Transformation of normal rat kidney (NRK) cells by an infectious retrovirus carrying a synthetic rat type α transforming growth factor gene

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ABSTRACT   We synthesized a gene for rat type α transforming growth factor (TGF-α) consisting of the leader sequence and the sequence coding for the mature 50-amino acid peptide without the C-terminal processed region. This gene was inserted into the retrovirus vector pSW272, which is derived from spleen necrosis virus, to obtain an infectious recombinant virus carrying the rat TGF-α gene. This recombinant virus can infect normal rat kidney (NRK) cells and allow these cells to grow in soft agar in the presence of TGF-β. Transformed cells isolated from colonies grown in soft agar contain an integrated form of the recombinant virus and secrete biologically active TGF-α into their medium. These results show that a biologically active TGF-α can be produced by a gene synthesized from only part of the coding region and that the infectious retrovirus carrying the TGF-α gene (SW355) can function as a transforming agent in NRK cells in the presence of TGF-β.

Two classes of transforming growth factors (TGF-α and TGF-β) have been purified to homogeneity from mouse (1), rat (2), and human (3, 4) cells; and their amino acid sequences have been determined (see ref. 5 for review of TGFs). TGF-α was initially isolated from a retrovirus-transformed cell line (6); then it was found in several nonvirally transformed cell lines (7) and also in the early rat embryo (8). Since TGF-α and epidermal growth factor (EGF) bind to the same receptor (9), the effects of TGF-α and EGF seem to be qualitatively indistinguishable from each other. Comparison of amino acid sequences reveals about 35% homology among these EGF-like peptides (rat, mouse, and human EGFs and rat and human TGF-α) (10). This implies that they originated from a common ancestral gene. TGF-β differs from TGF-α and EGF both in its sequence and in its unique receptor. TGF-α (EGF) alone (11) or the combination of TGF-α (EGF) and TGF-β allows the anchorage-independent growth of certain nontransformed cells in soft agar (12). Anchorage-independent growth of fibroblast cells in vitro correlates best with their tumorigenicity in vivo (13).

It has been shown that cells transformed by retroviruses carrying an oncogene (ras, mos, and fes) secrete TGF-α (2, 6, 14). By using a temperature-sensitive mutant of Ki-ras it was demonstrated that secretion of TGF-α is regulated by an oncogene product (14). Although the TGF-α gene has not been shown to be a transforming gene by standard methods, this could be simply due to inappropriate assay methods.

We wished to see whether we could activate TGF-α expression by inserting its gene into a retrovirus vector to generate an infectious transforming retrovirus that would lead to overexpression of TGF-α. This would be analogous to the relationship between platelet-derived growth factor and the sis gene in simian sarcoma virus found in nature (15, 16). We chose as our model system the rat TGF-α gene and the

NRK rat cell line. Although TGF-α is synthesized naturally as a 159-amino acid-long precursor with N- and C-terminal extensions (17), we inserted into the retrovirus vector a chemically synthesized gene encoding only the N-terminal leader sequence and the mature 50-amino acid TGF-α region. In this study, we show that recovered recombinant retroviruses carrying the TGF-α gene can induce NRK cells to grow in soft agar upon infection in the presence of TGF-β and that infected cells secrete biologically active TGF-α.

MATERIALS AND METHODS

Cells. NRK cells were grown in Dulbecco's modified Eagle's medium containing 7% (vol/vol) calf serum. Culture conditions for chicken embryo fibroblasts, Buffalo rat liver thymidine kinase-deficient cells, and selection of Buffalo rat liver thymidine kinase-positive cells were described previously (18). Transfection of cells was done by the calcium phosphate precipitation method described by Graham and Van der Eb (19).

Synthesis and Purification of Oligonucleotides. Oligonucleotides were synthesized on an automated DNA synthesizer (Applied Biosystems, Foster City, CA, model 380A). Those with the correct length were separated by acrylamide gel electrophoresis, eluted by diffusion, and concentrated by binding to a small DE-52 column. They were eluted from the column in 2 M ammonium bicarbonate, heated at 70°C for 15 min, and lyophilized. All of the oligonucleotides used were stored in deionized H_2O at −20°C.

