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Tropomyosin-2 cDNA Lacking the 3′ Untranslated Region Riboregulator Induces Growth Inhibition of v-Ki-*ras*-transformed Fibroblasts

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The levels of high molecular weight isoforms of tropomyosin (TM) are markedly reduced in *ras*-transformed cells. Previous studies have demonstrated that the forced expression of tropomyosin-1 (TM-1) induces reversion of the transformed phenotype of *ras*-transformed fibroblasts. The effects of the related isoform TM-2 on transformation are less clear. To assess the effects of forced expression of the TM-2 protein on *ras*-induced tumorigenicity, we introduced a TM-2 cDNA lacking the 3′ untranslated region riboregulator into *ras*-transformed NIH 3T3 fibroblasts. TM-2 expression resulted in a flatter cell morphology and restoration of stress fibers. TM-2 expression also significantly reduced growth rates in low serum, soft agar, and nude mice. The reduced growth rates were associated with a prolongation of G_0-G_1. To identify the mechanism of TM-2-induced growth inhibition, we analyzed the effects of TM-2 reexpression on ERK and c-jun N-terminal kinase (JNK) activities. Levels of ERK phosphorylation and activity in TM-2-transfected tumor cells were comparable to those in mock-transfected tumor cells. JNK activity was only modestly increased in *ras*-transformed cells relative to untransformed NIH 3T3 cells and only slightly reduced as result of forced TM-2 expression. We conclude that the partially restored expression of the TM-2 protein induces growth inhibition of *ras*-transformed NIH 3T3 cells without influencing ERK or JNK activities. Furthermore, the 3′ untranslated region riboregulator of the α-tropomyosin gene is not needed for the inhibition of *ras*-induced growth.

INTRODUCTION

Transformation of cells is accompanied by the reduced expression of cytoskeletal proteins such as α-actinin (Gluck *et al.*, 1993), vinculin (Rodriguez Fernandez *et al.*, 1992), and the tropomyosin (TM) isoforms 1, 2, and 3 (Matsumura *et al.*, 1983; Prasad *et al.*, 1993). Restoration of the expression of α-actinin (Gluck *et al.*, 1993), vinculin (Rodriguez Fernandez *et al.*, 1992), or TM-1 (Prasad *et al.*, 1993) by cDNA transfections suppressed the transformed phenotype of *ras*- or simian virus 40-transformed fibroblasts. Suppression of protein levels of either α-actinin, vinculin, or TM-1 with antisense constructs was sufficient to induce the transformed phenotype in nontransformed fibroblasts (Rodriguez Fernandez *et al.*, 1993; Gluck and Ben-Ze'ev, 1994; Boyd *et al.*, 1996). These data suggest that cytoskeletal proteins play an important regulatory role in malignant transformation of mesodermal cells.

The TM isoform TM-1 is encoded by the β-tropomyosin gene, and the isoforms TM-2 and TM-3 are encoded by the α-tropomyosin gene (Pittenger *et al.*, 1994). TMs form α-helical coiled-coil dimers that bind along actin filaments. These proteins are present in stress fibers in nonmuscle cells. Nonmuscle TM was recently shown to play a role in the localization of *oscar* mRNA, which is involved in anterior-posterior axis formation of *Drosophila* (Erdelyi *et al.*, 1995). Recently, TM-2 and TM-3 have been shown to play a role in the formation of stress fibers and focal adhesion plaques (Gimona *et al.*, 1996; Masuda *et al.*, 1996). Otherwise, little is known about the biochemical and functional properties of nonmuscle TM isoforms (Pittenger *et al.*, 1994).

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The mechanism by which TM gene expression modulates the malignant phenotype of transformed cells is unclear. Recent data suggest that a region in the 3′ untranslated region (UTR) of the TM transcript known as the riboregulator, rather than the TM protein, may be responsible for this effect. For example, Rastinejad *et al.* (1993) have shown that RNA from the α-tropomyosin 3′ UTR was sufficient to suppress tumorigenicity of the mutant myogenic cell line NMU2. Other studies have shown that transfection with TM-2 cDNA lacking the 3′ UTR coding region was not able to reduce anchorage independence of v-*raf*-transformed NRK cells (Takenaga and Masuda, 1994; Masuda *et al.*, 1996), suggesting that the 3′ UTR may be necessary to suppress tumorigenicity in these transformed cells. Indeed, all of the constructs used in the experiments cited above that resulted in tumor suppression contained the 3′ UTR (Rastinejad *et al.*, 1993). Therefore, the prospect that the effects induced by the reexpression or down-regulation of a particular cytoskeletal protein gene might be due to the 3′ UTR instead of the protein itself cannot be ruled out.

In the present report, we show that a TM-2 cDNA lacking all but the first 62 bp of the α-tropomyosin 3′ UTR is able to restore stress fibers and to suppress the growth of v-Ki-*ras*-transformed NIH 3T3 fibroblasts in low serum, soft agar, and nude mice. The reduced growth is due to retention of TM-2-transfected cells in G_0-G_1. These observations demonstrate that the 3′ UTR riboregulator of α-tropomyosin is not essential for the growth inhibition resulting from the transfection with TM-2 cDNA. Our results also suggest that the modulatory effects of TM-2 on the malignant phenotype of *ras*-transformed cells may be due to the interaction of TM-2 with factors involved in a Ras-dependent signaling pathway distinct from those involved in the ERK and JNK signaling cascades.

MATERIALS AND METHODS

Cell Culture

Nontransformed NIH 3T3 and v-Ki-*ras*-transformed NIH 3T3 fibroblasts (kindly provided by Dr. L. Feig, Tufts University, Boston, MA) were grown in DMEM containing 10% calf serum (BioWhittaker, Walkersville, MD).

Constructs and Transfections

The expression vector pCGE/TM-2 (Gimona *et al.*, 1995) was provided by Dr. D.M. Helfman (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY). This vector contains the human cytomegalovirus promoter, the herpes simplex virus (HSV) *tk* gene 5′ untranslated leader sequence and initiation codon, rabbit β-globin gene splicing and polyadenylation signals, and the replication origin of simian virus 40. The rat TM-2 cDNA is inserted downstream of the cytomegalovirus promoter and HSV *tk* leader sequence between the pCGE *Xba*I and *Bam*HI sites. This construct contains the entire TM-2 coding region with 62 bp of 3′ untranslated sequence and no 5′ UTR. Control vectors (pCGE) were created by removing the TM-2 cDNA by digestion with *Xba*I and *Bam*HI restriction enzymes and subsequent blunt-end ligation. The TM-2 cDNA encodes for a protein of 284 amino acids with an apparent M_r of 36,500. The rat TM-2 amino acid sequence is 100% identical to that of the mouse TM-2 protein (Takenaga *et al.*, 1988b; Goodwin *et al.*, 1991).

V-Ki-*ras*-transformed NIH 3T3 fibroblasts were transfected with pCGE or pCGE/TM-2 expression vectors in combination with the expression vector pHSVneo expressing the neomycin-resistance gene by using the Lipofectin transfection method. Colonies resistant to 500 μg/ml G418 (Geneticin, Life Technologies, Gaithersburg, MD) were isolated with cloning cylinders.

