Retroviral Expression of Transforming Growth Factor-Alpha Does Not Transform Fibroblasts or Keratinocytes

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Transforming growth factor α (TGFα) is a peptide so named because it helps to impart anchorage-independent growth to normal rat kidney (NRK) cells in vitro and is secreted by many rodent and human tumor cells. To directly investigate the transforming properties of this factor, we constructed a replication-defective murine retrovirus that expresses the human sequence coding for TGFα. Infection of NIH/3T3 cells with the TGFα retrovirus led to the integration of a transcriptionally active provirus and overexpression of biologically active TGFα, but failed to induce morphologic transformation. Similarly, the TGFα retrovirus failed to induce morphologic transformation of five other types of rodent fibroblasts.

We also investigated the effect of TGFα expression on the growth of BALB/MK mouse keratinocytes, which require epidermal growth factor (EGF) for proliferation. We show that exogenously added TGFα is an extremely potent mitogen for BALB/MK cells. However, retroviral expression of TGFα in BALB/MK cells failed to relieve dependence on exogenously added EGF (or TGFα) for cell growth. These results suggest that overexpression of TGFα does not, by itself, transform rodent fibroblasts or keratinocytes. *J Invest Dermatol 95:382–387, 1990*

Transforming growth factor alpha (TGFα) is a peptide that was originally discovered by virtue of its secretion into the culture medium by retrovirally transformed rodent cells [1]. TGFα is synthesized as a glycosylated and palmitoylated 160-amino acid transmembrane precursor that contains a 100-amino acid extracellular domain encoding the mature 50-amino acid polypeptide, a hydrophobic transmembrane domain, and a 35-amino acid cytoplasmic domain [2]. TGFα is detected in culture supernatants in different forms, ranging in size from 5 to 20 kD [3]. The larger species represent N-glycosylated forms of TGFα released after proteolytic cleavage of the extracellular domain of the precursor [2]. This peptide has both structural and functional homology to EGF [1,4,5] and binds to the EGF receptor [6], triggering a sequence of intracellular events that ultimately lead to stimulation of cell growth. TGFα has been detected in neoplastic tissues [7,8]. In particular, TGFα is often expressed in squamous cell carcinomas [9]. These results, and the finding that exogenous TGFα confers a transformed phenotype and anchorage-independent growth to NRK cells in culture [10], led to the proposal that TGFα may cause malignant transformation in vivo of cells releasing this factor [11].

The cloning of the cDNA for TGFα [12,13] has permitted a direct analysis of this growth factor. Introduction of the human TGFα gene into rat 1 cells and subsequent expression of TGFα protein was reported to enhance the tumorigenicity of the recipient cell lines [14]. However, high-level expression of TGFα does not confer the malignant phenotype to NIH/3T3 cells [15]. Recent work has shown that retroviral expression of a TGFα gene in primary mouse epidermal cells or papilloma cells stimulated the growth of skin papillomas in a skin graft reconstitution model, but did not cause neoplastic progression [16]. A role for TGFα in normal cellular growth and development is suggested by recent studies showing that bovine adenohypophysial cells [17] and human keratinocytes [18] synthesize TGFα.

Cloned BALB/MK-2 cell lines derived from BALB/c mouse keratinocytes have been developed [19]. These lines absolutely require EGF for cell proliferation [20] and thus provide a useful model system to test the effect of autocrine (effect of synthesis of a growth factor by a cell on that individual cell) expression of TGFα on epithelial cell growth. Prior studies have shown that retroviral transforming genes of the *src* and *ras* oncogene families can relieve the EGF requirement for BALB/MK-2 growth [20].

In the present study, we constructed and characterized a replication-defective murine retrovirus (ZipTGFα) that expresses the entire coding sequence of human TGFα. We used this retrovirus to introduce and express sequences encoding TGFα into six different fibroblast cell lines as well as BALB/MK-2 epithelial cells. We show that TGFα expression does not induce focus formation in any of the fibroblast cells analyzed nor does infection of BALB/MK-2 epithelial cells with ZipTGFα lead to EGF-independent growth. These findings further suggest that autocrine expression of TGFα alone is not sufficient for transformation.

MATERIALS AND METHODS

Cell Culture Fibroblast cell lines were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% calf serum (Colorado calf serum). The FR3T3 cell line was a gift from

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Abbreviations:
CFU: colony-forming units
DMEM: Dulbecco's modified Eagle's medium
EGF: epidermal growth factor
Neo: neomycin
NRK: normal rat kidney
TGFα: transforming growth factor alpha

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R. A. Weinberg (Whitehead Institute). The clonal mouse epidermal keratinocyte cell line, BALB/MK-2 [19], was grown in DMEM containing a calcium concentration of 0.05 mM and supplemented with 10% dialyzed fetal calf serum (GIBCO Laboratories) and, where indicated, 4 ng EGF (Collaborative Research, Inc.) per ml. Recombinant human TGFα was prepared as described previously [12].

DNA Transfections ψ2 cells [21] were transfected with 10 μg pZipTGFα [15] or pZipNeo [22] DNA and 40 μg calf thymus carrier DNA/100-mm petri dish using the calcium phosphate precipitation method [23,24]. After 10 h, the medium was changed to fresh serum-supplemented DMEM. Twenty-four hours later, cells were trypsinized and replated at a 1:100 dilution in medium containing 750 μg/ml G418 (GIBCO). G418-resistant colonies were picked and expanded after 2 weeks.

Viral Infections Infectious virus was collected as a 24-h supernatant from subconfluent virus producing ψ2 cells. To titer infectious virus, NIH/3T3 cells were plated at 5×10^5/60-mm dish on day 1. On day 2, the medium was changed to medium containing 2 μg/ml polybrene, and test viral samples were added. On day 3, the cells were split in medium containing 400 μg/ml G418. Colonies were counted after two weeks.