Oligonucleotide-Directed Mutagenesis. The oligonucleotide and single-stranded M13 DNAs were incubated with kinase and hybridized, and the oligonucleotide was extended by DNA polymerase I (Klenow fragment). The mixture was treated with ligase, and the products were used without further purification to transform competent cells made from a repair-deficient strain of Escherichia coli (HB2154). The transformed bacteria were plated using E. coli (HB2151) to make the lawn (20). Phages were isolated from 96 plaques and hybridized with the oligonucleotide used for mutagenesis by using published procedures (21, 22). The sequences of the clones were verified by the method of Sanger et al. (23).

Cellular DNA and RNA Analysis. Isolation of cellular DNA and RNA, as well as Southern analysis and S1 nuclease mapping analysis, have been described (18).

Soft Agar Assay and EGF Binding-Competition Assay. Both procedures have been described (12).

RESULTS

Chemical Synthesis of the Rat TGF-α Gene. The rat TGF-α gene that we synthesized consists of the mature rat TGF-α coding sequence (50 amino acids) and the leader sequence (38

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amino acids). We synthesized these two domains separately and ligated them later. Fig. 1 illustrates the steps involved in this construction. The amino acid sequence of the mature rat TGF-α (2) was converted to a nucleotide sequence (150 nucleotides) by choosing the preferred codons used by higher eukaryotes (24). Because the initial attempt to synthesize a gene for mature TGF-α in one step, by ligating eight 40-nucleotide-long fragments, was not successful, the mature TGF-α coding region was synthesized in two steps. This manipulation was necessary to reduce random hybridization of the 40-nucleotide-long oligonucleotides. First, we synthesized four oligonucleotides (nos. 1–4) to construct a core section lacking four regions, A–D (14–16 nucleotides), from the mature TGF-α sequence (Fig. 1). Then we inserted sequences A–D (Fig. 1), leading to a mature coding region (clone M13SW1). A BamHI site was inserted near the 5' end of this clone (resulting in M13SW8) to allow the insertion of the leader sequence. The rat TGF-α leader sequence has been published (17). We constructed this region by synthesizing eight 22–35-nucleotide-long oligonucleotides (L1–L8, Fig. 1) and ligating them into the M13 vector, resulting in M13SW5. Then, we inserted the leader sequence from M13SW5 into the BamHI site of M13SW8, resulting in M13SW13. This clone contained a BamHI site that does not exist in the cDNA clone and was repaired. The rat TGF-α coding region with the leader sequence from the resulting clone M13SW19 was inserted into the Xba I site of a retrovirus vector derived from the spleen necrosis virus (SNV), pSW272 (18). The rat TGF-α gene in this clone, pSW355, should be transcribed from the promoter in the long terminal repeat of the SNV vector and then translated. The signal peptide sequence should promote cellular secretion of rat TGF-α and should be cleaved by signal peptidase to release mature rat TGF-α into the medium.

Biological Properties of pSW355. Chicken embryo fibroblasts were transfected with plasmids pSW355 and cloned helper virus REV-A, pSW253 (18) to recover infectious recombinant retrovirus. Since the vector pSW272 contains the thymidine kinase gene from herpes simplex virus type 1 as a selectable marker, the titer of recombinant viruses was determined by infecting Buffalo rat liver thymidine kinase-deficient cells and selecting infected cells in hypoxanthine/aminopterin/thymidine medium. The titer of the virus stock for SW355 was approximately 2–3 × 10^5 thymidine kinase-transforming units per ml of culture medium at 4–5 days after transfection. The titer for the vector SW272 was similar to that of SW355 (data not shown).

NRK cells were infected with the same virus stock of SW272 and SW355 to test the ability of the infected NRK cells to grow in soft agar and to secrete TGF-α. We observed significant numbers of colonies in soft agar when NRK cells were infected with SW355, which contains rat TGF-α in the retrovirus vector, and were incubated in the presence of TGF-β (data not shown). Several colonies were isolated into 24-well dishes and expanded for further study.