Monoclonal Antibodies

The monoclonal antibody TM-311 (Sigma, St. Louis, MO) recognizes protein sequences encoded by exon 1a of the TM genes α and β (Stamm *et al.*, 1993) and, therefore, reacts with fibroblast TM isoforms TM-1 (encoded by the β gene) and TM-2 and TM-3 (both encoded by the α gene; Pittenger *et al.*, 1994). Anti-mitogen-activated protein kinase (MAPK) monoclonal antibody sc94 (Santa Cruz Biotechnologies, Santa Cruz, CA) reacts with ERK-1 (p44) and ERK-2 (p42). Both free anti-JNK-1 (sc 474-G) and agarose-conjugated anti-JNK-1 (sc 474-AC) monoclonal antibodies were obtained from Santa Cruz Biotechnologies. Horseradish peroxidase-conjugated goat anti-mouse IgG was purchased from Bio-Rad (Richmond, CA).

Immunofluorescence

Cells were grown on coverslips and fixed for 20 min in 3.7% formaldehyde in piperazine-*N*,*N*′-bis(2-ethanesulfonic acid)-buffered saline (PiBS, pH 7.4). The cells were permeabilized in 0.5% Triton X-100 in PiBS and subsequently blocked with 0.2% bovine serum albumin in PiBS. Preparations were incubated with the TM311 antibody followed by incubation with fluorescein isothiocyanate-conjugated goat anti-mouse IgG antibody (Boehringer Mannheim, Indianapolis, IN) and analyzed by fluorescence microscopy. To identify actin stress fibers, samples were incubated with rhodamine-conjugated phalloidin (Sigma).

Western Blotting and TM-2 Expression

Confluent layers of cells in a 100-mm dish were washed twice with ice-cold phosphate-buffered saline. The cells were incubated for 20 min with 1 ml of ice-cold RIPA buffer (20 mM Tris, pH 8.0, 150 mM NaCl, 1% Triton X-100, 0.1% SDS) containing protease inhibitors leupeptin (1 mM), aprotinin (1 mM), and phenylmethylsulfonyl fluoride (1 mM). Triton X-100-soluble proteins were removed and Triton X-100-insoluble material was scraped into 500 μl of cytoskeleton buffer (10 mM Tris, pH 7.4, 2 mM EDTA, 1% 2-mercaptoethanol, 2% SDS) containing the above protease inhibitors. The samples were transferred to microcentrifuge tubes and 1 ml of water was added to reduce viscosity of the solution. Samples were boiled for 5 min and subsequently centrifuged for 15 min in a microcentrifuge. Protein content was determined by the Pierce bicichoninic acid protein assay according to the manufacturer's instructions. Equal amounts of proteins (30–50 μg) were electrophoresed on a 12.5% gel and proteins were transferred to a nitrocellulose membrane. Membranes were blocked with 5% nonfat milk in TBS-T (10 mM Tris 8.0, 150 mM NaCl, 0.1% Tween 20) and incubated with the TM-311 monoclonal antibody in TBS-T followed by incubation with horseradish peroxidase-conjugated goat anti-mouse IgG. TM-2 was detected by enhanced chemiluminescence using the ECL kit from Amersham (Arlington Heights, IL). The intensity of the bands was determined by densitometry (LKB Ultroscan XL).

Northern Blotting

Cells were lysed with Trizol (Life Technologies) and total RNA was isolated with chloroform extraction. Equal amounts of RNA were electrophoresed on a formaldehyde/agarose gel and transferred to

a nitrocellulose membrane. To identify endogenous TM-2 mRNA, membranes were probed with a 449-bp region of the mouse 3′ UTR of TM-2 (192–641 bp downstream of the stop codon). To identify endogenous TM-1, mRNA membranes were probed with rat TM-1 cDNA. The probes were labeled with a random prime labeling kit (Ready to Go, Pharmacia, Piscataway, NJ).

Proliferation and Serum Independence Assays

Cells were plated in 6-well tissue culture plates (Costar, Cambridge, MA) at 5×10^5 cells/well in DMEM with 10% calf serum. For the serum independence assays, this medium was replaced after 1 d with medium containing only 0.25% or 0.5% calf serum as indicated. Cell numbers were counted after trypsinization using a hemocytometer.

Anchorage Independence Assays

One thousand cells were seeded into 0.33% semisolid agar medium (DMEM containing 10% calf serum), which was subsequently poured on top of a 0.6% agar bottom layer. New 0.33% semi-solid agar medium was added every 2 or 3 d. After 3 wk, the numbers of colonies larger than 50 μm were counted.

In Vivo Tumor Growth

Cells (1×10^6) were injected s.c. into the flanks of female athymic BALB/c mice, ages 6–8 wk (Charles River Labs, Wilmington, MA). Mice were killed, and the tumors were excised and weighed when the largest tumor reached a size of 1 cm^3.

Cell Cycle Analysis

Cells were serum starved by reducing the concentration of serum in the medium to 0.5% thirty h before analysis. Cells were trypsinized and cell pellets were resuspended in 70% EtOH at 4°C and incubated overnight. Cells were then pelleted and incubated for 30 min at 4°C in a propidium iodide solution (50 μg/ml propidium iodide, 100 Kunitz U/ml RNase A, 1 mg/ml glucose in phosphate-buffered saline). Cells were analyzed on a FACScan (Becton Dickinson, San Jose, CA), and distribution of the cell cycle phases was analyzed using ModFit software (Verity Software, Topsham, ME) in which G_1 and G_2-M were mathematically fitted to Gaussian curves.

In-Gel MAP Kinase Assay

This assay was performed according to the protocol of Kameshita and Fujisawa (1989). Cells were serum starved by reducing the concentration of serum in the medium to 0.5% twenty h before each experiment. Cells were washed twice with ice-cold PBS. Subsequently, whole-cell extracts of the cells were prepared by scraping the cells into ice-cold RIPA buffer (20 mM Tris, pH 8.0, 137 mM NaCl, 5 mM EDTA, 10% glycerol, 1% Triton X-100) containing protease and phosphatase inhibitors (1 mM leupeptin, 1 mM aprotinin, 1 mM phenylmethylsulfonyl fluoride, 1 mM Na_3VO_4, 1 mM EGTA, 10 mM NaF, 1 mM sodium pyrophosphate, 1 mM β-glycerophosphate). Samples were centrifuged for 15 min at 4°C and the supernatant was transferred to new tubes. Equal amounts of protein (30–50 μg) were electrophoresed on a 10% SDS-polyacrylamide minigel in which the MAPK substrate myelin basic protein (MBP) was copolymerized (0.5 mg/ml). SDS was washed away by rinsing the gel in 20% isopropanol in 50 mM Tris (pH 8.0) for 30 min at room temperature (RT). These rinses were repeated with fresh buffer four times. Proteins were then denatured first by incubating the gel for 1 h in buffer A (50 mM Tris, pH 8.0, 5 mM 2-mercaptoethanol) at RT followed by two changes of buffer A containing 6 M guanidine hydrochloride, each for 30 min at RT. Renaturation was then accomplished by incubating the gel in five changes of buffer A containing 0.04% Tween 40 for 16 to 20 h in total at 4°C. The kinase reaction was performed by placing the gel for 1 h at RT in 20 ml of kinase buffer (40 mM HEPES, pH 7.8, 2 mM dithiothreitol, 0.1 mM EGTA, 5 mM $MgCl_2$, 50 μM ATP) containing 50 μCi of [γ-^{32}P]ATP. After the in-gel kinase reaction, the gels were extensively washed with multiple changes of washing buffer (5% trichloroacetic acid, 1% sodium pyrophosphate) until no radioactivity was detectable in the washing buffer. The gel was dried, and phosphorylated MBP was visualized by exposing the gel to x-ray film.

