β-actin-specific probes. *B,* Northern blot analysis of *Col1A2* transcripts. The same RNAs (10 μg/lane) were hybridized to ^{32}P-labeled HER1KA1/11 (COL1A2) RACE product- and β-actin-specific probes. *Lanes 1* and *2* are without and *lanes 3* and *4* with PD153035.

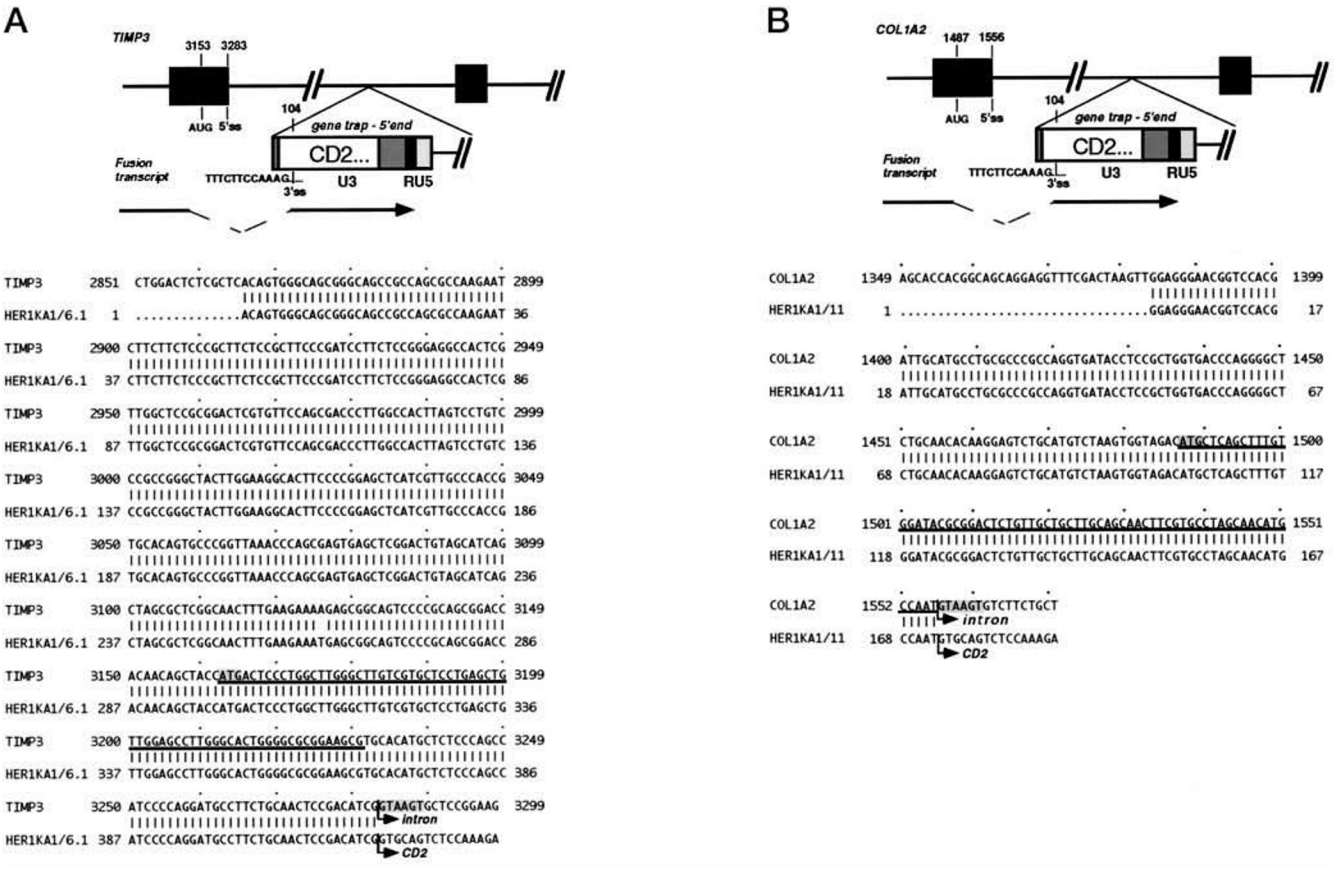

FIG. 3. **Gene trap activating mechanism in HER1KA1/6.1 and HER1KA1/11 cells.** *A* (*top*), predicted gene trap integration site in the first intron of the mouse *Timp3* gene (18). In frame fusion of *Timp3* and *CD2* sequences is achieved by splicing nucleotide 3283 of *Timp3* to nucleotide 104 of the provirus which eliminates the *CD2*-AUG. The 3′-splice consensus sequence of *CD2* is shown *below* the U3 region of the provirus. *Black boxes* are *Timp3* exons, and *solid lines* are *Timp3* introns. *Bottom*, sequence homology between the 5′-RACE amplification product of HER1KA1/6.1 cells and the first exon of *Timp3* (GenBank™ accession number U19462). The exon/intron junction of *Timp3* and the site of *Timp3*/*CD2* gene fusion are indicated by *arrows*. The AUG of the fusion transcript and the 5′-splice consensus sequence of *Timp3* are *shaded*. *B* (*top*), predicted gene trap integration site in the first intron of the mouse *Col1A2* gene (34). In frame fusion of *Col1A2* and *CD2* sequences is achieved by splicing nucleotide 1556 of *Col1A2* to nucleotide 104 of the provirus which eliminates the *CD2*-AUG. *Black boxes* are *Col1A2* exons, and *solid lines* are *Col1A2* introns. *Bottom*, sequence homology between the 5′-RACE amplification product of HER1KA1/11 cells and the first exon of *Col1A2* (GenBank™ accession number K01832). The exon/intron junction of *Col1A2* and the site of *Col1A2*/*CD2* gene fusion are indicated by *arrows*. The AUG of the fusion transcript and the 5′-splice consensus sequence of *Timp3* are *shaded*. Signal sequences for both genes are *underlined*.