The morphology of cells infected with SW355 in monolayer culture was different from that of uninfected NRK cells and was similar to the morphology of NRK cells growing in medium containing 10 ng of EGF per ml (data not shown). Growth of cells infected with SW355 in a monolayer was
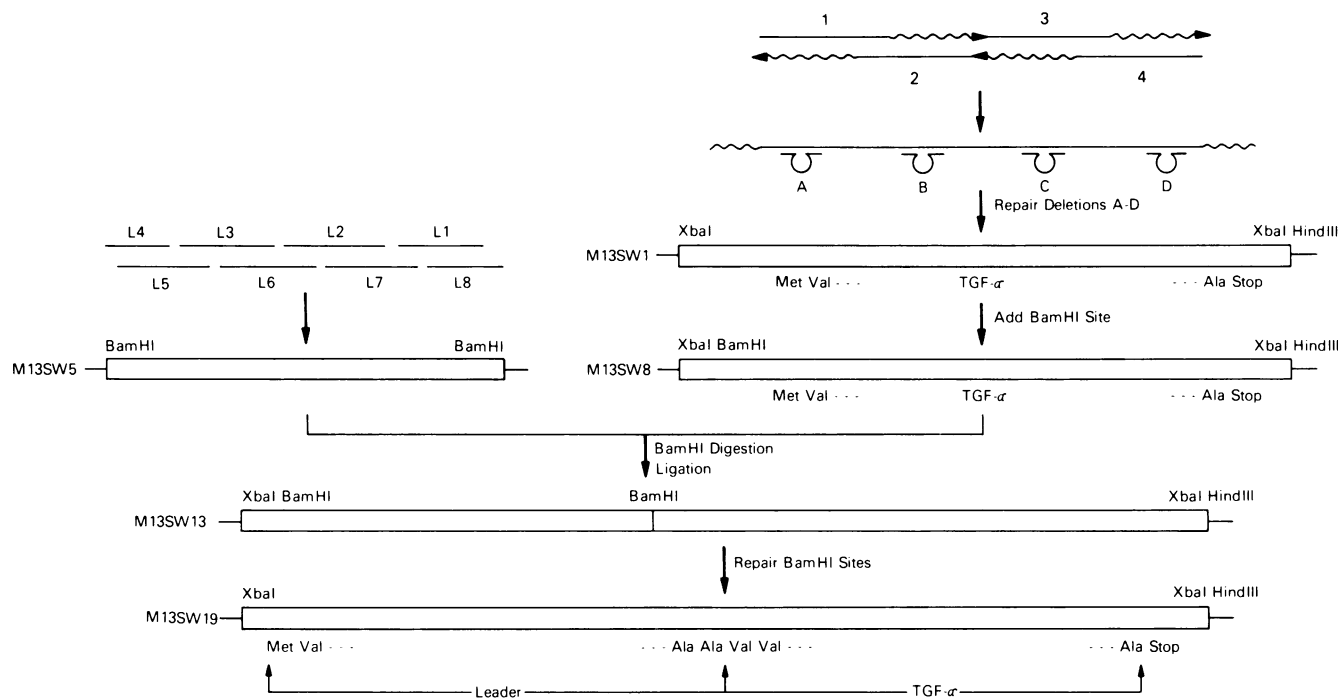

Fig. 1. Schematic diagram of chemical synthesis of the rat *TGF-α* gene. The four initial 40-nucleotide-long oligonucleotides (nos. 1–4) contained overlapping regions (10 bp) that were able to hybridize to each other. After all fragments were treated with kinase, they were hybridized and incubated with *E. coli* DNA polymerase I (Klenow fragment) and dNTPs, as indicated by ∿, to synthesize the complementary strand. The products were cloned into vector M13mp18. The correct sequence of the core section of the rat *TGF-α* gene was confirmed by the method of Sanger *et al.* (23). The four sequences (A–D) in this clone were inserted by oligonucleotide-directed mutagenesis, resulting in the clone M13SW1. M13SW1 was modified by an oligonucleotide-directed mutagenesis to introduce a *Bam*HI site 5' to the ATG initiation codon, resulting in M13SW8. Oligonucleotides L1–L8 (22–35 nucleotides long) were synthesized for both strands with overlapping regions, as pictured. The 5' ends of L4 and L8 had cohesive ends for cloning in a *Bam*HI site. After L1–L4 and L5–L8 were pooled and treated with kinase, they were all mixed and hybridized. The products were cloned into the *Bam*HI site of M13mp18. The clone containing the correct leader sequence was named M13SW5. Replicative forms M13SW8 and M13SW5 were digested with *Bam*HI and ligated to combine the rat leader sequence and the mature TGF-α sequence. Construction of the correct clone (M13SW13) was confirmed by DNA sequencing. The junction between the signal peptide region and the mature TGF-α region in M13SW13 contained a *Bam*HI site that does not exist on the cDNA clone, and this was repaired by oligonucleotide-directed mutagenesis, resulting in M13SW19.

better than that of NRK cells in the medium containing 7% (vol/vol) calf serum. Regardless of the presence or absence of the helper virus sequence, cells infected with SW355 reached a higher saturation density in a shorter doubling time than NRK cells (data not shown). Thus, SW355, which carries a rat *TGF-α* gene, allows NRK cells to grow as if the medium contained a mitogen.