Solid-Phase JNK Assay

JNK-1 was immunoprecipitated from whole-cell lysates with anti-JNK-1 monoclonal antibodies conjugated to agarose beads (sc 474-AG, Santa Cruz Biotechnology). Beads were washed three times with phosphate-buffered saline/1% Triton X-100 and once with kinase buffer (50 mM Tris, pH 7.4, 10 mM $MgCl_2$, 1 mM dithiothreitol). Beads were incubated with 30 μl of kinase buffer containing 0.1 μCi/μl [γ-^{32}P]ATP, 50 μg/μl glutathione *S*-transferase coupled to c-jun(1–78) [GST-c-jun(1–78)] (kindly provided by Dr. E. Paulson, Tufts University), and 25 μM ATP at 30°C for 15 min. A mutant GST-c-jun(1–78) in which the serine residues at positions 63 and 73 were replaced by alanine (also provided by Dr. E. Paulson) was used as a control. Reactions were stopped by adding 10 μl of a 5× Laemmli buffer and samples were boiled for 3 min. Samples were loaded on a 10% SDS-polyacrylamide minigel. The gel was dried, and phosphorylated c-jun was visualized by exposing the gel to x-ray film. The amount of JNK-1 in each immunoprecipitate was determined by running aliquots of the samples on a separate SDS-polyacrylamide gel. Western blots were probed with anti-JNK-1 antibody (sc 474-G, Santa Cruz Biotechnology) and JNK-1 was visualized by chemiluminescence as described above.

RESULTS

Restoration of TM-2 Expression in ras-transformed Fibroblasts

Western blot analyses using the TM311 antibody showed TM-2 to be the predominant high molecular weight TM isoform in the particular NIH 3T3 fibroblast cell line used in our studies (Figure 1). In each cell type examined (nontransformed NIH 3T3, *ras*-transformed cells, and TM-2 transfectants), each of the three high molecular weight TM isoforms was predominantly associated with the Triton X-100-insoluble (cytoskeletal) fraction. The relative expression levels of TM isoforms in the Triton X-100-soluble fraction (cytoplasm) were similar to those in the cytoskeletal

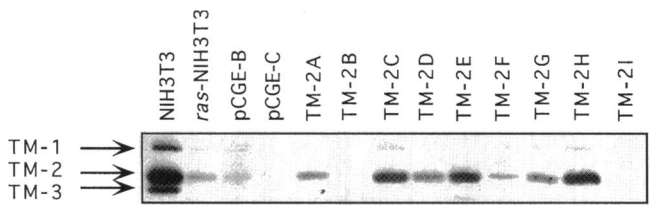

Figure 1. TM protein levels in NIH 3T3 fibroblasts. Western blots of Triton X-100-insoluble (cytoskeletal) proteins of the indicated clones (nontransformed NIH 3T3, *ras*-transformed NIH 3T3, control-transfected tumor cell lines pCGE-B and pCGE-C, and TM-2-transfected tumor cell lines TM-2A through TM-2I) were stained with the TM-311 monoclonal antibody, which recognizes the TM-1, TM-2, and TM3 isoforms. Arrows indicate the positions of the various isoforms.

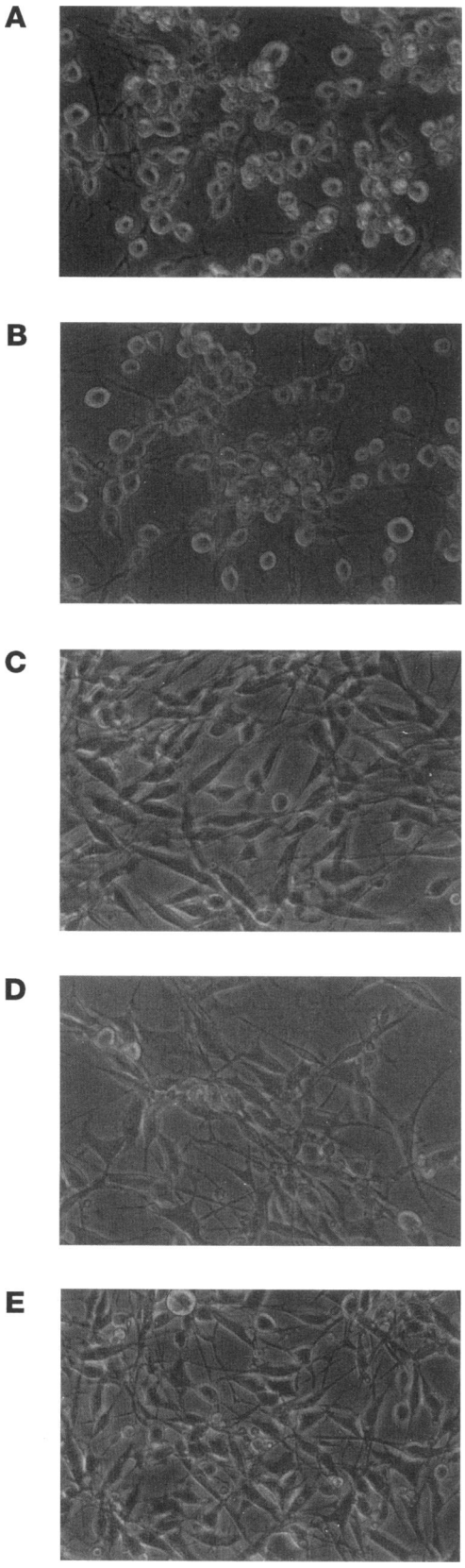

Figure 2.

fraction. Figure 1 shows the expression levels of the TM isoforms in the Triton X-100-insoluble (cytoskeletal) fraction of the cell.

The expression of isoforms TM-1, TM-2, and TM-3 is greatly reduced in v-Ki-*ras*-transformed NIH 3T3 fibroblasts relative to nontransformed cells (Figure 1). TM-2 protein levels in *ras*-transformed NIH 3T3 fibroblasts are in fact less than 1% of the levels present in nontransformed cells, as determined by densitometry. Transfection of v-Ki-*ras*-transformed fibroblasts with TM-2 cDNA lacking the 3′ UTR riboregulator resulted in two low expressors (clones TM-2A and TM-2F) with TM-2 protein levels of approximately 5% of that of nontransformed cells, three intermediate expressors (clones TM-2C, TM-2D, and TM-2G) with TM-2 protein levels of 10–25% of that of nontransformed cells, and two high expressors (clones TM-2E, and TM-2H) with TM-2 protein levels of 40–60% of that of nontransformed cells. The TM-2 level in TM-2E was not maintained with protracted cell culture. TM-2 levels of clones TM-2B and TM-2I were comparable to that of the nontransfected *ras*-transformed cells (Figure 1). No clones were isolated with expression levels equal to or higher than those in nontransformed NIH 3T3 cells. Cell lines transfected with the empty pCGE vector (clones pCGE-B and pCGE-C) had TM levels comparable to that of the parental cell line (Figure 1).

