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Expression cDNA Cloning of a Transforming Gene Encoding the Wild-Type Gα12 Gene Product

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Using an expression cDNA cloning approach, we examined human tumor cell lines for novel oncogenes that might evade detection by conventional techniques. We isolated a transforming sequence that was highly efficient in transforming NIH 3T3 mouse fibroblasts. DNA sequence analysis identified the gene as the human homolog of a recently cloned α subunit of mouse GTP-binding protein Gα12. NIH 3T3 cells transfected with Gα12 cDNA grew in soft agar and were tumorigenic in nude mice. There were no apparent mutations in the cloned cDNA in comparison with a Gα12 cDNA clone isolated from a normal human epithelial cell library, implying that overexpression alone was sufficient to cause NIH 3T3 cell transformation. The observed altered growth properties mediated by Gα12 showed a certain degree of dependency on serum factors, and its mitogenic potential was also potently inhibited by suramin treatment.

The prevalent view of human carcinogenesis postulates a multistep process involving the activation of cellular proto-oncogenes and inactivation of tumor suppressor genes (29, 32). By means of genomic DNA transfection-transformation assays utilizing NIH 3T3 mouse fibroblasts, various oncogenes have been isolated from both human and rodent tumors. They represent diverse classes of growth-regulatory molecules, including growth factors, growth factor receptors, mitogenic signal transducers, and transcription factors (8). Frequently, mutational alterations inflicted on coding sequences or transcriptional elements of normal cellular proto-oncogenes have been shown to be responsible for oncogenic activation. This approach has generated substantial knowledge concerning the molecular mechanisms of malignant transformation but is limited with respect to oncogene detection. First, oncogenic sequences of considerable size (>100 kb) may not be detected because of inefficiencies in gene transfer techniques (27). Second, promoter/enhancer elements of transforming genes may not function optimally in fibroblast cells. To overcome these inherent difficulties, we decided to take advantage of an expression cloning system in which a high proportion of full-length cDNA can be synthesized and cloned directionally into a phagemid expression cloning vector containing a strong retroviral promoter (22). Following transfection of library cDNA into NIH 3T3 cells, transformed foci can be identified, and plasmids containing oncogenic sequences can be efficiently rescued by using this vector system (22).

Soft tissue sarcomas are a highly heterogeneous group of malignant neoplasms arising from mesodermal tissue such as fat, muscle, and fibrous tissue (17). Previous studies have indicated that specific chromosomal translocations are tightly associated with particular tumor types or subtypes (5), suggesting that mutations of growth-regulatory genes may be primary events in the genesis of these tumors. To date, relatively few oncogenes have been identified in such tumors. Thus, we sought to test the efficiency of our expression cloning approach to the identification of potential oncogenes in such tumors. We report here the isolation of a transforming gene from a soft tissue sarcoma expression cDNA library that represents an α subunit of a guanine nucleotide-binding protein (G protein), Gα12.

MATERIALS AND METHODS

Cell lines. Cell line NIH 3T3 and human synovial tumor-derived cell line A2095 were established in this laboratory. A Ewing's sarcoma cell line, RD-ES-1, was obtained from the American Type Culture Collection (ATCC/HTB166). All cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% calf serum (CS). NIH 3T3 cells carrying different plasmid constructs were derived by transfecting 1.5×10^5 cells with 1.0 μg of DNA by the standard calcium phosphate precipitation method (33). Transfectants were selected in Geneticin (750 μg/ml) and were passaged twice prior to characterization of growth properties in vitro and in vivo.

Construction of a cDNA library. A cDNA library was constructed from A2095 and RD-ES-1 cell lines in the pCEV27 vector (22) by the automatic directional cloning method (23). Transfection of the library DNA, focus identification, and plasmid rescue procedures were performed as described previously (22).