For focus assays on fibroblast cell lines, cells were seeded overnight at a density of 1.5×10^5 cells/60-mm dish in medium containing 2 μg polybrene/ml. The next day, the cells were infected with serial virus dilutions and the cells were processed as described [25]. For generation of clonally infected NIH/3T3 cell lines, cells were infected with serial dilutions of virus in the presence of 2 μg/ml polybrene. Forty-eight hours later, the medium was changed to medium containing 400 μg/ml G418. Isolated colonies were picked and expanded 2 weeks later.

Focus assays on BALB/MK-2 keratinocytes plated at 1×10^5 cells/60-mm plate were performed as described [20]. Where indicated, 48 h after infection the medium was changed to include 10 μg/ml G418 and/or 4 ng/ml EGF or TGFα.

To generate virally infected BALB/MK-2 cell lines, 1×10^5 cells were seeded/60-mm dish and infected with either 10^5 or 10^6 colony-forming units in the presence of 2 μg/ml polybrene. Forty-eight hours later, the cells were selected in 10 μg/ml G418 as mass cultures.

Analysis of Proviral Integration DNA samples were digested with XbaI, electrophoresed in 1% agarose gels, and transferred to nitrocellulose as described [25]. Filters were hybridized with DNA fragments labeled with α[^{32}P]-dATP by nick translation [27]. Hybridization was performed in 50% formamide, 5 × SSC, at 42°C for 18 h. Filters were then washed twice for 20 min with 2 × SSC at room temperature, once at 65°C for 20 min in 0.1 × SSC, and 0.1% SDS, then for 20 min at 65°C in 0.1 × SSC. The neo probe used was the HindIII to SmaI fragment of pSV2 neo [28]. The TGFα cDNA probe was the ClaI to ClaI fragment of pSVTGFα [15].

Transcriptional Analysis Total cellular RNA were isolated by a modification of the guanidine hydrochloride extraction method [29] and analyzed in agarose-formaldehyde gels [30]. The gel was then incubated for 30 min at 24°C in 50 mM NaOH before transferring the RNA to nitrocellulose filters in a 1 M ammonium acetate solution. Filters were hybridized and washed as described above for Southern analysis.

Measurement of TGFα in Cell Lysates and Medium All samples were assayed using a sandwich ELISA for TGFα, with TGFα monoclonal antibody as solid phase antibody and polyclonal rabbit anti-TGFα serum as detecting antibody [31]. Human and mouse EGF were not detectable in the ELISA assay. For assay of TGFα production by virally infected BALB/MK-2 cells, BALB/MK-2 cell lines were grown to confluence in two 175 cm² flasks. Twenty-four-h supernatants were collected in 50 ml medium supplemented with 10% dialyzed fetal calf serum, and the cells and the medium were processed as described [9]. Fetal calf serum did not contain detectable TGFα.

Table I. Expression of TGFα by NIH/3T3 Cells Infected by a TGFα Recombinant Retrovirus

Cell Line	Human TGFα Secreted[a] (ng/ml)
ZipNeo infected	
neo-1-3	<0.1
ZipTGFα infected	
ZipTGF-1	3.2
ZipTGF-2	2.0
ZipTGF-3	7.4
ZipTGF-4	1.8
ZipTGF-5	3.2
ZipTGF-6	2.5
ZipTGF-7	1.9
ZipTGF-8	1.7
ZipTGF-9	1.3
ZipTGF-10	2.2

[a] Confluent 10-cm plates were washed 3 times with DMEM and supernatants collected after a 24 h incubation with 5 ml DMEM.

Thymidine Incorporation Assay BALB/MK-2 cells (6×10^3 in 0.18 ml of DMEM, 10% fetal calf serum, 0.05 mM Ca^{++}, 4 ng/ml of EGF) were seeded into each well of a 96-well plate and incubated at 37°C. After 1–2 days, the cells were confluent and the medium was changed to serum-free medium lacking EGF. Forty-eight hours later, 180 μl of the indicated growth factors or conditioned media was added. After seventeen h, [^{3}H]thymidine (80 Ci/mmol, New England Nuclear) was added to the cultures (1 μCi in 20 μl DMEM). Six hours later, the medium was aspirated, the cells washed 3 times with 5% trichloroacetic acid (200 μl/well), acid-insoluble radioactivity extracted with 0.25 M NaOH (150 μl), and the extract counted in Aquasol (New England Nuclear).

RESULTS

Characterization of a Murine Retroviral Vector Which Expresses Human TGFα To generate a murine retrovirus which expresses human TGFα, we utilized the retroviral construct pZipTGFα [15]. In this construct (pZipTGFα), the entire coding region of the TGFα cDNA was cloned into the BamHI site of the Moloney murine leukemia virus (Mo-MuLV)-based vector pZipNeoSV (X) 1 [22]. This vector contains the bacterial transposon TN5 neomycin-resistance gene, conferring G418 resistance in mammalian cells [28].

Infectious ZipTGFα virus was produced by transfecting pZipTGFα DNA into the packaging cell line ψ2 [21]. G418-resistant colonies were expanded and analyzed for their ability to secrete TGFα into the medium. Five of five ZipTGFα ψ2 cell lines secreted TGFα at concentrations ranging from 6 to 22 ng/ml. Supernatants were collected and titered for their ability to transmit G418 resistance to NIH/3T3 cells. Viral titers ranged from 10^5 to 10^6 G418-resistant colony-forming units (cfu) per ml.