Endogenous levels of TM-1 and TM-2 mRNA were not affected by transfection with TM-2 cDNA (our unpublished data).

TM-2 Induces Reversion of the Transformed Phenotype of ras-transformed Fibroblasts

To assess the effects of forced TM-2 expression on the phenotype of *ras*-transformed fibroblasts, we compared the morphology, proliferation rates in low serum, anchorage-independent growth, and in vivo tumor growth in TM-2- and pCGE-transfected v-Ki-*ras*-transformed NIH 3T3 cells.

Light microscopic analysis showed that cells of the TM-2-transfected tumor cell lines have a flatter morphology than cells transfected with control vectors, pCGE-B and pCGE-C, which have a round morphology (Figure 2).

The flatter morphology correlates with the restoration of stress fibers in TM-2-transfected cells. Immunofluorescent staining of cells grown on coverslips with the anti-TM antibody TM311 shows that TM is organized in stress fibers in nontransformed NIH 3T3

Figure 2 (cont). Morphology of mock-transfected clones pCGE-B (A) and pCGE-C (B) and TM-2-transfected clones TM-2C (C), TM-2G (D), and TM-2H (E). Cells were grown in DMEM with 10% calf serum. TM-2-transfected clones shown are representative of all TM-2-transfected clones with increased TM-2 protein levels.

cells but not in parental tumor cells or in tumor cells transfected with the control vector only (Figure 3,A and B). Transfection of the tumor cells with TM-2 cDNA restored the organization of TM in stress fibers (Figure 3C). Similar results were obtained with the use of rhodamine-conjugated phalloidin in which case the TM-2-transfected, but not the *ras*-transformed, cells were shown to have stress fibers.

The proliferation rates of TM-2-transfected cell lines did not differ significantly from those of the control clones when the cells were grown in medium with 10% serum. However, growth of TM-2-transfected clones was significantly reduced compared with that of control clones when the concentration of serum was lowered to 0.5 or 0.25% (Figure 4). By d 5 of culture in low serum, the untransformed NIH 3T3 cells were dead, whereas the mock-transfected tumor cells had reached densities of 2.1–2.7 million cells. The proliferation rates of pCGE-transfected tumor cells grown in medium with 0.5 or 0.25% serum were not significantly different from those of untransfected controls. TM-2-transfected cells did not die in medium with reduced serum but showed a delayed onset of proliferation and a decreased rate of growth. By d 5 the TM-2-transfected cells had reached densities of 0.6–2.1 million cells in medium supplemented with 0.5% serum and 0.5–1.25 million cells in medium supplemented with 0.25% serum. The reduced growth rates of the TM-2-transfected cell lines in low serum correlate with a prolongation of G_0-G_1 (Figure 5). After serum starvation for 30 h, an average of 39% of the cells of the control cell lines (range, 37.2–40.3%) were in the S-phase or G_2-M. In contrast, only 17% of the cells of the TM-2-transfected cell lines (range, 11.7–21.2%) were in the S-phase or G_2-M phase. Ten percent of nontransformed NIH 3T3 cells were in the S-phase or G_2-M phase.

Anchorage-independent growth in soft agar was also affected by TM-2 expression, although this effect was predominantly manifested as reduced colony size rather than in a reduction in the number of colonies formed. Several lines tested behaved in a manner suggesting that TM-2 expression suppressed colony formation. For example, TM-2H (a high expressor) and TM-2D and TM-2G (both intermediate expressors) formed significantly fewer colonies than the control clones pCGE-B, pCGE-C, and TM-2B (no TM-2 expression), especially in low (5%) serum, and the lines with low TM-2 levels (TM-2A and TM-2F) were highly clonogenic (Figure 6). However, TM-2C (an intermediate expressor) also formed large numbers of colonies, suggesting that the perceived differences in clonogenicity among the various TM-2 transfectants may be attributable to factors other than variable TM-2 expression. However, it should be pointed out that, despite the moderate level of TM-2 expression observed in TM-2C cells grown in monolayers, the levels

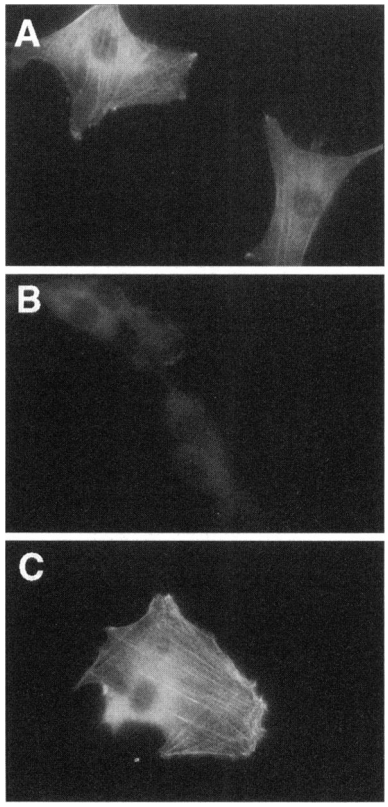

Figure 3. Cytoskeletal stress fiber formation in NIH 3T3 fibroblasts. Cells were grown in medium supplemented with 10% serum on coverslips and stained for TM (see MATERIALS AND METHODS). (A) Nontransformed NIH 3T3 fibroblasts. (B) *ras*-transformed clone pCGE-B. (C) TM-2-transfected tumor cell line TM-2H (representative of all TM-2-transfected clones except TM-2F, which did not show stress fibers).

detected in TM-2C cells isolated directly from soft agar colonies were much lower and in fact were comparable to that of *ras*-transformed cells. In fact, this was true for all TM-2-transfected clones grown in soft agar. This finding suggests that growth in soft agar may have selected for TM-2 subpopulations less able to express TM-2.

In contrast to the inconsistent results obtained from studies in which the number of colonies generated in agar served as end point, the results obtained from colony size measurements clearly demonstrate that TM-2 expression interferes with anchorage-independent growth. Day 21 colonies from all cell lines with intermediate or high TM-2 protein levels were significantly smaller on average than those of the control cell lines pCGE-B, pCGE-C, and TM-2B ($p < 0.001$; Student's *t* test), especially when the soft agar assays were performed in 5% instead of 10% serum (Figures 7 and 8, A–D). This serum dependency was less apparent for the control cell lines, which formed colonies of the same size in both 5 and 10% serum. The cell line