SW355-Infected NRK Cells Contain an Intact Rat *TGF-α* Gene and Express its RNA. Nine clones isolated from soft agar were examined for the presence of an integrated form of SW355 in their chromosomal DNA. Genomic DNAs (10 μg) from NRK cells and nine clones were digested with *Sac* I, separated in an agarose gel, transferred to nitrocellulose paper, and hybridized with a ^{32}P-labeled probe made from pSW355. Fig. 2A shows the hybridization pattern of SW355-infected clones and parental NRK cells. No significant hybridization was seen with DNA from NRK cells. Hybridization of the probe with DNA from SW355-infected NRK cells at 0.88, 1.85, and 2.0 kilobase pairs (kbp), which were generated from SW355 (Fig. 2B), shows that all of the clones isolated from soft agar were indeed infected with recombinant retrovirus carrying the rat *TGF-α* gene and were not spontaneously transformed clones. The absence or the presence of 0.78- and 7.4-kbp fragments generated from the helper virus REV-A (Fig. 2B) that hybridize to the probe shows that each clone was infected with either SW355 alone or both SW355 and REV-A. Since clone 1/28 Cl.3 did not hybridize with the probe at the 0.78- and 7.4-kbp bands, it must have been infected with only SW355. The rest of the clones isolated from soft agar were infected with both SW355
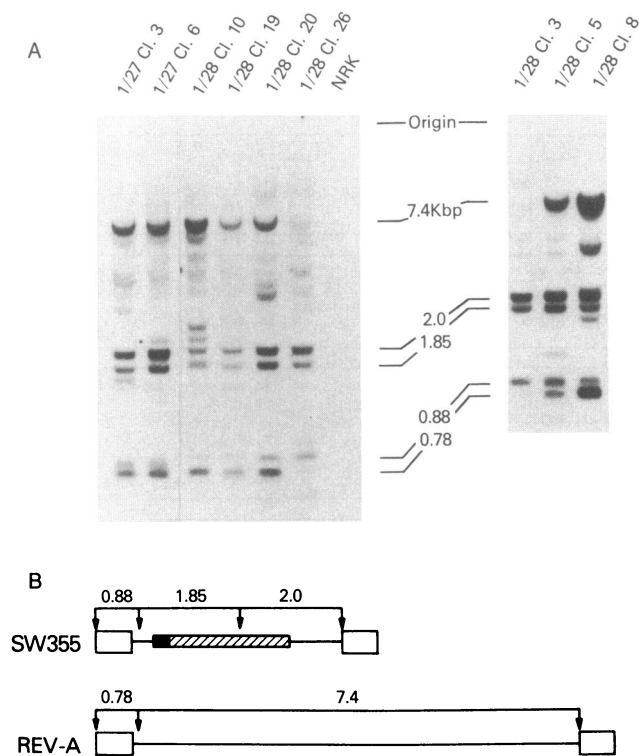
and REV-A. Additional bands that hybridize to the probe could be due to rearrangement in some viruses.

Total cellular RNAs were isolated from the same set of clones used for Fig. 2 to determine whether they transcribe the rat *TGF-α* coding region without any alteration in the sequence. A small *Sal* I–*Xba* I fragment was initially subcloned in vector M13tg131 for preparation of the probe (Fig. 3B). If cells were expressing the rat *TGF-α* gene without any alteration of the sequence, the entire *Sal* I–*Xba* I region (360 nucleotides) of the probe must hybridize to RNA. Even though this probe may hybridize to endogenous rat TGF-α