To demonstrate that TGFα was expressed by ZipTGFα virus, we infected NIH/3T3 cells with ZipTGFα virus and isolated 10 clonally derived G418 resistant cell lines as described in *Materials and Methods*. ZipTGFα-infected cells secreted TGFα at concentrations ranging from 1.3 to 7.4 ng/ml, as judged by a TGFα ELISA (Table I). The amounts of TGFα released were at least five times higher than those secreted by transformed cell lines reported to be high producers of TGFα [7,10]. Thus, the ZipTGFα virus could both induce G418 resistance and direct the synthesis of large amounts of TGFα by infected fibroblasts. As expected, the ZipNeo control virus induced G418 resistance, but not TGFα production, in NIH/3T3 cells.

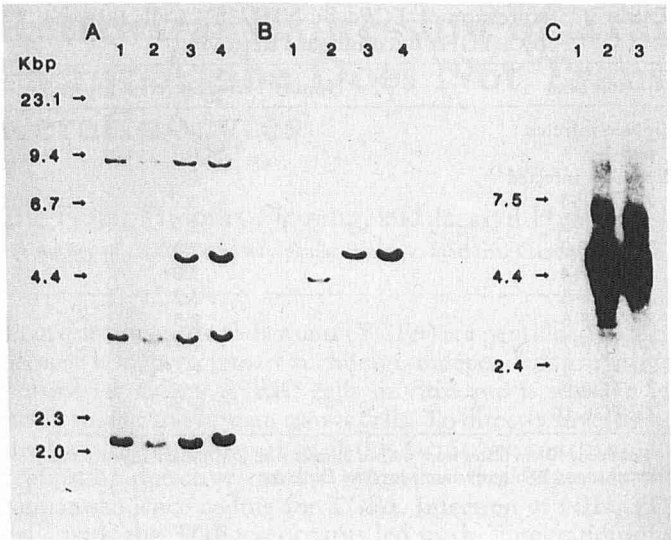

Figure 1. Analysis of proviral integration and virus-specific mRNA in virally infected NIH/3T3 cells. High-molecular-weight DNA was digested with XbaI and analyzed by Southern blotting with *A*, a TGFα cDNA probe and *B*, a neo^R gene probe. *Lane 1,* NIH/3T3 cells; *lane 2,* ZipNeo-infected cell line neo-1-3; *lanes 3* and *4*, ZipTGFα-infected cell lines ZipTGFα-1, ZipTGFα-3. *C* Total RNA from the following cell lines was electrophoresed on a denaturing gel and analyzed by Northern blotting with a TGFα cDNA probe: *lane 1,* NIH/3T3 cells; *lanes 2* and *3*, ZipTGFα-infected cell lines ZipTGFα-3, ZipTGFα-1. Each *lane* contains 15 μg total RNA.

Analysis of Viral Integration and Transcription in ZipTGFα-Infected NIH/3T3 Cells To demonstrate proviral integration in ZipTGFα-infected NIH/3T3 cells, genomic DNA from clonally infected cell lines was digested with XbaI and analyzed by Southern blotting. Hybridization to a TGFα cDNA probe (Fig 1A) revealed an extra fragment, 4.8 kb long, in ZipTGFα-infected cells (*lanes 3* and *4*), compared with uninfected NIH/3T3 cells *(lane 1)* or ZipNeo-infected cells *(lane 2)*. The extra fragment in ZipTGFα-infected cells corresponds to the ZipTGFα provirus, because XbaI cleaves the ZipTGFα construct once in each LTR [22]. Hybridization of these same DNA to a neo^R probe (Fig 1B) revealed, as expected, the same size proviral fragment in ZipTGFα-infected NIH/3T3 cells (*lanes 3* and *4*), whereas uninfected NIH/3T3 DNA did not hybridize. DNA from the ZipNeo-infected cells contained the expected 4.2-kb XbaI fragment *(lane 2)*.

The expression of TGFα-containing RNA transcripts in ZipTGFα-infected NIH/3T3 cell lines was examined by Northern blot hybridization analysis (Fig 1*C*). ZipTGFα- (*lanes 2* and *3*), but not ZipNeo- *(lane 1)* infected cells contained one major mRNA species of approximately 5.1 kb, corresponding to the size of viral genomic RNA, which hybridized to the TGFα cDNA probe.

Effect of ZipTGFα Viral Infection on the Phenotype of Fibroblast Cell Lines To assess the transforming potential of ZipTGFα virus, we infected various fibroblast cell lines. Infected cultures were scored for foci formation or selected in G418-containing medium. As shown in Table I, ZipTGFα virus did not induce detectable foci in any of the cell lines. These results were not due to inability of these cell lines to be infected with ZipTGFα virus, because at least 100 colonies were observable in every plate after selection of infected cells in G418-containing medium (Table II). Moreover, selected cultures infected with undiluted virus (10^6 cfu/ml) grew uniformly denser (data not shown), suggesting that when large numbers of cells were infected with ZipTGFα, sufficient TGFα was secreted into the medium to exert a growth-promoting effect on the entire cell population. Infection of the same fibroblast cell lines with 10^6 cfu/ml control ZipNeo virus had no effect on the density of cell growth. These results are consistent with previous work, which showed that TGFα-expressing NIH/3T3 cells grew to high saturation density in culture, but TGFα exerted little, if any, effect on the individual cell synthesizing this factor [15].