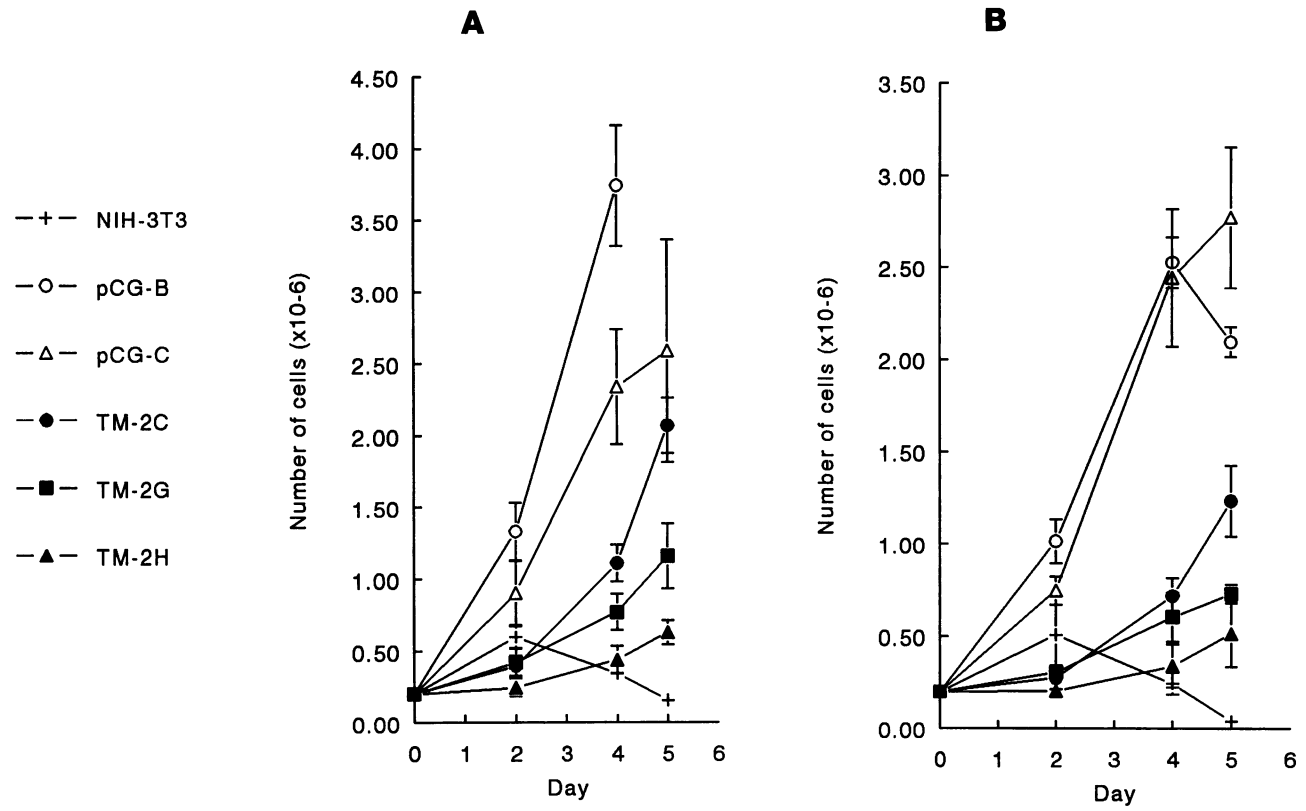

Figure 4. Serum-independence assays. Approximately 250,000 cells of the indicated clones were seeded in duplicates in a 6-well plate in DMEM with 10% calf serum. The following day, the medium was replaced with DMEM with either 0.5% (A) or 0.25% (B) calf serum. The cells were then trypsinized and counted with a hemocytometer. On each day, two wells were counted in duplicate. Means ± SD are plotted.

TM-2F, with low TM-2 protein level, is especially illustrative of this serum dependency. In 10% serum, the average colony diameter was comparable to that of the control cell lines, but in 5% serum, the colonies formed by TM-2F cells were significantly smaller ($p < 0.001$) than those of the control cell lines (Figures 7 and 8, E and F).

To test tumor growth in vivo, cells of TM-2-transfected, mock-transfected, and parental *ras*-transformed clones were injected into the flanks of nude mice. Although the cell lines TM-2C, TM-2G, and TM-2H were still able to induce tumor growth, these tumors were significantly smaller than those induced by the control cell lines (Table 1).

Effects of TM-2 Reexpression on ERK and JNK Activation in ras-transformed Fibroblasts

Two of the major signaling pathways downstream of Ras are the Raf/ERK and the Rac/JNK pathways. Both pathways have been implicated in *ras*-induced transformation. The kinases ERK and JNK are dually phosphorylated on their threonine and tyrosine residues by MEK and SEK1, respectively. Phosphorylation of both residues is required for activation of the kinases. To study the possible effects of TM-2 reexpression on the ERK and JNK pathways in *ras*-transformed fibroblasts, ERK in-gel kinase activity assays and JNK solid-phase assays were performed as described in MATERIALS AND METHODS. To prevent possible activation of both pathways by serum components, cells were serum starved for 20 h prior to analysis.

In-gel kinase activity assays showed that *ras*-transformed cells constitutively express ERK-1 and ERK-2 kinase activities, whereas the nontransformed NIH 3T3 cells contained little or no ERK-1 or ERK-2 kinase activities (Figure 9, lanes 1 and 2). Transfection of tumor cells with either pCGE or pCGE/TM-2 expression vectors induced a slight increase in ERK-1 and ERK-2 kinase activities compared with the parental *ras*-transformed cells (Figure 9, lanes 2–7). However, there was no difference in the levels of ERK-1 or ERK-2 kinase activities between TM-2 transfected tumor cells and pCGE-transfected tumor cells.

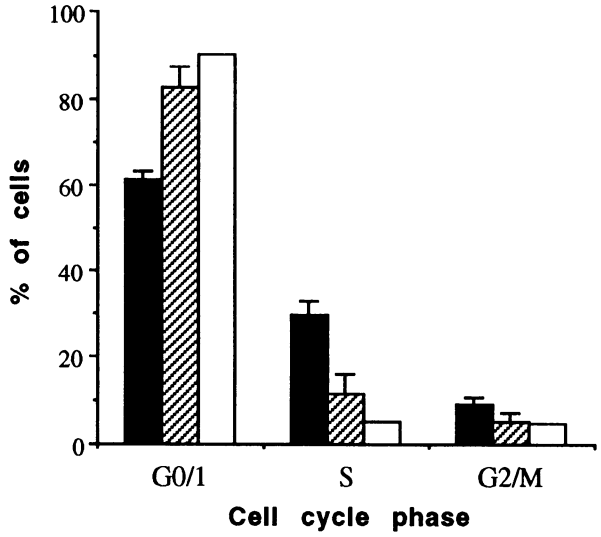

Figure 5. Cell cycle analysis. Cell cycle phase distribution was determined as described in MATERIALS AND METHODS. The average ± SD of each cell cycle phase is plotted for control transfected clones (average of clones pCGE-B and pCGE-C; solid bars), TM-2-transfected clones (average of clones TM-2A, TM-2D, TM-F, TM-2G, and TM-2H; striped bars), and nontransformed NIH 3T3 cells (open bars).

Solid-phase JNK assays revealed only a twofold to threefold increase in JNK activity in *ras*-transformed NIH 3T3 cells relative to untransformed fibroblasts, and this increase was only slightly diminished by forced expression of TM-2 (Figure 10). The extent of JNK activation in 3T3 cells associated with *ras* transformation is quite modest relative to that achieved by exposure to sorbitol, which typically increases JNK activity 20-fold or more. Assays similar to those shown in Figure 10 were carried out with a mutant GST-c-jun(1–78) fusion protein in which the serine residues at positions 63 and 73 were replaced by alanines. This mock JNK substrate was not phosphorylated by the JNK precipitated from the various *ras*-transformed cells.