HER1KA1/11 amplification products revealed a 100% homology to the first exon of the *Timp3* gene (18) and to the first exon of the *Col1A2* gene (Fig. 3) (19). Interestingly, both RACE products exhibited a 103-nucleotide deletion that included the cell DNA-provirus junctions, the translation initiating AUG of *CD2*, and the signal peptide encoding sequence of *CD2*. This resulted in an in frame fusion of the cellular sequence to the remaining *CD2* sequence, suggesting that the gene trap was activated by mRNA splicing. Indeed, as has been previously observed with other marker genes inserted into U3 regions,[3] the *CD2* gene contains a 3′-splice consensus sequence exactly at the site of fusion (Fig. 3). Thus, in both cases the gene trap must have integrated into the first introns of the respective cellular genes (Fig. 3). Moreover, the fusion proteins were still capable of LFA3 and anti-CD2 antibody binding. This suggested that the CD2 moiety was directed to the membrane by the fused signal sequences of the secreted TIMP3 and COL1A2 proteins. Indeed, as shown in Fig. 3, both RACE products contained the signal sequences of the respective proteins (Fig. 3, *underlined*) (34, 35).

Repression of TIMP3 and COL1A2 Requires EGF—To test whether TIMP3 and COL1A2 repression was linked to EGF or instead was a consequence of cellular transformation, we ana-

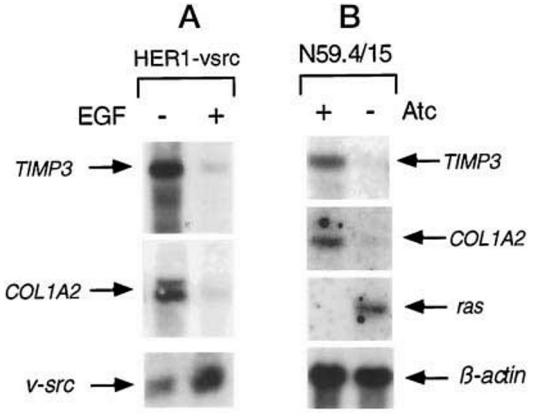

FIG. 4. ***TIMP3* and *COL1A2* expression in transformed cells.** *A,* Northern blot analysis of v-SRC transformed cells. HER1v-SRC cells were obtained by electroporating pMSsrc into HER1 cells as described previously (12) and by selecting for transformed clones. Transformed HER1v-SRC cells were exposed to 25 ng/ml EGF for 48 h. RNAs were hybridized on Northern blots to HER1KA1/6.1 (TIMP3) RACE product- (*top*), HER1KA1/11 (COL1A2) RACE product- (*center*), and v-SRC (*bottom*)-specific probes. *B*, Northern blot analysis of Ha-*ras*-transformed N59.4/15 cells. N59.4/15 were propagated in 10 ng/ml anhydrotetracycline (Atc) for 5 days to switch off *ras* expression. RNAs were hybridized on Northern blots to ^{32}P-labeled TIMP3 (*top*), COL1A2 (*center*), and β-actin-specific probes.