In vitro transcription and translation. Plasmid pCEV27-Gα12 was linearized with restriction enzyme *Bst*EII to act as a template for SP6 RNA polymerase as described by the manufacturer (Promega). Purified RNA was quantitated by a spectrophotometer, and approximately 3 μg was added to an in vitro translation reaction using rabbit reticulocyte lysate in the presence of 40 μCi of [^{35}S]methionine (10 mCi/ml; specific activity, 1,078 Ci/mmol; Dupont, NEN) as described by the manufacturer (Promega). Five percent of the reaction mixture was boiled in Laemmli sample buffer, and protein products were resolved by sodium dodecyl sulfate (SDS)–10% polyacrylamide gel electrophoresis (PAGE) under reducing conditions. Gels were dried and exposed to X-ray films at −70°C for 6 to 12 h.

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Northern (RNA) analysis. Total RNAs were isolated as described previously (20). After separation of samples by electrophoresis on a 1% denaturing formaldehyde agarose gel, RNAs were transferred to nitrocellulose filters (20). Blots were hybridized at 42°C for 12 h with a ^{32}P-labeled DNA probe in 40% formamide–6× saline sodium citrate (SSC; 1× SSC is 0.15 M NaCl plus 0.015 sodium citrate)–5× Denhardt's solution–1% SDS–10% dextran sulfate–sonicated salmon sperm DNA (50 μg/ml). After the hybridization reactions, filters were washed twice in 1× SSC–0.1% SDS at room temperature and then in 0.1× SSC–0.1% SDS at 50°C. Filters were air dried and exposed to X-ray films for 12 to 24 h at −70°C.

Cell proliferation assay. For analysis of proliferation in semisolid medium, 10^4 and 10^3 cells were suspended in 0.4% agarose (SeaPlaque; FMC) in 10% CS as described elsewhere (11). Colonies were stained and scored after 2 weeks. For analysis of tumor-forming capacity, about 1×10^5 to 2.5×10^5 cells were injected subcutaneously into athymic nude mice as described previously (15). Tumor occurrence and size were monitored at least weekly. For measurement of doubling time and saturation density, NIH 3T3 transfectants were plated at 3×10^4 cells per well in six-well Costar plates in Dulbecco's modified Eagle's medium supplemented with 10% CS. After 12 h, medium was switched to 5% CS and replenished every 2 days. At indicated time points, cells were trypsinized and counted by a hemocytometer. For low-serum growth studies, cells were plated at the density of 10^3 cells per well on duplicated 60-mm-diameter culture dish precoated with fibronectin (1 μg/cm^2) and cultured in the presence of serum. Approximately 12 h after plating, medium was switched to 0%, 0% (with 10 μg of insulin per ml), 0.5%, or 5.0% CS and was replaced every 2 days with fresh medium. Colonies were Giemsa stained at 2 weeks.

Suramin treatment. Approximately 3×10^4 cells were plated per well in fibronectin-coated 24-well Costar plates and cultured in the presence of 10% CS. At 12 h after plating, medium was switched to serum-free medium with or without the addition of 100 μM suramin. DNA synthesis was measured over a period of 5 h with 1 μCi of [^{3}H]thymidine per well starting 24 h after the addition of suramin. Trichloroacetic acid-insoluble DNA was collected and counted as described elsewhere (26).

Nucleotide sequence accession number. The sequence reported has been assigned GenBank accession number L01694.

RESULTS

Expression cloning of a transforming gene from a human sarcoma cDNA library. An expression cDNA library was constructed from a pool of poly(A)$^+$ RNA derived from two human tumor cell lines, A2095 and RD-ES-1. Following transfection of the library DNA into NIH 3T3 cells, four morphologically distinct foci were selected for further analysis. To identify the cDNA sequences responsible for the transformed phenotypes, integrated plasmid DNAs were first released from the mouse genome by *Not*I digestion followed by circularization for transformation of bacterial cells. Individual rescued plasmids were then tested for their transforming potential in NIH 3T3 cells. This strategy led to the isolation of a plasmid, 58-1, that demonstrated high-titered transforming activity (>10^4 focus-forming units/pmol) on NIH 3T3 cells.