DISCUSSION

Cellular transformation by *ras* oncogenes is associated with a marked decrease in the expression of several cytoskeletal proteins, including the high molecular weight TM isoforms (TM-1, TM-2, and TM-3; Matsumura *et al.*, 1983). The restoration of TM-1 levels resulting from the transfection of TM-1 cDNA into *ras*-transformed fibroblasts was recently shown to induce tumor suppression (Prasad *et al.*, 1993). Suppression of protein levels of TM-1 with antisense constructs was sufficient to induce the transformed phenotype in nontransformed fibroblasts (Boyd *et al.*, 1996). These observations indicate that the diminution in TM expression associated with an activated *ras* oncogene may be essential for the expression of the malignant phenotype in *ras*-transformed cells.

The exact functions of the various TM isoforms, in particular TM-2, in nonmyogenic cells are poorly understood (Pittenger *et al.*, 1994). It has recently been shown that restoration of TM-2 levels in v-Ki-*ras*-transformed NRK cells restores stress fibers and vinculin-containing focal adhesion plaques and increases

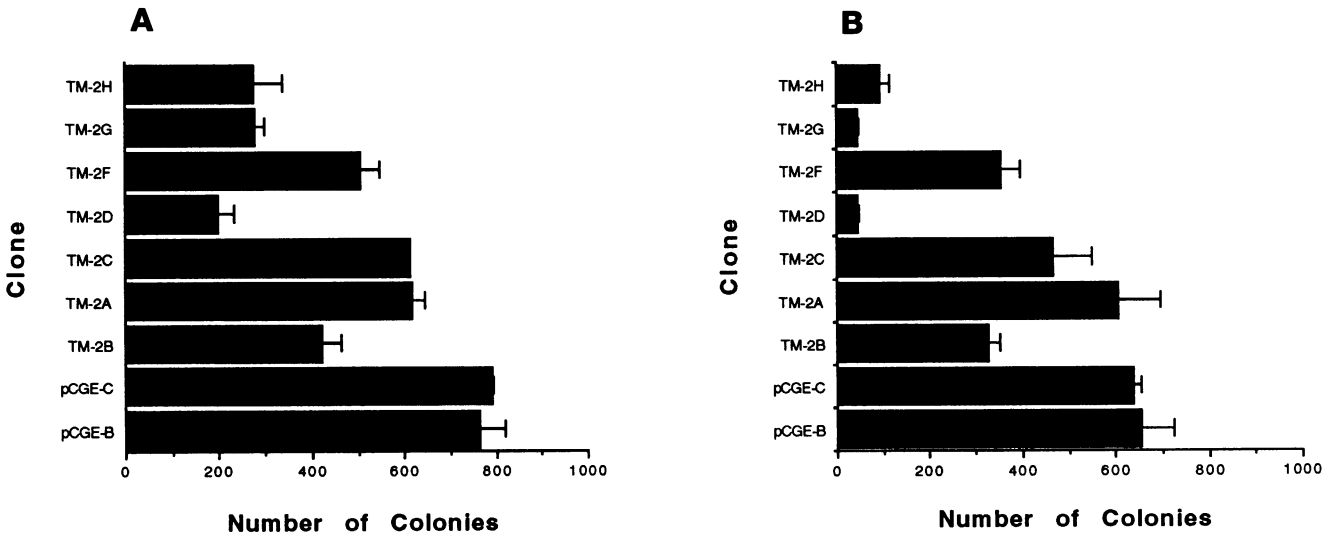

Figure 6. Anchorage-independence assay. Approximately 1000 cells of the indicated clones were seeded in 0.33% semi-solid agar medium on top of a 0.6% agar bottom layer. Cells were fed every 2 or 3 d. After 21 d, the numbers of colonies larger than 25 μm were counted. (A) Number of colonies in medium with 10% serum. (B) Number of colonies in medium with 5% serum.