[3] H. E. Ruley, personal communication.

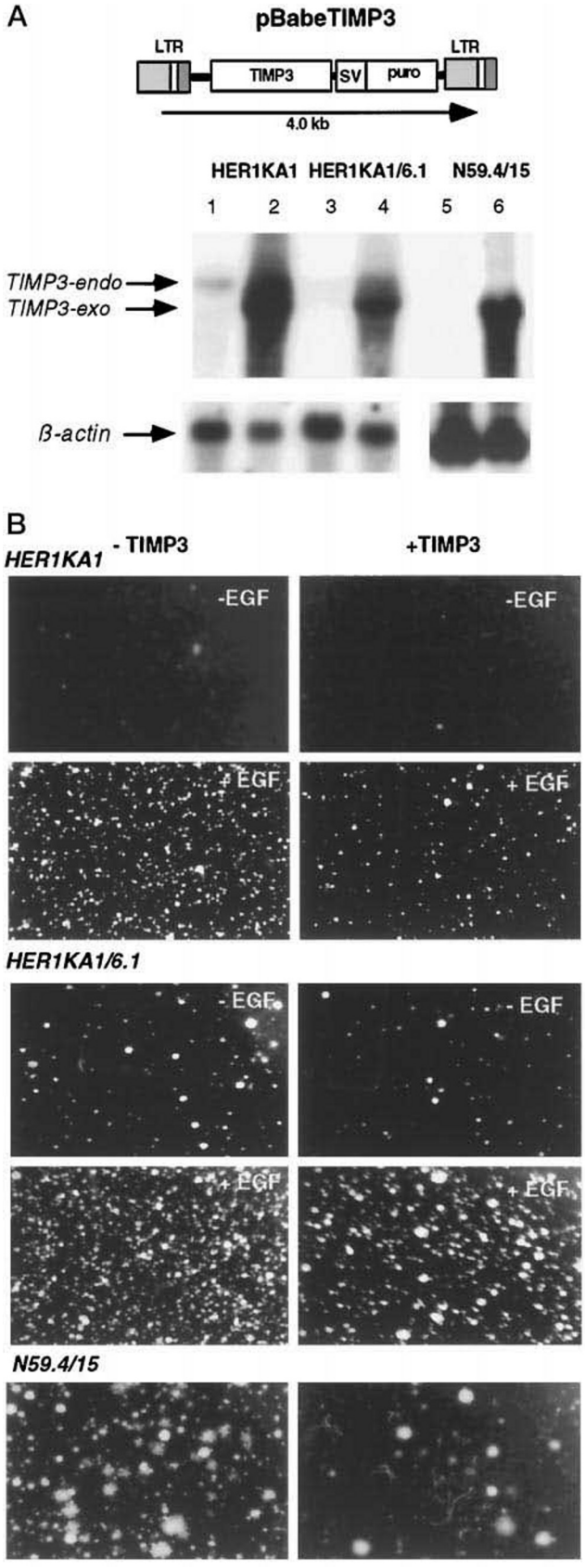

FIG. 5. **Exogenous *TIMP3* expression in HER1KA1-, HER1KA1/6.1-, and N59.4/15 cells.** *A* (*top*), structure of pBabeTIMP3 proviruses and predicted transcript. *Bottom*, Northern blot analysis of proviral TIMP3 transcripts. RNAs were hybridized to ^{32}P-labeled *Timp3* and β-actin probes. *Even* and *odd lanes* contain RNAs from cells infected with pBabeTIMP3 and pBabepuro, respectively. Note the almost complete repression of endogenous TIMP3 transcripts (*lanes 1, 3*, and *5*). *B,* colony formation in soft agar in presence and absence of exogenous TIMP3. HER1KA1-, HER1KA1/6.1-, and N59.4/15 cells were transduced with *Timp3* cDNA using the retroviral expression vector Babepuro (44). Cells obtained after selecting for 7 days in 5 μg/ml puromycin were grown in agar cultures as described under "Materials and Methods." Colonies were evaluated after incubating for 14 days using dark field microscopy at 40 × magnification.

lyzed their expression in presence of the EGF receptor kinase-specific antagonist PD153035 (36). As shown in Fig. 2 (*lanes 3* and *4*), PD153035 prevented *Timp3* and *Col1A2* transcripts from responding to EGF, indicating that EGFR signaling is required for repression.