FIG. 2. Southern blot analysis of DNA from SW355- and SW253-infected NRK cells able to grow in soft agar in the presence of TGF-β. Ten micrograms of DNA from each clone was digested with *Sac* I, separated by 0.8% agarose gel electrophoresis, transferred to nitrocellulose filter paper, and hybridized to a probe made from pSW355 nick-translated with [α-^{32}P]dCTP. (A) Hybridization pattern of DNA from the clones with the probe. (B) Locations of *Sac* I sites on SW355 and REV-A, with distances (kbp) between each *Sac* I site. □, Long terminal repeat of retrovirus; ■, rat *TGF-α* gene insert; ▨, thymidine kinase gene of herpes simplex virus type 1.
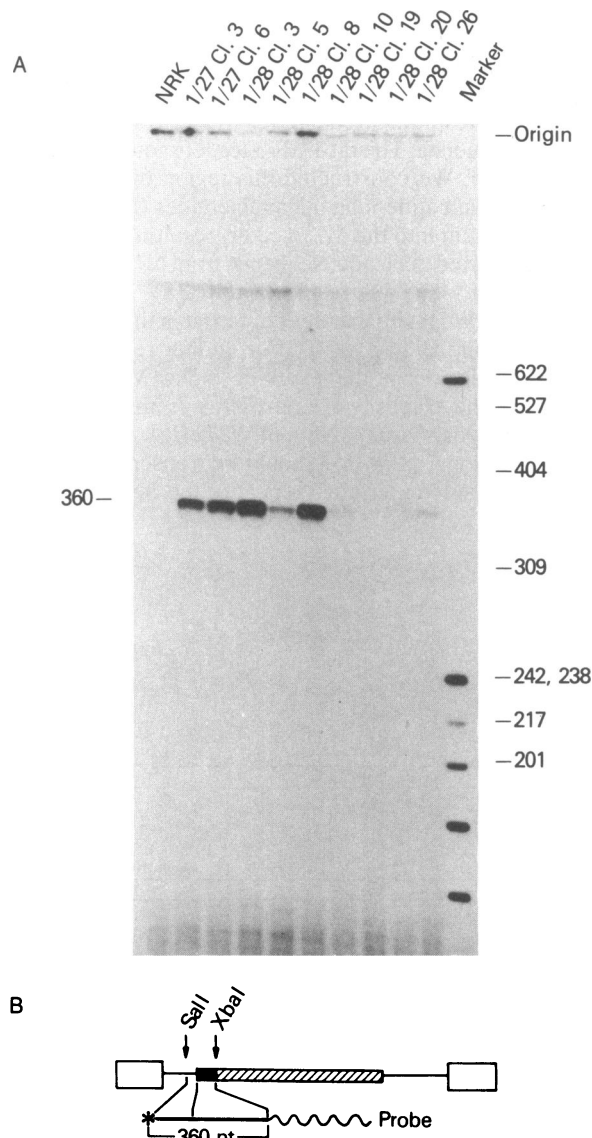
FIG. 3. S1 nuclease protection analysis of RNA isolated from SW355- and SW253-infected NRK cells able to grow in soft agar in the presence of TGF-β. After 100 μg of total cellular RNA from nine clones and NRK cells were annealed with the ^{32}P-labeled probe, hybrids were digested with S1 nuclease. S1 nuclease-resistant materials were separated on 5% denaturing polyacrylamide gel electrophoresis. (A) Autoradiogram of S1 nuclease-resistant fragments separated in a gel with molecular weight markers, indicated as numbers of nucleotides. The size of the primary S1 nuclease-resistant fragment (360 nucleotides) was determined by molecular weight markers. (B) Location of the ^{32}P-labeled *Sal* I site. After the *Sal* I site was filled in with [α-^{32}P]dCTP and DNA polymerase I (Klenow fragment), the DNA was digested with *Bgl* II, which is 10 bp away from this *Sal* I site on the M13 vector M13tg131. □, Long terminal repeats; ■, TGF-α insert; ▨, thymidine kinase gene; ▬, TGF-α coding sequence with the virus sequence attached; ∿, M13 sequence.

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RNA, the labeled *Sal* I site will not hybridize since the *Sal* I site is from the viral sequence of the vector (Fig. 3*B*). The single band of 360 nucleotides, which is S1 nuclease resistant, shows that cells are expressing the correct rat TGF-α sequence. Fig. 3*A* shows that all the clones except the parental NRK cells express the entire rat *TGF-α* gene but at different levels. We do not know if the difference in expression level is due to different integration sites or due to some other reason.