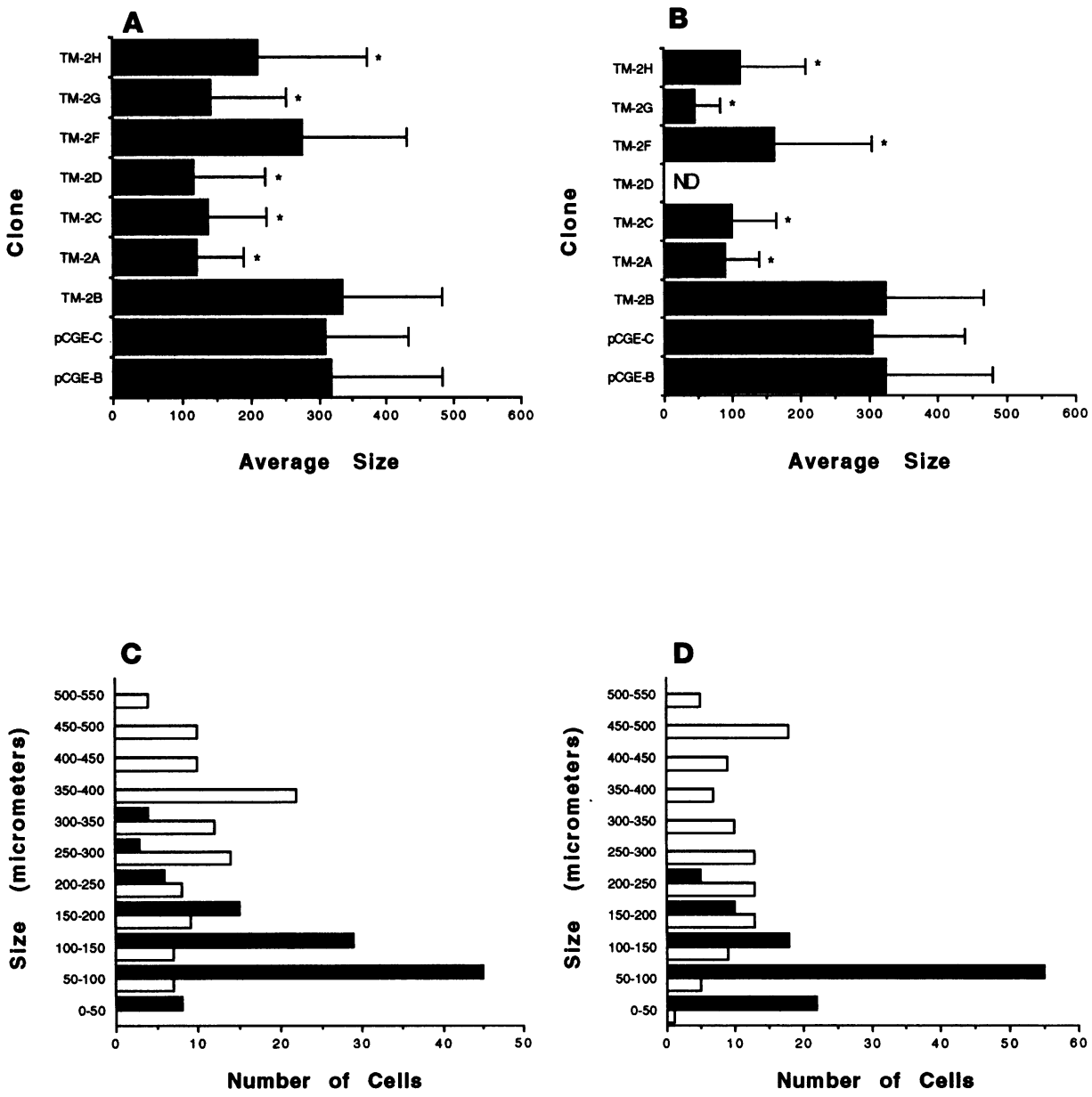

Figure 7. Colony size. Assays were performed as described in the legend of Figure 6. Colony diameters were determined with a micrometer. More than 100 colonies were analyzed for each clone if possible. Otherwise, all clones were analyzed. (A) Size of colonies in medium with 10% serum. *Significantly different from control cell lines pCGE-B, pCGE-C, and TM-2B ($p < 0.001$; Student's *t* test). (B) Size of colonies in medium with 5% serum. *Significantly different from control cell lines ($p < 0.001$; Student's *t* test). ND, not determined. (C) Distribution of the sizes of the colonies of clones pCGE-C (open bars) and TM-2A (solid bars) in soft agar with 10% serum. (D) Distribution of the sizes of the colonies of clones pCGE-C (open bars) and TM-2A (solid bars) in soft agar with 5% serum.

cell spreading (Gimona *et al.*, 1996; Masuda *et al.*, 1996). Since the different TM isoforms are highly homologous, one would expect that they might be similar or even interchangeable in their biological functions. However, despite the aforementioned effects of TM-2 on cellular morphology, there is little evidence to date suggesting that TM-2 shares with TM-1 the ability to reverse a malignant phenotype. In fact, in previously published studies, forced TM-2 expression failed to restore the anchorage dependence of v-*raf*- or v-*ras*-transformed NRK cells (Takenaga and Masuda, 1994; Masuda *et al.*, 1996) or of v-*ras*-transformed NIH 3T3 (DT) cells (Braverman *et al.*, 1996).

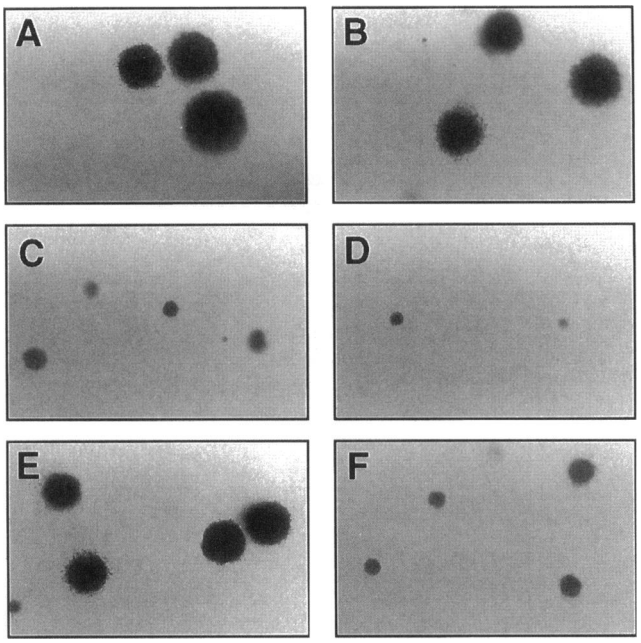

Figure 8. Colony morphology. (A and B) Clone pCGE. (C and D) Clone TM-2H (high level of TM-2 protein). (E and F) Clone TM-2F (low level of TM-2 protein). Soft agar in A, C, and E contained 10% serum, whereas soft agar in B, D, and F contained 5% serum.

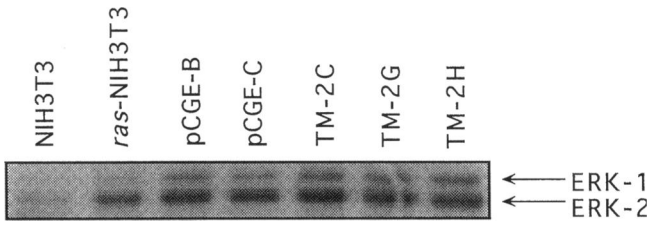

Figure 9. MAPK in-gel kinase activity assay. Whole-cell lysates from the indicated clones were electrophoresed in 10% SDS-polyacrylamide gel in which MBP was copolymerized. Renatured gels were incubated in kinase buffer with [γ-^{32}P]ATP. Arrows indicate the locations of MBP phosphorylated by ERK-1 and ERK-2. The results shown are representative of two separate experiments.

In the present report, we show that TM-2 is able to induce phenotypic reversion in v-*ras*-transformed NIH 3T3 cells, as previously demonstrated with TM-1. Compared with the *ras*-transformed parental cells, the TM-2-transfected cells had a flatter morphology and stress fibers, which would otherwise not be present in *ras*-transformed cells. Although TM-2 transfectants grew normally as a monolayer in the presence of serum, they grew only after a latent period when the serum concentration was reduced and then at a rate substantially slower than that of the *ras*-transformed parental cells. Although the TM-2 transfectants were clonogenic, the colonies produced were slow to appear and small, especially in agar containing only 5% serum. Furthermore, despite the intermediate to high levels of TM-2 detected in the transfected cells propagated as monolayers, cells isolated directly from soft

Table 1. Tumor growth in athymic mice

	Tumor mass (g)					
	Control lines			TM-2-transfected lines		
	Ras	pCGE-B	pCGE-C	TM-2C	TM-2G	TM-2H
Individual tumors	1.03	1.71	0.73	0.09	0.14	0.48
	0.58	1.19	0.57	0.08	0.04	0.22
	0.33	0.49	0.57	0.01	0.03	0.20
	0.24	0.46	0.54	0.01	0.02	0.07
		0.38	0.50	0.01	0.02	0.05
		0.23	0.39	0.00	0.02	0.00
		0.01	0.38			
			0.18			
			0.14			
			0.07			
Mean ± SEM	0.55 ± 0.18	0.64 ± 0.23	0.41 ± 0.07	0.03 ± 0.02[a]	0.05 ± 0.02[a]	0.17 ± 0.07[b]
Overall mean ± SEM		0.51 ± 0.09			0.08 ± 0.03[c]	

Mice were inoculated s.c. with 1×10^6 cells of the indicated cell lines. All animals were sacrificed on d 12, and tumors were excised and weighed. TM-2-transfected clones gave rise to tumors that were smaller than those derived from control clones. All statistical analyses were performed with the nondirectional Mann–Whitney *U* test.

[a] $p < 0.002$.

[b] $p < 0.05$.

[c] $p < 0.002$.