Clone 58-1 contained a ~4.0-kb cDNA insert. Sequence analysis from its 5′ end revealed strong similarity to a recently cloned mouse cDNA which encodes for an α subunit of a G protein, Gα12^{mu} (31). Available information derived from the mouse sequence indicated that clone 58-1 possessed the entire coding region in addition to 6 bp of 5′ untranslated region and ~3 kb of 3′ untranslated sequence. The predicted amino acid sequence of clone 58-1 diverged from Gα12^{mu} in only six residues, strongly suggesting that it was the human homolog (Gα12^{hu}) of the mouse sequence (Fig. 1). To identify the translational product of this cDNA clone, we performed in vitro transcription-translation analysis. This study revealed a major protein species with a relative molecular mass of ~45 kDa (Fig. 2), consistent with the size of 44 kDa calculated from its predicted coding sequence and resembling the sizes of other α subunits of known high-molecular-weight G proteins.

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hu    1 MSGVVRTLSRCLLPAEAGGARERRAGSGARDAEREARRRSRDIDALLARERRAVRRLVKIL 61
mu    1 ------------------*-------*A-------------------------------- 59
            A
hu   62 LLGAGESGKSTFLKQMRIIHGREFDQKALLEFRDTIFDNILKGSRVLVDARDKLGIPWQYS 122
mu   60 ------------------------------------------------------------H- 120

hu  123 ENEKHGMFLMAFENKAGLPVEPATFQLYVPALSALWRDSGIREAFSRRSEFQLGESVKYFL 183
mu  121 ------------------------------------------------------------ 182
                                                        C
hu  184 DNLDRIGQLNYFPSKQDILLARKATKGIVEHDFVIKKIPFKMVDVGGQRSQRQKWFQCFDG 244
mu  182 ------------------------------------------------------------ 242
              E                                                     G
hu  245 ITSILFMVSSSEYDQVLMEDRRTNRLVESMNIFETIVNNKLFFNVSIILFLNKMDLLVEKV 305
mu  243 ------------------------------------------------------------ 303
                                                          I
hu  306 KTVSIKKHFPDFRGDPHRLEDVQRYLVQCFDRKRRNRSKPLFHHFTTAIDTENVRFVFHAV 366
mu  304 -S----------K-----------------------------------------I------- 364

hu  367 KDTILQENLKDIMLQ 381
mu  365 --------------- 379
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FIG. 1. Amino acid sequence comparison between the human (hu) and mouse (mu) Gα12 coding regions. -, identical residues; *, residues not found in the mouse sequence. Boxed sequences represent domains conserved among proteins.

Transforming properties of Gα12. To ascertain whether Gα12^{hu} cDNA could act as a classical oncogene, plasmid 58-1 was transfected into NIH 3T3 cells, and marker-selected mass cultures were obtained. As controls, parallel cultures were transfected with a known oncogene, c-*sis*/PDGF-B, and with the vector alone. As shown in Fig. 3, Gα12^{hu} transformed foci were stellate in appearance, with aggregation of cells at high density in the center of the focus,

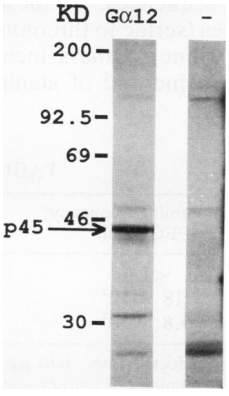

FIG. 2. In vitro translation of Gα12^{hu} RNA. Samples with either 3 μg of in vitro-transcribed Gα12^{hu} RNA (left lane) or no addition of RNA (right lane) were translated with rabbit reticulocyte lysate in the presence of [^{35}S]methionine. Approximately 1% of the reaction mixtures was analyzed on by SDS–10% PAGE. The gel was dried and exposed to X-ray film for 10 h. The 45-kDa protein species encoded by Gα12^{hu} is indicated by an arrow.