Thus, we established that the ability of the NRK colonies to grow in soft agar in the presence of TGF-β after infection with SW355 is indeed due to the infection and not due to spontaneous transformation and that these cells transcribe the rat *TGF-α* gene without any change in the sequence.

SW355-Infected NRK Cells Secrete Rat TGF-α into the Medium. We used soft agar assays and EGF receptor binding-competition assays to confirm that SW355-infected NRK clones (1/28 Cl.3 and 1/28 Cl.8) were synthesizing biologically active rat TGF-α and to evaluate the level of its secretion.

Fig. 4 shows the results of soft agar assays with conditioned media prepared from 1/28 Cl.8 and NRK cells. NRK cells do not produce any TGF-α-like activity to support NRK cell growth in soft agar in the presence of TGF-β. The amount of TGF-α-like activity secreted from infected cells was found to be approximately 1.2 ng per ml of conditioned medium by comparison with an EGF standard curve. Somewhat lower amounts of TGF-α-like activity (0.8 ng per ml of medium) were secreted into the medium conditioned by 1/28 Cl.3 cells (data not shown). Therefore, the activity can be attributed to the rat TGF-α insert in SW355 and not to the helper virus sequence.

The amount of TGF-α released by the transformed cells was also determined from an EGF receptor binding-competition assay. Various amounts of conditioned media (equivalent to 0.1–10 ml of original conditioned media) were assayed for their ability to compete with ^{125}I-labeled EGF in binding to the EGF receptor. Fig. 5 shows the EGF standard

FIG. 5. EGF radioreceptor assay with conditioned media from 1/28 Cl.8 and NRK cells. Lyophilized conditioned media prepared as described in the legend to Fig. 4 were dissolved in binding assay solution. Binding of ^{125}I-labeled EGF (140,000 cpm) to NRK cells in the presence of various amounts of concentrated conditioned media or EGF was carried out in 24-well dishes at room temperature. ●, EGF standard curve; ○, conditioned media from 1/28 Cl.8 cells; ▲, conditioned media from NRK cells.

curve and curves obtained from conditioned media prepared from NRK and 1/28 Cl.8 cells. The amount of EGF competing activity in 1/28 Cl.8 was approximately 0.3 ng per ml of conditioned medium.

The nature of the activity was also determined by a radioimmunoassay using a rat TGF-α-specific antibody that does not recognize EGF (data not shown). The amount of rat TGF-α secreted from 1/28 Cl.8 cells was approximately 0.3 ng per ml of conditioned medium and the amount for 1/28 Cl.3 was somewhat lower than that for 1/28 Cl.8. Therefore, both 1/28 Cl.3 and 1/28 Cl.8 cells secreted biologically active rat TGF-α from the integrated form of SW355.

DISCUSSION

Recent progress in chemical synthesis of oligonucleotides has made it possible to synthesize genes (25). The rat *TGF-α* gene was synthesized by a combination of enzymatic and chemical methods and was expressed in a retrovirus vector. Our construct contains the leader sequence and the mature TGF-α sequence, but it does not have the 3'-processed sequence. This sequence is the most conserved between the human and rat TGF-α coding regions.

It has been observed that virally transformed cells can grow in low serum medium but that nontransformed cells require more serum for their growth (26, 27). The concept that transformed cells synthesize growth factors, release, and respond to them has been proposed and termed "autocrine" secretion (28), and such autocrine stimulation of cell growth has been associated with tumor growth *in vivo* (29). Since all cells of 1/28 Cl.3 or 1/28 Cl.8 are genetically identical (clones), we expect that all cells are secreting TGF-α at similar levels and that TGF-α secreted from these clones interacts with the EGF receptor by way of an autocrine mechanism.