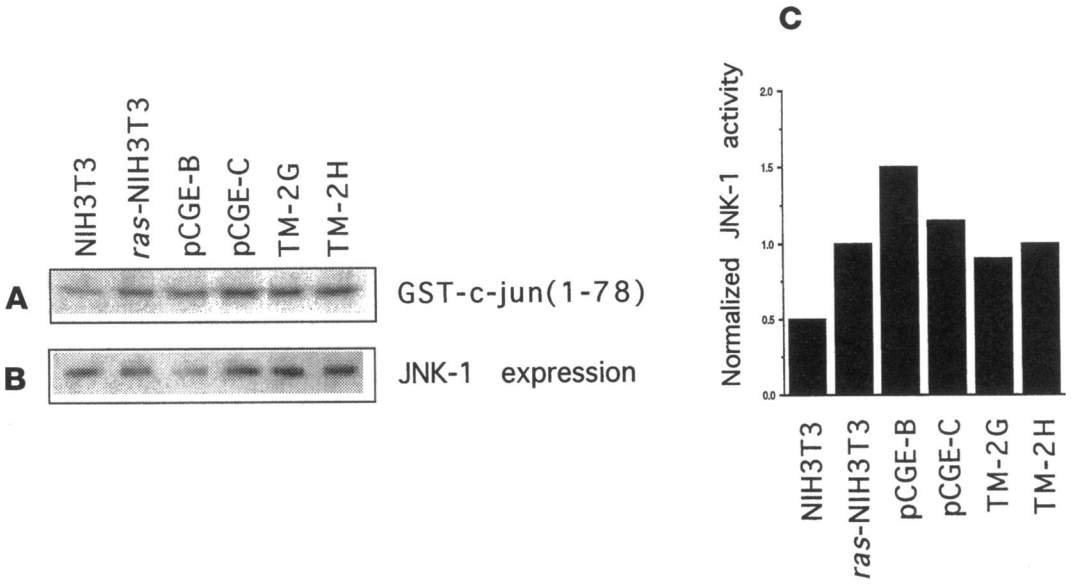

Figure 10. JNK solid-phase activity assay. (A) Phosphorylation of GST-c-jun(1–78) by JNK-1. JNK-1 immunoprecipitates of whole-cell lysates from the indicated clones were incubated with GST-c-jun(1–78) in the presence of [γ-^{32}P]ATP. GST-c-jun was electrophoresed on a 10% SDS-polyacrylamide gel. Phosphorylation of GST-c-jun(1–78) was visualized by exposing the dried gel to film. (B) Amount of JNK-1 in each assay. Aliquots of each immunoprecipitation were electrophoresed on a 10% SDS-polyacrylamide gel. Western blots of the gels were probed with anti-JNK-1 antibody. JNK-1 was visualized by using chemiluminescence. (C) JNK-1 activity normalized for amounts of JNK-1. Intensities of the bands in Figure 10, A and B, were determined by densitometry. Normalized JNK-1 activity was determined by dividing the intensities of the bands in Figure 10A by those of the bands in Figure 10B.

agar colonies had TM-2 levels that were comparable to those in *ras*-transformed cells. Collectively, these findings indicate that the restoration of TM-2 levels in *ras*-transformed fibroblasts partially restores the serum dependency characteristic of nontransformed cells and interferes with anchorage-independent growth. The findings are also supported by biochemical evidence showing that TM-2-transfected tumor cells are retained in G_0-G_1 when cells are grown in medium with low concentrations of serum.

Some of our data differ from those of Braverman *et al.* (1996), who were unable to demonstrate that TM-2 is able to reduce anchorage-independent growth or tumorigenicity in nude mice. The divergent results reported in the literature on the effects of various TM constructs may be due to differences in cell types used in these studies (NIH 3T3 versus NRK) or type of transformation [double v-*ras*-transformed (Braverman *et al.*, 1996) versus single v-*ras*-transformation in our studies] rather than intrinsic differences between TM isoforms. The double *ras*-transformed NIH 3T3 cells (DT cells) used by Braverman *et al.* (1996) are much more tumorigenic than our single *ras*-transformed NIH 3T3 cells. This difference is reflected in the fact that an inoculum of a mere 2000 DT cells is sufficient to induce tumors in nude mice (Prasad *et al.*, 1993), whereas at least 100,000 cells of single *ras*-transformed NIH 3T3 fibroblasts are required (our unpublished finding). In addition, in our variant of the NIH 3T3 cell line, TM-2 is the dominant high molecular weight TM isoform expressed, whereas in studies in which TM-2 transfection failed to induce growth inhibition (Braverman *et al.*, 1996), TM-1 and TM-2 were equally expressed. These different NIH 3T3 cell lines, and consequently their different *ras*-transformed counterparts, may vary in their response to forced TM-2 re-expression.

Recent studies have shown that transfection of the 3′ UTR of the α-tropomyosin gene is sufficient to inhibit tumorigenicity of transformed cells (Rastinejad *et al.*, 1993). This effect was attributed to sequences in the middle of the 3′ UTR (214–594 bp downstream of the stop codon) referred to as the riboregulator. Our studies, which were carried out with a construct lacking the 3′ UTR riboregulator, show that the 3′ UTR riboregulator is not absolutely required for the suppression of the transformed phenotype of *ras*-transformed cells resulting from TM-2 transfection. Although the possibility exists that the 5′ 62 bp of the 3′ UTR present in our construct may have properties similar to the 3′ UTR riboregulator, we believe this is unlikely. This 62-bp 3′ UTR sequence was not identified in the search for differentiation-inducing genes in which the TM riboregulator was found (Rastinejad *et al.*, 1993). Furthermore, the tumor suppressive effects of the 3′ UTR sequences of the α-tropomyosin gene are questionable in the case of *ras*-transformed tumor cells, as the low molecular weight TM isoforms TM-5a and

TM-5b, which share the same 3′ UTR as TM-2, are up-regulated in *ras*-transformed cells (Takenaga *et al.*, 1988a).

It is unclear how the enforced reexpression of TM-2 in *ras*-transformed cells inhibits tumor growth. To address this issue, we examined the effects of TM-2 reexpression on two of the best characterized signaling pathways downstream of Ras, namely activation of ERK and JNK (Derijard *et al.*, 1994; McCormick, 1994; Burgering and Bos, 1995; Coso *et al.*, 1995) ERK activity is known to be necessary for transformation by oncogenic *ras* (Cowley *et al.*, 1994; Khosravi-Far *et al.*, 1995). Recently, it has been shown that Ras induces JNK activity via activation of Rac (Coso *et al.*, 1995; Macara *et al.*, 1996). Rac is also known to be necessary for transformation by oncogenic *ras* (Khosravi-Far *et al.*, 1995; Qiu *et al.*, 1995). JNK mediates phosphorylation and activation of c-jun (Derijard *et al.*, 1994), whose transcriptional activity is necessary for *ras*-induced transformation (Johnson *et al.*, 1996). The forced expression of TM-2 had no effect on ERK activity and only a minor inhibitory effect on JNK activity despite the obvious change in phenotype and tumorigenicity of TM-2-transfected tumor cells. This is somewhat surprising considering the fact that TM-2 transfection partly arrests cells in G_0-G_1. These data indicate that TM-2 exerts its inhibitory effects on tumorigenicity either downstream of ERK and JNK or through a distinct Ras-dependent pathway. Possible TM targets are the serine-threonine kinases $P70^{S6}$ kinase, ROKα, and $p160^{ROCK}$, which may play a role in the cell cycle and whose activities are controlled by Rac independent of JNK activity (Chou and Blenis, 1996; Joneson *et al.*, 1996; Lamarche *et al.*, 1996). Whether TM-2 selectively suppresses the malignant phenotype of *ras*-transformed cells due to a critical interaction with these kinases or another pathway remains to be determined.

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