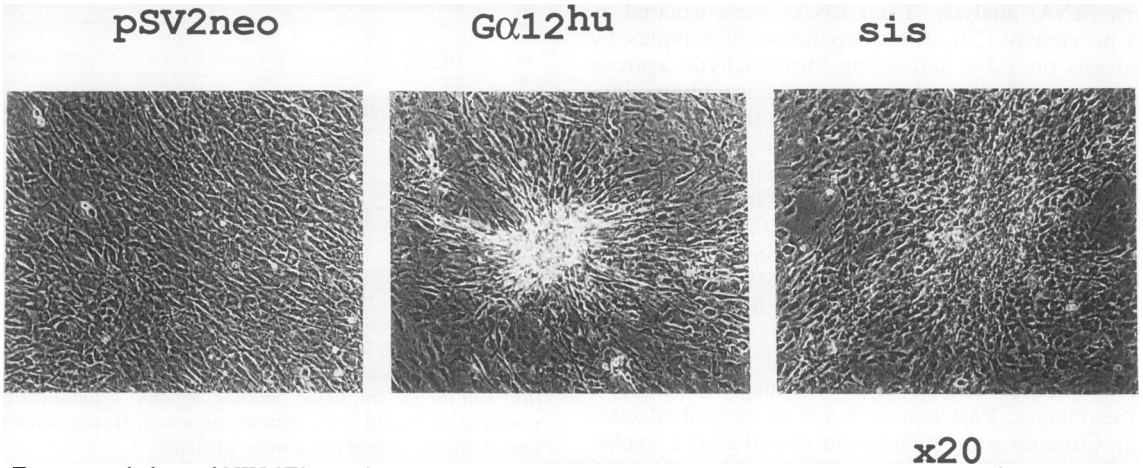

FIG. 3. Focus morphology of NIH 3T3 transfectants. Expression plasmids representing pSV2neo and the Gα12^{hu} and *sis* genes (1 μg of each) were transfected separately into NIH 3T3 cells, and morphologically transformed foci were photographed after 2 weeks in culture. Magnification, ×20.

resulting in an overall punctate morphology. Gα12^{hu} transformants also showed a decreased doubling time and increased saturation density compared with control pSV2neo transfectants (Table 1). Another property of transformed cells is the ability to grow in semisolid medium. As shown in Table 1, Gα12^{hu}-transformed cells formed large, progressively growing colonies in soft agar. Finally, inoculation of Gα12^{hu} transfectants subcutaneously into athymic nude mice induced tumor formation at high frequency, while transfectants containing the control plasmid pSV2neo had substantially lower incidence of tumors under the same assay conditions (Table 1). All of these findings indicated that Gα12^{hu} transformation of NIH 3T3 cells both induced morphological alterations and enhanced proliferation both in vitro and in vivo.

Overexpression of wild-type Gα12 is sufficient for transformation. Because our Gα12^{hu} cDNA clone was derived from human tumor cells, we investigated whether its oncogenic activation was due to mutations within the coding sequence as has been reported for several other α subunits of G proteins (18, 19). On the basis of our sequence, we identified six amino acid positions that showed divergence between the human and mouse sequences. Of these, three represented conservative changes (serine to threonine, lysine to arginine, and isoleucine to valine). Others included substitution of histidine 119 by tyrosine and of alanine 26 by serine and glycine as well as a glycine addition in position 18 (Fig. 1). To determine whether these disparities were due to species differences or tumor-specific mutations, we screened a cDNA library constructed from a normal human mammary epithelial cell line (21) for additional Gα12^{hu} cDNAs. A total of 33 positive clones were isolated, and cDNAs that contained the entire coding region were identified by polymerase chain reaction. Sequence analysis revealed no differences at the candidate sites for mutations between cDNA clones derived from either normal or tumor cell lines. Moreover, examination of the biological activities of several normal cDNA clones revealed that each exhibited high-titered transforming activity comparable to that of clone 58-1 (data not shown).