Most of the oncogenes discovered thus far have been isolated by transfection of cellular DNA from tumors into
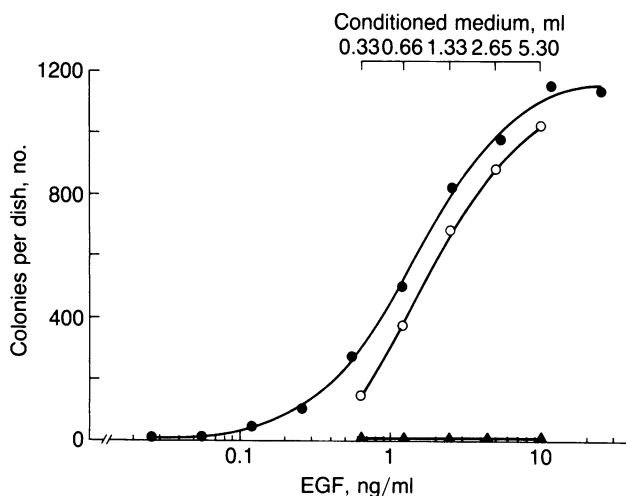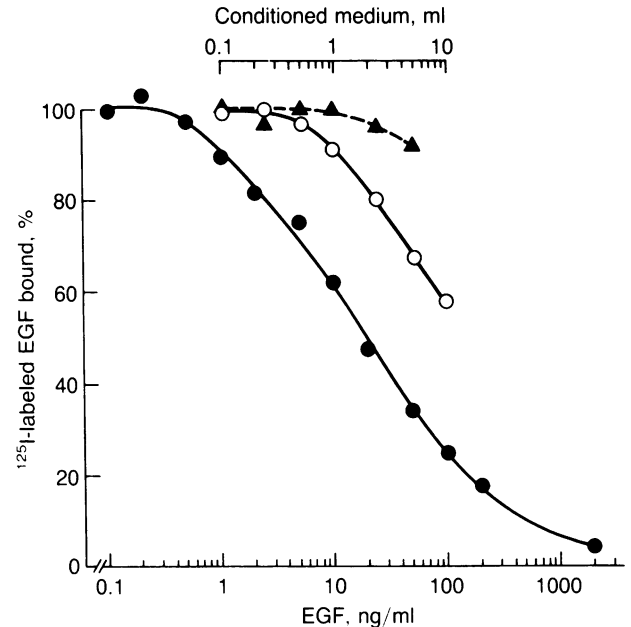

FIG. 4. Soft agar assay of the conditioned media from NRK cells and 1/28 Cl.8. Serum-free conditioned media from NRK and 1/28 Cl.8 cells were prepared by incubating cells in serum-free Dulbecco's modified Eagle's medium for approximately 18 hr after preincubation in the same medium for 3 hr. After extensive dialysis of the conditioned media against 1 M acetic acid, the media were lyophilized and dissolved in 4 mM HCl containing bovine serum albumin at 1 mg/ml. Various amounts of concentrated conditioned media and 4.2 × 10³ NRK cells were mixed in 0.5% soft agar containing TGF-β at 1 ng/ml in 35-mm dishes. Colonies (>62 μm) were counted after 7 days of incubation by using an Omnicon automated image analyzer. ●, EGF standard curve; ○, conditioned media from 1/28 Cl.8 cells; ▲, conditioned media from NRK cells.

NIH 3T3 cells or were carried on transforming viruses (for review, see refs. 30 and 31). Transforming genes carried on retroviruses were transduced from cellular genes by unknown mechanisms. We have shown that the *TGF-α* gene, normally induced by known oncogenes, can be expressed by inserting it into the retrovirus vector in our NRK system. It is important to study whether not only the infected cells producing TGF-α but also the recombinant virus SW355 itself can be tumorigenic *in vivo* where the additional factors might be supplied in the bloodstream. A similar approach using a retrovirus vector was taken to introduce the granulocyte-macrophage colony stimulating factor (GM-CSF) gene into a GM-CSF-dependent murine cell line (32). The distinction between oncogenes and genes coding for growth factors and their receptors is diminishing rapidly after the findings that platelet-derived growth factor, the EGF receptor, and the GM-CSF receptor are related to the oncogene products of *sis, erbB,* and *fms,* respectively (15, 16, 33, 34). All of these oncogenes were found in transforming retroviruses. It is also known that the EGF receptor and the *src* protein phosphorylate some common substrates for their kinase activity (35). These similarities indicate a common pathway in growth stimulation and transformation. Thus, other growth factor genes could be "transforming genes" under appropriate conditions—i.e., in the right cell type and with the right supplements.

Note Added in Proof. After this manuscript was submitted, it was shown that cells expressing a cDNA clone of the human *TGF-α* gene in an SV40 vector were tumorigenic in nude mice (36).

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