To address the possibility of nonspecific repression, we transformed the original EGFR overexpressing HER1 cells with the v-*SRC* oncogene using the expression plasmid pMSsrc (26). Highly transformed cells were isolated from a focus, exposed to EGF, and analyzed by Northern blotting. Fig. 4*A* shows that HER1v-src cells continued to express *Timp3* and *Col1A2*. More importantly, both transcripts were readily repressed by EGF, indicating that v-*src*-induced transformation does not modify the response to EGF. We conclude that down-regulation of *Timp3* and *Col1A2* requires EGFR activation.

Since downstream EGFR signaling involves at least two distinct pathways, one of which includes the protooncogene c-*ras* (37, 38), we wanted to determine whether activated RAS could mimic the EGF-mediated suppression of *Timp3* and *Col1A2* expression. To this end, the transforming human Ha-*RAS*$^{Leu-61}$ oncogene (39) was transduced in NIH3T3 cells using an expression vector with a tetracycline-repressible promoter (24). A cell line (N59.4/15) expressing a tightly controlled Ha-RAS^{Leu-61} was used to analyze *Timp3* and *Col1A2* transcription. Withdrawal of anhydrotetracycline (Atc) resulted in oncogenic transformation associated with a 20-fold increase in p21$^{Ha-RAS-Leu-61}$ (data not shown) and in a significant reduction of *Timp3*- and *Col1A2* mRNA (Fig. 4*B*). A similar repression was observed in cell lines conditionally transformed with oncogenic c-*raf1* which is a downstream target of ras (data not shown). These results suggest that EGF engages c-*ras* to suppress *Timp3* and *Col1A2* expression.

TIMP3 Antagonizes the Transformed Phenotype of EGF and RAS-transformed Cells—While overexpression of COL1A2 has recently been shown to repress *ras*-induced transformation in NIH3T3 cells (40), it is still unclear whether TIMP3 has a similar effect in fibroblasts. Since TIMP3 has been implicated in both oncogenic transformation and tumor suppression in systems ranging from chicken embryo fibroblasts to human colon carcinoma cells (18, 41–43), it was important to determine whether TIMP3 would revert a transformed phenotype induced by EGFR signaling. To this end, *Timp3* coding sequences were cloned into the retrovirus expression vector pBabepuro (44) to obtain pBabeTIMP3 (Fig. 5*A*). pBabeTIMP3 was packaged into virus particles by transient transfection into BOSC23 cells (12) and supernatants thereof were used to infect HER1KA1, HER1KA1/6.1, and tetracycline-inducible Ha-RAS expressing N59.4/15 cells (Fig. 5*A*). Mixed populations of infected cells were recovered following selection in puromycin and assayed for focus formation and anchorage-independent growth in the presence or absence of EGF or tetracyclin. A 2–3-fold reduction of colony growth was observed in TIMP3 expressing HER1KA1 and N59.4/15 cells in which endogenous *Timp3* expression was repressed by EGF and activated *ras*, respectively. However, this effect was reduced in HER1KA1/6.1 cells both in the presence or absence of EGF (Table I, Fig. 5*B*). Since these cells exhibited a transformed phenotype even in absence of EGF (Table I, Fig. 5*B*), it is likely that mutations acquired by other genes antagonize the tumor suppressor func-

TABLE I

Repression of focus formation and colony growth in soft agar by TIMP3

10^5 cells were analyzed for focus formation and growth in soft agar as described under "Experimental Procedures." EGF concentrations were 25 ng/ml. Ha-RAS^{Leu-61} was induced in N59 4/15 cells by removing anhydrotetracycline (Atc) from the culture medium. Control cells were obtained by infecting with pBabepuro and selecting in puromycin. TIMP3 expressing cells were obtained by infecting with pBabeTIMP3 and selecting in puromycin. Results are means ± S.D. of 2–4 independent experiments.