To further elucidate the mechanism of Gα12^{hu} transformation, we performed Northern blot analysis using total RNA from different human cell lines and NIH 3T3 transfectants. Using Gα12^{hu} cDNA as probe, we detected a 4.4-kb transcript in both A2095 and RD-ES-1 tumor lines, with the latter expressing a higher level of this RNA species (Fig. 4). The same transcript was also present in RNA prepared from a normal human epithelial cell line, B5/589 (30). NIH 3T3 cells transfected with the Gα12^{hu} construct showed the highest transcript levels, with species ranging in size from 4.0 to 5.0 kb. In contrast, NIH 3T3 cells transfected with control plasmid pSV2neo exhibited only very low levels of the

TABLE 1. Transforming properties of NIH 3T3 transfectants

Plasmid	Transforming frequency[a] (FFU/pmol)	Soft agar growth[b] (%)	Cell doubling time[c] (h)	Saturation density[d] (10^6 cells)	Tumorigenicity[e] (frequency)
pSV2neo	<1.0	1.2	30	2.3	1/6
sis	18×10^4	21.2	26	4.5	5/5
Gα12^{hu}	9.8×10^4	19.9	23	4.8	6/6

[a] NIH 3T3 cells were transfected with ~0.01 μg of each plasmid, and the number of focus-forming units (FFU) was scored after 3 weeks in cultures. All three plasmids produced similar numbers of marker-selectable colonies.

[b] NIH 3T3 transfectants were suspended in 0.4% soft top agar in the presence of 10% CS. Colonies of >0.2 mm were scored after 14 days, and data represent average values of duplicate plates.

[c] Approximately 3×10^4 cells were plated in duplicate in 60-mm-diameter plates and cultured in the presence of 5% CS. Cells were counted each day for 6 consecutive days, and the result was used in the calculation of exponential doubling time.

[d] NIH 3T3 transfectants were plated as described in footnote *c*. Cells were allowed to grow to confluence until cell numbers did not alter after three consecutive counts. Data represent average values of duplicate samples.

[e] Approximately 1×10^5 to 2.5×10^5 cells were introduced subcutaneously into athymic nude mice. Data indicate incidence of tumors 5 weeks after inoculation. All tumors generated by Gα12^{hu} were of well-differentiated fibrosarcomas.

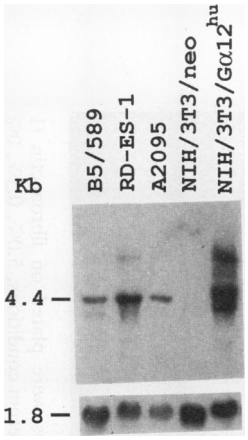

FIG. 4. Northern analysis of the Gα12^{hu} transcript. Total RNAs (20 μg of each) isolated from B5/589, RD-ES-1, A1095, and NIH/3T3 cells transfected with pSV2neo or a Gα12^{hu} plasmid were electrophoresed in a 1% denaturation agarose gel, transferred to a nitrocellulose filter, and hybridized with a Gα12^{hu} cDNA probe (upper gel). To normalize for the amount of RNA loaded in each lane, the same filter was hybridized with a mouse β-actin cDNA probe (lower gel).

endogenous Gα12^{mu} transcript. All of these findings indicated that overexpression of the normal Gα12^{hu} in NIH 3T3 cells was sufficient to confer the malignant phenotype.