BALB/MK-2 Cells Infected with ZipTGFα Virus are Not Relieved of Their EGF Requirement for Cell Growth BALB/MK-2 is a clonally derived keratinocyte cell line that requires EGF for growth [19]. Because TGFα has both structural and functional homology to EGF [4,5] and binds to the same receptor as EGF [6], we initially examined whether exogenously added TGFα could replace the BALB/MK-2 requirement for EGF for proliferation. As seen in Fig 2, TGFα is a potent mitogen for BALB/MK-2 cells. A fivefold stimulation in [^{3}H]thymidine incorporation was observed upon addition of only 0.1 ng/ml TGFα. Addition of 10 ng/ml TGFα led to ~80 times stimulation in [^{3}H]thymidine incorporation. At all concentrations tested, TGFα stimulated DNA synthesis as well as, or significantly better than, EGF. These results were consistent with other studies indicating that TGFα and EGF are comparable in their mitogenicity for BALB/MK cells [32].

Because TGFα is a potent mitogen for BALB/MK cells, we reasoned that infection of BALB/MK-2 cells with ZipTGFα virus might lead to factor-independent growth via an autocrine mechanism. Therefore, we infected BALB/MK-2 cells with ZipTGFα virus. As shown in Fig 3, *plate C,* Kirsten-MSV induced EGF-independent foci, in agreement with previous results [20]. In striking contrast, infection with the ZipTGFα virus failed to yield any EGF-independent foci (Fig 3, *plate A*). This lack of EGF-independent growth by ZipTGFα-infected cells did not affect the ability of the ZipTGFα virus to infect BALB/MK-2 cells because selection of infected cells in medium containing EGF and G418 allowed the growth of many colonies (Fig 3, *plate B*). Furthermore, BALB/MK-2 cells grew well in medium containing 4 ng/ml recombinant TGFα (Fig 3, *plate D*), indicating that externally added TGFα was functionally interchangeable with EGF in stimulating the growth of BALB/MK-2 cells.

Several mechanisms could account for the inability of ZipTGFα to abrogate the EGF dependence of BALB/MK-2. It was possible that the ZipTGFα virus was not expressed in the epithelial cells.

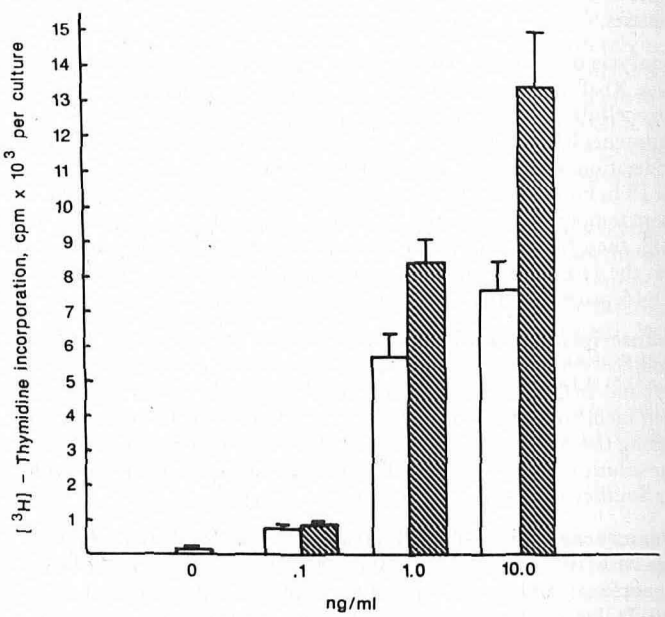

Figure 2. TGFα mitogenic effects on BALB/MK-2 keratinocytes. The mitogenic response of BALB/MK-2 cells to indicated concentrations of EGF (□) or TGFα (▨). The incorporation of [^{3}H]thymidine into DNA (trichloroacetic acid-insoluble radioactivity) was determined as described in *Materials and Methods.* Data (mean ± SD) are from a representative experiment.

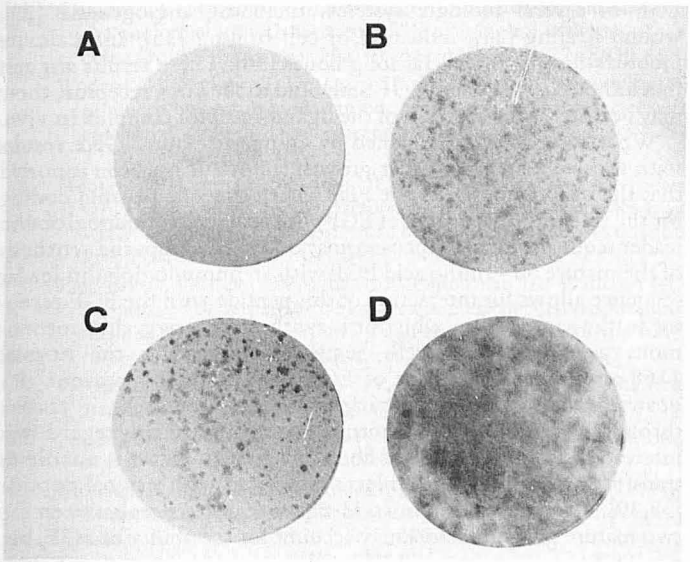

Figure 3. Focus assay following infection of BALB/MK-2 cells by retroviruses. BALB/MK-2 cells were plated at 1×10^5 cells/60-mm dish. After virus infection, the cells were maintained in low-calcium growth medium containing the indicated additions. Plates were stained with Giemsa after 2 weeks. *A*, ZipTGFα virus; *B*, ZipTGFα virus + 4 ng/ml EGF + 10 μg/ml G418; *C*, Kirsten-MSV; *D*, uninfected + 4 ng/ml TGFα.