Cells	−TIMP3			+TIMP3		
	Focus formation	Growth in soft agar	Efficiency of colony formation in soft agar	Focus formation	Growth in soft agar	Efficiency of colony formation in soft agar
			%			%
HER1KA1						
−EGF	15 ± 7	0	0	3 ± 1	0	0
+EGF	205 ± 7	40,000 ± 2,000	40	100 ± 22	21,000 ± 2,000	21
HER1KA1/6.1						
−EGF	64 ± 14	940 ± 85	0.94	38 ± 17	700 ± 28	0.70
+EGF	393 ± 30	54,000 ± 2,000	54	325 ± 65	44,000 ± 2,000	44
N59 4/15						
−Atc	0	0	0	0	0	0
−Atc	900 ± 82	59,000 ± 7,000	59	350 ± 147	21,000 ± 7,000	21

tion of TIMP3. Such mutations could have been induced by one or more of the multiple proviruses that had integrated in this clone. However, this seems quite unlikely because we and others (2, 33, 45) have previously shown that only one in 200–500 proviral integrations disrupt expressed cellular genes. In line with this, HER1KA1/6.1 cells expressed only one cell-provirus fusion transcript (Fig. 1*C*). Nevertheless, the ability of TIMP3 to suppress focus formation and anchorage-independent growth of all cell lines tested strongly suggests that TIMP3 has a growth inhibitory function in this system.

DISCUSSION

In the present study, a gene trap strategy has been used to identify genes that are repressed by an oncogenic signal. NIH3T3 cells undergoing EGF-mediated transformation were infected with a retrovirus carrying coding sequences for human *CD2* and *SEAP* in the U3 region. By selecting for and against CD2 expression, two cell clones were isolated in which the gene traps had disrupted known genes with tumor suppressor function. These genes encoded the secreted proteins TIMP3 and COL1A2, which are both components of the extracellular matrix. The extracellular matrix is increasingly implicated in the pathology of degenerative and/or neoplastic diseases. For example, TIMP proteins, which normally inactivate matrix metalloproteinases involved in degrading the structural components of the extracellular matrix (*e.g.* collagens, fibronectins, and gelatins), were found to prevent cell migration and metastasis (Refs. 46 and 47 and reviewed in Ref. 48). Moreover, depending on the experimental model, TIMP proteins can either promote or inhibit oncogenic transformation, suggesting that their function is cell type-specific (18, 41–43, 49). Also, inactivating mutations in TIMP3 lead to cell growth abnormalities (50) that can cause degenerative eye disease (51). Similarly, collagens participate in a variety of cellular processes such as differentiation, tumorigenesis, and apoptosis (52). Mutations in collagen genes can lead to a variety of degenerative disorders ranging from scorbut and osteogenesis imperfecta and in mice they cause recessive lethal phenotypes (reviewed in Ref. 53). In addition, overexpression of COL1A2 suppresses tumorigenesis induced by RAS-transformed NIH3T3 cells (40).

Thus, the gene trap strategy described here is well suited for tagging genes with tumor suppressor function, and it substantiates previous observations showing that putative tumor suppressor genes are often repressed in transformed cells (42, 54–56). Interestingly, the gene trap employed here seems to require a signal sequence for its activation, thereby enriching for integrations into genes encoding membrane and/or secreted proteins. A similar gene trap has been successfully used to identify membrane and secreted proteins important for mouse development (57).

In addition to antagonizing transformation in NIH3T3 cells, the present experiments have shown that *Timp3* and *Col1A2* are specific targets of the EGF/EGFR signal transduction pathway. Since EGF-mediated repression of both genes could be duplicated by oncogenic *ras* but not by v-*src*, it is likely that TIMP3 and COL1A2 are downstream targets of c-*ras*. Moreover, the persistence of *Timp3* and *Col1A2* expression in cells transformed by v-*src* seems to suggest that transformation by v-*src* does not require RAS (58). In summary, *Timp3* and *Col1A2* are specifically repressed by EGFR signaling, thereby facilitating oncogenic transformation. Therefore, it is likely that TIMP3 and COL1A2 interfere with the progression of human carcinomas expressing aberrant forms of one or more EGFR family receptor tyrosine kinases (14, 15).

A collection of cell lines with gene trap integrations in genes repressed by transformation are potentially highly useful as targets in automated screening assays for antineoplastic drugs. In particular, the easily quantifiable CD2 and SEAP marker proteins qualify such lines as faithful indicators of gene expression. Moreover, since the reporter genes are expressed from the normal chromosomal location of the trapped genes, the indicator lines avoid the problems encountered with conventional targets transcribed from random chromosomal sites. Finally, since the assays are based on gene activation, only target inducing drugs such as the EGFR antagonist PD153035 described in this study will score positively in a screen. This effectively eliminates all compounds with nonspecific toxic effects.

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