Serum dependence of Gα12^{hu} transformants. To gain further insight into the role of Gα12^{hu} gene product in propagating proliferation signals and the extent of growth autonomy acquired by NIH 3T3 transformants, we examined the ability of Gα12^{hu}-transformed cells to proliferate under various conditions of growth restriction. NIH 3T3 cells expressing the Gα12^{hu}, c-*sis*/PDGF-B, and *neo* genes were plated at a low cell density (~10^3 cells per 60-mm-diameter dish) under four different serum concentrations: 5.0%, 0.5%, 0%, and 0% supplemented with insulin (10 μg/ml). The three transfectants showed comparable colony-forming efficiencies in 5.0% serum but differed significantly under low-serum conditions (Fig. 5A). Whereas c-*sis* transfectants produced similar numbers of colonies in 5.0 or 0.5% serum and smaller colonies in 0% medium or 0% medium with insulin (10 μg/ml), the growth of Gα12^{hu} transformants was drastically impaired when cells were cultured in 0.5% serum. In the total absence of serum factors, the ability of Gα12^{hu} cells to form colonies was completely abolished, and only slowly growing colonies were detected with the addition of insulin. Similar results were observed with the control pSV2neo transfectants (Fig. 5A). When Gα12^{hu}-transfected cells were cultured under subconfluent conditions (~10^5 cells per 60-mm-diameter dish) in 10% serum and subjected to serum starvation (0%) for 24 h, their transformed phenotype reverted to a more flattened morphology resembling that of the contact-inhibited parental NIH 3T3 cells (Fig. 5B). In contrast, serum withdrawal failed to induce morphological reversion of c-*sis*/PDGF-B transformants; instead, a more transformed phenotype had emerged. We infer from these results that the Gα12^{hu} protein expressed in NIH 3T3 cells retained significant dependence on exogenous serum factors for expression of the transformed phenotype.

DNA synthesis in Gα12^{hu} transformants is potently inhibited by suramin. It has been documented that suramin inhibits certain mitogens by interrupting interaction between growth factors and their cell surface receptors (7). The apparent dependence of Gα12^{hu} transformation on serum factors prompted us to study the effect of suramin on Gα12^{hu}-transformed cells. It has been demonstrated that suramin is a potent inhibitor of proliferation and induces morphological reversion of v-*sis* transformants (12). As shown in Fig. 6, DNA synthesis in Gα12^{hu} transfectants was reduced by ~80% within 24 h of incubation with 100 μM suramin. In contrast, DNA synthesis by NIH 3T3 cells expressing the c-*erb B2* gene, a constitutively activated, ligand-independent growth factor receptor oncogene, was not markedly affected under the same conditions. These results are consistent with the possibility that mitogenic signalling and transforming properties of Gα12^{hu} are dependent on exogenous stimulation by some serum factor(s).

DISCUSSION

G proteins of the high-molecular-weight class constitute a group of heterotrimeric molecules composed of α, β, and γ subunits implicated in channeling external stimuli such as hormones, cytokines, or light, with the ultimate effect of eliciting a wide variety of physiological responses, including cell growth (28). Specific mutations in two Gα subunits, α_s and α_{i2}, have been described in a subset of human endocrine tumors (18, 19). These mutations resemble the classical point mutations detected in *ras* gene family members (4) in that they impair the intrinsic GTPase activity of the G proteins, leading to the constitutive activation of adenylate cyclase. In model systems, introduction of α_{i2} with analogous mutations has been reported to induce transformation in rodent fibroblasts (14, 16, 24). Similar observations have also been reported for a constitutively activated G-protein-coupled α_{1B}-adrenergic receptor (2), providing further support for the concept that G proteins play a role in some malignancies.

In this study, we identified a transforming cDNA from a soft tissue sarcoma-derived expression library. This cDNA encoded a member of a new class of Gα subunits designated Gα12. Our findings demonstrate that at least one member of this class of Gα has the ability to act as an oncogene in inducing transformed foci in rodent fibroblasts, as well as anchorage-independent growth and tumor formation in animals. The possibility that mutational activation was responsible for its transforming activity was excluded by our demonstration of the same sequence of the Gα12^{hu} coding region in cDNAs isolated from a normal human epithelial cell library as well as the highly efficient transforming activity of Gα12^{hu} cDNAs derived from both tumor and normal cell libraries.