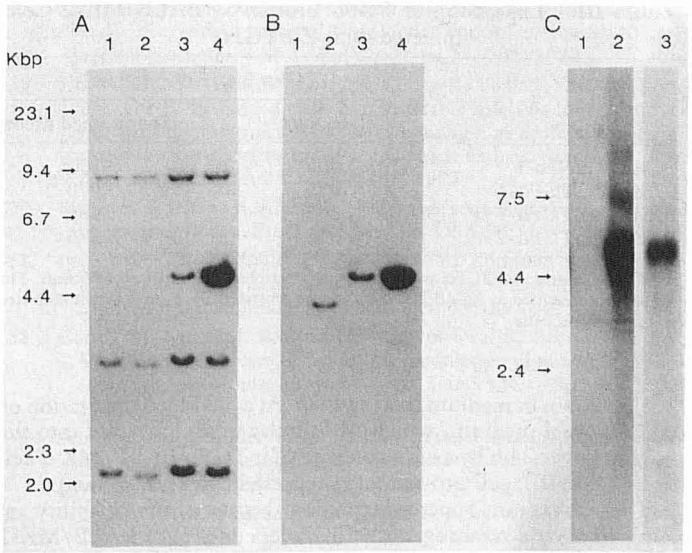

Figure 4. Analysis of proviral integration and virus-specific mRNA in virally infected BALB/MK-2 cells. High molecular weight DNA was digested with XbaI and analyzed by Southern blotting with A a TGFα cDNA probe and B, a neo^R gene probe: *lane 1,* BALB/MK-2 cells; *lane 2,* ZipNeo-infected cell line B/MK-ZipNeo-1; *lanes 3* and *4*, ZipTGFα-infected cell lines B/MK-ZipTGFα-1, B/MK-ZipTGFα-2. *C*, Total RNA from the following cell lines was electrophoresed on a denaturing gel and analyzed by Northern blotting using a TGFα cDNA probe: *lane 1,* BALB/MK-2 cells; *lanes 2* and *3*, ZipTGFα-infected cell lines B/MK-ZipTGFα-2, B/MK-ZipTGFα-1. Each *lane* contains 20 μg total RNA.

Alternatively, the growth-promoting effects of TGFα might not be exerted specifically on the individual BALB/MK-2 cell infected with ZipTGFα, but instead act on the entire cell population in a paracrine manner after secretion into the medium. If the latter were the case, the number of ZipTGFα-infected cells plated might not secrete a sufficient amount of TGFα into the medium to cause the culture to grow in an EGF-independent manner.

To confirm that ZipTGFα-infected BALB/MK-2 cells contained integrated proviruses with the expected structure, we isolated genomic DNA from several mass cultures of virally infected BALB/MK-2 cells that had been selected in G418. Southern blot hybridization analysis of XbaI-digested DNA with a TGFα cDNA probe revealed an extra 4.8-kb fragment in cells infected with 10^5 cfu ZipTGFα (Fig 4*A, lane 3*), compared with uninfected (Fig 4*A, lane 1*) BALB/MK-2 cells. BALB/MK-2 cells that had been infected with 10^6 cfu ZipTGFα demonstrated evidence of multiple proviral inserts per cell (Fig 4*A, lane 4*) when the intensity of hybridization of the proviral fragments to the endogenous TGFα fragments was compared. Hybridization of the same DNA to a neo^R probe confirmed these results (Fig 4*B*).

To establish that ZipTGFα-infected BALB/MK-2 cells were expressing TGFα transcripts, we examined total RNA from mass cultures of infected cells by Northern blot hybridization analysis. As shown in Fig 4*C*, ZipTGFα- *(lanes 2, 3)* but not ZipNeo- *(lane 1)* infected cells contained one predominant RNA species of approximately 5.1 kb, which hybridized to the TGFα cDNA. This size RNA corresponds to the viral genomic RNA and was identical in size to the transcript detected in NIH/3T3 cells infected with the same virus. Finally, mass cultures of ZipTGFα virally infected cells, which on average contained multiple proviral inserts, expressed more TGFα RNA than cells containing approximately one proviral insert per cell (Fig 4*C, lane 2* versus *lane 3*).

To demonstrate that ZipTGFα-infected BALB/MK-2 cells produced functional TGFα protein, we analyzed cell extracts and conditioned media by the TGFα ELISA (Table III). Both ZipTGFα-infected cell lines showed significant levels of TGFα in the conditioned media, whereas no TGFα protein was detectable in ZipNeo-infected cells. None of the cell lines displayed detectable TGFα in the cell extracts, suggesting that TGFα expressed by BALB/MK-2 cells is predominantly secreted. The TGFα ELISA utilized here detects TGFα that can bind to the EGF receptor but does not detect TGFα with incorrectly formed disulfide bridges [31], suggesting that the TGFα secreted by BALB/MK-2 cells was functionally active. To confirm these results, we tested whether conditioned media from ZipTGFα-infected cells was mitogenic for BALB/MK-2 cells. Supernatants from near-confluent ZipTGFα-infected, but not ZipNeo-infected, cells stimulated [^{3}H]thymidine incorporation in control BALB/MK-2 cells at least twofold (data not shown).

To determine whether the level of TGFα expression by ZipTGFα-infected BALB/MK-2 cells was sufficient to support BALB/MK-2 cell growth, we added TGFα to control BALB/MK-

Table II. Infection of Different Cell Lines by ZipTGFα Virus: Incidence of Foci Formation and G418-Resistant Colonies

	Viral Dilution[a]	Number of Foci in Normal Medium[b]	Number of G418-Resistant Colonies[c]
BALB/3T3	10^0	0	TMC[d]
	10^{-1}	0	>500
Fisher rat	10^0	0	TMC
	10^{-1}	0	>500
Fisher rat embryo	10^0	0	TMC
	10^{-1}	0	>300
FR3T3	10^0	0	TMC
	10^{-1}	0	>500
NRK	10^0	0	TMC
	10^{-1}	0	>100
NIH/3T3	10^0	0	TMC

[a] Viral titer of undiluted virus on NIH/3T3 cells was 10^6 cfu/ml.
[b] Cells were maintained in media containing 5% calf serum. Plates were scored 16 d after infection.
[c] Seventy-two hours after infection, the medium was changed to that containing 400 μ/ml G418. Colonies were counted 14 d later.
[d] *TMC,* too many to count.

Table III. Expression of TGFα Protein by BALB/MK-2 Cells Infected with ZipTGFα

Cell Line	TGFα Expression (ng/ml)[a]	
	Cell Extract	Conditioned Media
B/MK-ZipNeo-1	<.005	<.005
B/MK-ZipTGFα-1	<.005	.070
B/MK-ZipTGFα-1	<.005	.085
B/MK-ZipTGFα-2	<.005	.275

[a] The expression of TGFα was assessed as described in *Materials and Methods*. The results are presented as ng of TGFα/ml unconcentrated 24-h supernatant collected from confluent cells.

2 cells grown in medium lacking EGF. At a TGFα concentration of 0.275 ng/ml medium, which was similar to that secreted into the medium over 24 h by a near-confluent ZipTGFα BALB/MK-2 cell line (Table III), cell growth was supported (data not shown).

There was an apparent paradox between the inability of ZipTGFα virus to abrogate the EGF dependence of BALB/MK-2 and the ability of ZipTGFα-infected BALB/MK-2 cells to secrete sufficient TGFα into the medium to support the growth of control BALB/MK-2 cells. This can be explained by noting that the focus assays in Fig 3 were performed by plating 1×10^5 BALB/MK-2 cells/dish, whereas conditioned medium was collected from G418-selected near-confluent cultures containing 10^7 virally infected BALB/MK-2 cells. If sparsely plated BALB/MK-2 cells that synthesized TGFα processed the growth factor through the cell as an inactive precursor or in a different cellular compartment from its receptor, and/or the growth factor were rapidly secreted and diffused, it may exert little or no direct effect on the growth of that cell. Conversely, near-confluent cultures of G418-selected virally infected cells would have a high cell-to-ml medium ratio, allowing detectable levels of TGFα to accumulate in the medium.

DISCUSSION

We report here the characterization of a murine retrovirus (ZipTGFα) that expresses the entire normal coding sequence of human TGFα. NIH/3T3 cells infected with this retrovirus were shown to have integrated a transcriptionally active provirus of the expected size, and to secrete TGFα into the medium. However, infection of NIH/3T3 cells with ZipTGFα did not induce foci formation. Moreover, we demonstrated that the inability of ZipTGFα virus to transform NIH/3T3 cells was not specific to this cell line, because similar results were obtained upon infection of five fibroblast cell lines. These results are consistent with earlier work showing that a high-level expression of TGFα by NIH/3T3 cells led to a growth-promoting effect on the entire cell culture, but did not confer the tumorigenic phenotype to cells synthesizing this factor [15]. Our results are consistent with recent work indicating that expression of TGFα by primary mouse epidermal cells or papilloma cells increased the size of tumors formed in vivo after skin grafting, but did not appear to influence tumor progression directly [16].

We also asked whether the expression of TGFα by a keratinocyte cell line that requires an analogous growth factor, EGF, for growth could lead to factor-independent growth. We showed that BALB/MK-2 cells infected with ZipTGFα remained dependent on EGF, whereas a viral ras gene led to loss of EGF dependence. This result was not due to an inability of BALB/MK-2 cells to respond to TGFα, because control BALB/MK-2 cells grew well in medium in which TGFα was the primary growth-factor supplement. Moreover, by enriching the infected population for only those cells which express the TGFα retrovirus, we were able to demonstrate that ZipTGFα-infected BALB/MK-2 cells secrete biologically active TGFα at a level that supports the growth of control BALB/MK-2 cells.

At high concentrations, TGFα appeared to be a more potent mitogen than EGF for BALB/MK-2 cells. The reasons for these differences are unknown. TGFα is known to be more potent than EGF in several biologic systems, including angiogenesis [33], wound healing [34], induction of cell ruffling [35], and calcium mobilization from fetal rat long bones [36]. These results suggest that although TGFα and EGF both bind to the same receptor, there may be differences in action of the ligand-receptor complex in vivo.

What insight may be gained by comparing our TGFα results with those obtained for other growth factors? It has been reported that the introduction into the FR3T3 cell line of a plasmid coding for the fully processed form of EGF with an added immunoglobulin leader sequence induces focus formation [37]. Perhaps the synthesis of the mature 50-amino acid EGF with an immunoglobulin leader sequence allows for interaction of this peptide with the EGF receptor in the same cell in which it is synthesized, triggering autonomous growth of that cell. Sequences present in the normal 1168-amino acid precursor of EGF may ordinarily prevent this growth factor from interacting with its receptor as it traffics through the cell during its normal processing. In this regard it is interesting to note that basic fibroblast growth factor is unable to transform NIH/3T3 cells unless synthesized with a signal peptide [38,39]. Alternatively, amino acid sequence differences between the two mature growth factors may account for the ability of EGF, but not TGFα, to transform FR3T3 cells.

The autocrine model [11] postulates that constitutive release of a mitogenic growth factor by a cell that possesses receptors for it leads to cellular transformation. TGFα is a potent mitogen for fibroblasts [15] and keratinocytes (Fig 2). The inability of TGFα to trigger autocrine transformation of fibroblasts or keratinocytes suggests that this model may not be applicable if the growth factor is rapidly secreted and/or processed in a different compartment from its cellular receptor and thus unable to mitogenically trigger the same cell in which it is synthesized. However, the expression of TGFα may confer a selective growth advantage to a tumor or preneoplastic lesion in vivo. To produce a tumor, neoplastic cells must counteract normal tissue conditions that restrict clonal expansion. In this regard, recent work has shown that expression of TGFα by papilloma cells in vivo can stimulate tumor growth, particularly when tumor growth is suppressed by normal tissue [16].