Only three members of the G12 class of α subunit have so far been described. Gα12 and Gα13 were cloned from mouse brain RNA by polymerase chain reaction using degenerate primers (31). A related *Drosophila* gene, concertina, is believed to be involved in ventral furrow formation and posterior midgut invaginations during gastrulation (25). Therefore, it is tempting to speculate that in addition to the ability to induce cell proliferation, members of this class of α subunit may also be involved in directing cell migration during organogenesis.

Cellular transformation by oncogenes often results in abrogation of serum requirements (1). However, this ability was only partially demonstrated by Gα12^{hu}-transformed cells in that they exhibited relatively retarded growth rate and morphological reversion in the absence of serum. There was also marked inhibition of DNA synthesis in Gα12^{hu}-transformed cells by suramin, a known inhibitor of ligand-receptor interactions (7). These findings may indicate that in

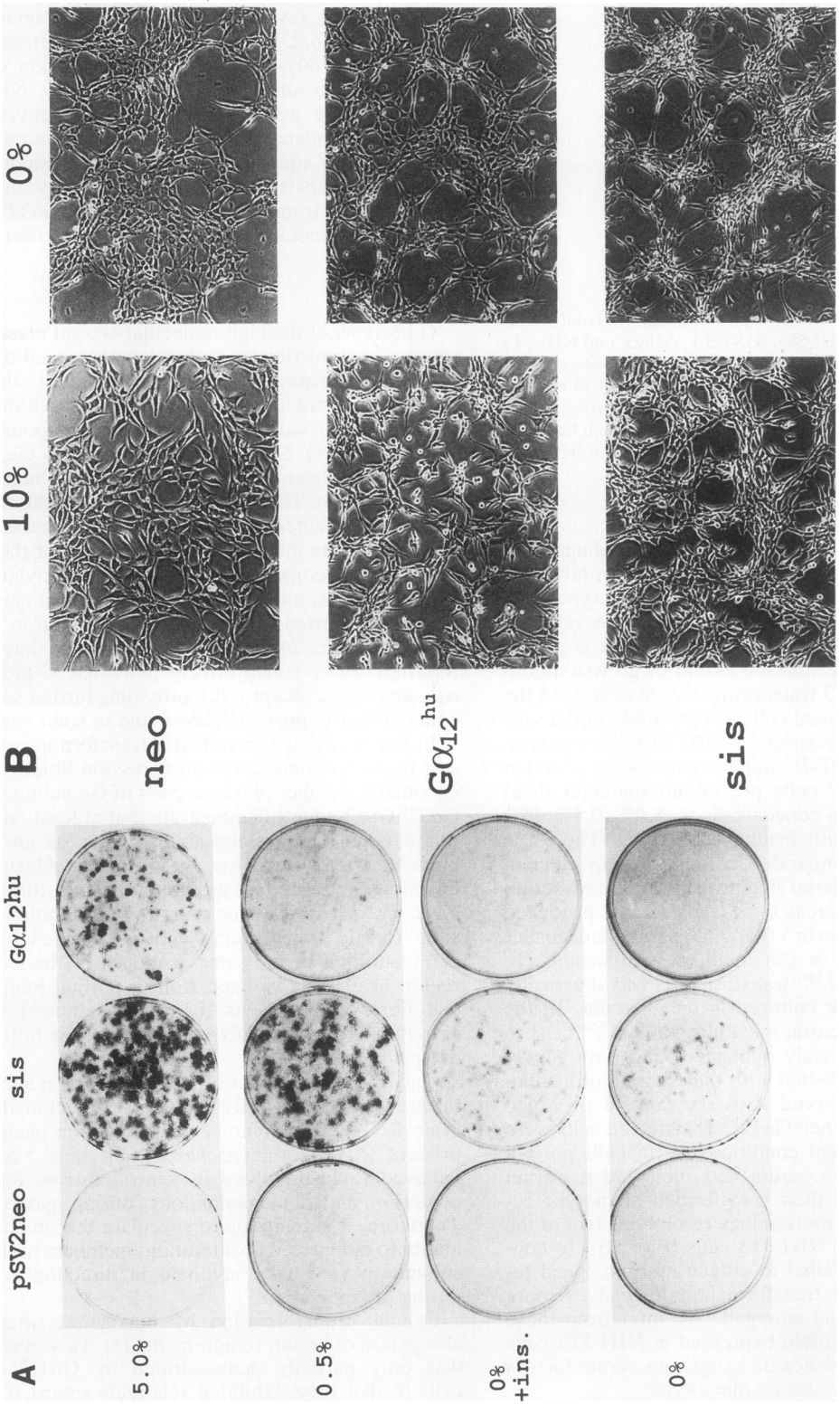

FIG. 5. Low-serum growth of various NIH 3T3 transfectants. (A) Approximately 10^3 cells from mass-selected cultures were plated on fibronectin (1 $\mu g/cm^2$)-coated 60-mm-diameter tissue culture dishes in the presence of 10% serum. Cells were then cultured in four different serum conditions, 5.0%, 0.5%, 0% (plus insulin), or 0% for 2 weeks, and surviving colonies were visualized with Giemsa stain. (B) NIH 3T3 transfectants were plated at subconfluent levels ($\sim 10^5$) under the conditions described above. Morphologies of cells growing in the presence of full serum (10%) or in serum-free medium (0%) for 24 h are shown. Magnification, ×20.