Overexpression of EGF receptors has been observed in cells derived from squamous cell carcinomas [9,41,42], mammary cell carcinomas [9,43,44], renal cell carcinomas [9], and gliomas [45]. TGFα is commonly coexpressed in these tumor types [9,46]. It has been reported that under conditions of 100–200 times overexpression of the EGF receptor by NIH/3T3 cells, TGFα coexpression was able to induce the transformed phenotype [46]. Expression of the EGF receptor above a critical threshold may be necessary for autocrine stimulation by TGFα to deliver an effective mitogenic signal to the cell in which it was synthesized. Thus, dysregulation of expression of TGFα and its receptor may contribute to the tumor phenotype in vivo.

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REFERENCES

1. Todaro GJ, De Larco JE, Cohen S: Transformation by murine and feline sarcoma viruses specifically blocks binding of epidermal growth factor to cells. Nature 264:26–31, 1976
2. Bringman TS, Lindquist PB, Derynck R: Different transforming growth factor-α species are derived from a glycosylated and palmitoylated transmembrane precursor. Cell 48:429–440, 1987
3. Derynck R: Transforming growth factor-α. Cell 54:593–595, 1988
4. Marquardt H, Hunkapiller MW, Hood LE, Twardzik DR, De Larco JE, Stephenson JR, Todaro GJ: Transforming growth factors produced by retrovirus transformed rodent fibroblasts and human melanoma cells: amino acid sequence homology with epidermal growth factor. Proc Natl Acad Sci USA 80:4684–4688, 1983
5. Massague J: Epidermal growth factor-like transforming growth factor. II. Interactions with epidermal growth factors in human placenta membranes and A431 cells. J Biol Chem 258:13614–13630, 1983