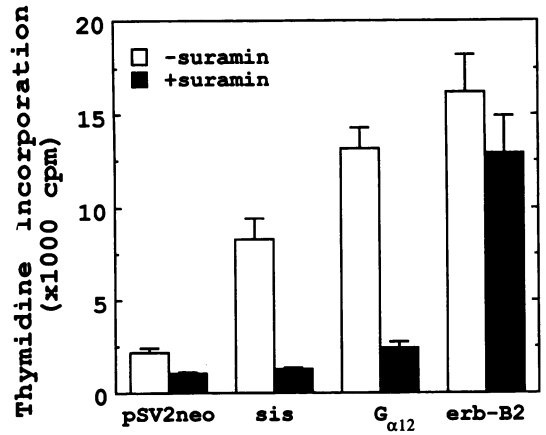

FIG. 6. Effects of suramin on NIH 3T3 transfectants. Approximately 3×10^4 of each NIH 3T3 transfectant was plated on 24-well fibronectin (10 μg/cm^2)-coated culture plates. Cells were then exposed to serum-free medium with (filled) or without (open) 100 μM suramin for 24 h. Each data point is the mean ± standard deviation of triplicate measurements.

the absence of external stimulation, wild-type Gα12^{hu} expressed in NIH 3T3 cells is still under certain normal feedback regulation through its own intrinsic GTPase activity or by the GTPase-activating protein activity of its effector molecule (3, 6, 9, 13). In this regard, it would be of interest to determine whether GTPase-deficient mutants of Gα12^{hu} with a constitutively increased activity can completely abrogate serum requirements for proliferation.

We have no direct evidence that Gα12^{hu} overexpression was involved in the malignant conversion of either of the soft tissue sarcomas from which the cDNA library was generated. One sarcoma, RD-ES-1, showed a higher level of Gα12^{hu} transcript than did any of the normal cell types analyzed, but this level was significantly lower than the levels expressed by Gα12^{hu}-transformed NIH 3T3 cells. If this gene was overexpressed in RD-ES-1 cells, we detected no evidence of any gross gene rearrangement by Southern blot analysis (10) that might account for increased transcript levels. Nonetheless, our present findings demonstrating the oncogenic potential of this new class of Gα subunits warrants a search for evidence of genetic alterations that cause overexpression or constitutive activation of this gene in human malignancies.

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