6. Carpenter G, Stoscheck CM, Preston YA, De Larco JE: Antibodies to the epidermal growth factor block the biological activities of sarcoma growth factor. Proc Natl Acad Sci USA 80:5627–5630, 1983
7. Todaro GJ, Fryling C, De Larco JE: Transforming growth factors produced by certain human tumors: polypeptides that interact with epidermal growth factor receptors. Proc Natl Acad Sci USA 77:5258–5262, 1980
8. Roberts AB, Lamb LC, Newton DL, Sporn MB, De Larco JE, Todaro GJ: Transforming growth factors: isolation of polypeptides from virally and chemically transformed cells by acid/ethanol extraction. Proc Natl Acad Sci USA 77:3494–3498, 1980
9. Derynck R, Goeddel DV, Ullrich A, Gutterman JR, Williams RD, Bringman TS, Berger WH: Synthesis of messenger RNAs for transforming growth factor α and β and the epidermal growth factor receptor by human tumors. Cancer Res 47:707–712, 1987
10. De Larco JE, Todaro GJ: Growth factors from murine sarcoma virus transformed cells. Proc Natl Acad Sci USA 75:4001–4005, 1978
11. Sporn MB, Todaro GJ: Autocrine secretion and malignant transformation of cells. N Engl J Med 303:878–880, 1980
12. Derynck R, Roberts AB, Winkler ME, Chen EY, Goeddel DV: Human transforming growth factor-α: precursor structure and expression in *E. Coli.* Cell 38:287–297, 1984
13. Lee DC, Rose TM, Webb NR, Todaro GJ: Cloning and sequence analysis of a CDNA for rat transforming growth factor-α. Nature 313:489–491, 1985
14. Rosenthal A, Lindquist PB, Bringman TS, Goeddel DV, Derynck R: Expression in rat fibroblasts of a transforming growth factor-α CDNA results in transformation. Cell 46:301–309, 1986
15. Finzi E, Fleming T, Segatto O, Pennington CY, Bringman TS, Derynck R, Aaronson SA: The human transforming growth factor type α coding sequence is not a direct acting oncogene when overexpressed in NIH/3T3 cells. Proc Natl Acad Sci USA 84:3733–3737, 1987
16. Finzi E, Kilkenny A, Strickland JE, Balaschak M, Bringman TS, Derynck R, Aaronson SA, Yuspa SH: TGFα stimulates growth of skin papillomas by autocrine and paracrine mechanisms but does not cause neoplastic progression. Mol Carcinogenesis 1:7–12, 1988
17. Kobrin MS, Asa SL, Samsoondar J, Kudlow JE: α-Transforming growth factor in the bovine anterior pituitary gland: secretion by dispersed cells and immunohistochemical localization. Endocrinology 121:1412–1416, 1987
18. Coffey RJ, Derynck R, Wilcox JN, Bringman TS, Goustin AS, Moses HL, Pittelkow MR: Production and autoinduction of transforming growth factor-α in human keratinocytes. Nature *(London)* 328:817–820, 1987
19. Weissman B, Aaronson SA: BALB and Kirsten murine sarcoma viruses alter growth and differentiation of EGF-dependent BALB/c mouse epidermal keratinocyte lines. Cell 32:599–606, 1983
20. Weissman B, Aaronson SA: Members of the *src* and *ras* oncogene families supplant the epidermal growth factor requirement of BALB/MK-2 keratinocytes and induce distinct alterations in their terminal differentiation program. Mol Cell Biol 5:3386–3396, 1985
21. Mann R, Mulligan RC, Baltimore D: Contruction of a retrovirus packaging mutant and its use to produce helper-free defective retroviruses. Cell 33:153–159, 1983
22. Cepko CL, Roberts BE, Mulligan RC: Construction and applications of a highly transmissible retrovirus shuttle vector. Cell 37:1053–1062, 1984
23. Graham FL, van der Eb AJ: A new technique for the assay of infectivity of human adenovirus 5 DNA. Virology 52:456–467, 1973
24. Wigler M, Pellicer A, Silverstein S, Axel R, Urlaub G, Chasin L: DNA-mediated transfer of the adenine phosphoribosyltransferase locus into mammalian cells. Proc Natl Acad Sci USA 76:1373–1376, 1979
25. Gazit A, Pierce JH, Kraus MH, Di Fiore PP, Pennington CY, Aaronson SA: Mammalian cell transformation by a murine retrovirus vector containing the avian erythroblastosis virus *erb*B gene. J Virol 60:19–28, 1986
26. Southern EM: Detection of specific sequences among DNA fragments separated by gel electrophoresis. J Mol Biol 98:503–517, 1975
27. Rigby PWJ, Dieckmann M, Rhodes C, Berg P: Labeling deoxyribonucleic acid to high specific activity *in vitro* in nick translation with DNA polymerase 1. J Mol Biol 113:237–251, 1977
28. Southern PJ, Berg P: Transformation of mammalian cells to antibiotic resistance with a bacterial gene under control of the SV40 early region promoter. J Mol Appl Genet 1:327–341, 1982
29. Adams SL, Sobel ME, Howard BH, Olden K, Yamada KM, de Crombrugghe B, Pastan I: Levels of translatable mRNAs for cell surface protein, collagen precursors, and two membrane proteins are altered in Rous sarcoma virus transformed chick embryo fibroblasts. Proc Natl Acad Sci USA 74:3399–3403, 1977
30. Maniatis T, Jeffrey A, Kleid D: Nucleotide sequence of the rightward operator of phage. Proc Natl Acad Sci USA 72:1184–1188, 1975
31. Bringman TS, Lindquist PB, Derynck R: Different transforming growth factor-α species are derived from a glycosylated and palmitoylated transmembrane precursor. Cell 48:429–440, 1987
32. Coffey RJ, Sipes NJ, Bascom CC, Graves-Deal R, Pennington CY, Weissman BE, Moses HL: Growth modulation of mouse keratinocytes by transforming growth factors. Cancer Res 48:1596–1602, 1988
33. Schreiber AB, Winkler ME, Derynck R: Transforming growth factor-α is a more potent angiogenesis mediator than epidermal growth factor. Science 232:1250–1253, 1986
34. Schultz GS, White M, Mitchell R, Brown G, Lynch J, Twardzik DR, Todaro GJ: Epithelial wound healing enhanced by transforming growth factor-α and vaccinia growth factor. Science 235:350–352, 1987
35. Myrdal SE, Twardzik DR, Auersperg N: Cell-mediated co-action of transforming growth factors: incubation of type B with normal rat kidney cells produces a soluble activity that prolongs the ruffling response to type α. J Cell Biol 102:1230–1234, 1986
36. Stern PH, Krieger NS, Nissenson RA, Williams RD, Winkler ME, Derynck R, Strewler GJ: Human transforming growth factor-α stimulates bone resorption in vitro. J Clin Invest 76:2016–2019, 1985
37. Stern DF, Hare DL, Cecchini MA, Weinberg RA: Construction of a novel oncogene based on synthetic sequences encoding epidermal growth factor. Science 235:321–324, 1987
38. Rogel S, Weinberg RA, Funning P, Klagsbrun M: Basic fibroblast growth factor fused to a signal peptide transforms cells. Nature 331:173–175, 1988
39. Blam SB, Mitchell R, Tischer E, Rubin JS, Silva M, Silver S, Fiddes JC, Abraham JA, Aaronson SA: Addition of a growth factor secretion signal to basic fibroblast growth factor results in cell transformation and secretion of aberrant forms of the protein. Oncogene 3:129–136, 1988
40. Gazit A, Igarashi A, Chiu I-M, Srinivasan A, Yaniv A, Tronick SR, Robbins KC, Aaronson SA: Expression of the normal human *sis*/PDGF-2 coding sequence induces cellular transformation. Cell 39:89–97, 1984
41. Hendler FJ, Ozanne B: Human squamous cell lung cancers express increased epidermal growth factor receptors. J Clin Invest 74:647–651, 1984
42. Yamamoto T, Kamata N, Kawano A, Shimizu S, Kuroki T, Toyoshima K, Rikimaru K, Nomura N, Ishizaki R, Pastan I, Gamon S, Shimizu N: High incidence of amplification of the epidermal growth factor receptor gene in human squamous carcinoma cell lines. Cancer Res 46:414–416, 1986
43. Filmus J, Pollak MN, Cailleau R, Buick RW: MOA-468, a human breast cancer cell line with a high number of epidermal growth factor (EGF) receptors, has an amplified EGF receptor gene and is growth inhibited by EGF. Biochem Biophys Res Commun 128:898–905, 1985
44. King CR, Kraus MH, Williams LT, Merlino GT, Pastan JR, Aaronson SA: Human tumor cell lines with EGF receptor gene amplification in the absence of aberrant sized mRNAs. Nucleic Acids Res 13:8477–8486, 1985
45. Wong AJ, Bigner SH, Bigner DD, Kinzler KW, Hamilton SR, Vogelstein B: Increased expression of the epidermal growth factor receptor gene in malignant gliomas is invariably associated with gene amplification. Proc Natl Acad Sci USA 84:6899–6903, 1987
46. Di Marco E, Pierce JH, Fleming TP, Kraus MH, Molloy CJ, Aaronson SA, Di Fiore PP: Autocrine interaction between TGF-α and EGF-receptor: quantitative requirements for induction of the malignant phenotype. Oncogene 4:831–838, 1989