Expression of epidermal growth factor receptor (EGF-R) antisense RNA results in a drastic reduction of EGF-R levels in the human carcinoma KB cell line and induces a reversal of their transformed phenotype (Moroni, M. C., Willingham, M. C., and Beguinot, L. (1992) J. Biol. Chem. 267, 2714–2722). We used parental and EGF-R antisense KB clones as a genetic system to study, in the same cell line, the role of transforming growth factor α (TGF-α) in the establishment and maintenance of the transformed phenotype. KB cells produce TGF-α mRNA, and their conditioned medium is able to sustain growth of antisense cells, mimicking the effect of exogenous EGF or TGF-α. In antisense cells there is a marked reduction of TGF-α mRNA steady-state levels. In addition, the decrease in TGF-α parallels the levels of residual EGF-R in the various antisense clones, indicating a direct correlation between receptors and growth factor levels. The addition of exogenous TGF-α (10 ng/ml) to antisense clones induces TGF-α levels. The half-life of TGF-α mRNA is 40–60 min in antisense cells and more than 8 h in parental KB cells, as determined by actinomycin D decay curves. This result indicates a predominant regulation of TGF-α mRNA at the post-transcriptional level. Nuclear run-on experiments show that there is only a marginal effect at the transcriptional level. We conclude that the autocrine loop responsible for the transformed phenotype of the human carcinoma KB cell line is dependent on both elevated levels of EGF-R and the presence of TGF-α. In addition, TGF-α is able to induce its own mRNA via a signal due to activation of the EGF-R acting predominantly at the post-transcriptional level.

An autocrine stimulatory loop sustaining transformation has been clearly demonstrated in a variety of cell lines in culture (2–6). The autocrine loop requires the production and the correct expression of both the receptor and the growth factor by the same cell. Regardless of whether activation of the receptor by its ligand occurs intracellularly, both on their route to the plasma membrane, or at the cell surface, it is still a subject of debate. A similar autocrine loop is also present in KB cells as a result of the overexpression of the epidermal growth factor receptor (EGF-R) together with expression of one of its physiological ligands, transforming growth factor α (TGF-α) (7). This loop is considered responsible for the serum-independent proliferation and the fully transformed phenotype of KB cells. Amplification of and overexpression of the TGF-R have been shown in many tumors of epidermal origin; however, little evidence has been reported so far for the presence of a similar loop in human tumor growth in vivo (35, 36).

KB cells, derived from a human oral carcinoma, express about 2 × 10⁶ EGF-R/cell and are tumorigenic when injected into nude mice. The stable expression of antisense EGF-R RNA in KB cells drastically reduces EGF-R levels and restores serum- and anchorage-dependent growth, reverting their transformed phenotype (1). These results support the primary role played by the EGF-R in maintaining the transformed phenotype of this tumoral cell line. However, overexpression of the EGF-R alone is not sufficient to induce transformation unless the ligand is also provided (8–10). Since TGF-α mRNA is also produced in KB cells we have now investigated its role in maintaining the uncontrolled cell growth leading to the KB cell line transformed phenotype. Self-induction has been proposed to serve as a mechanism for signal amplification and has been observed for other growth factors such as platelet-derived growth factor, TGF-β, and interleukin 1 (11–13).

In the present study, we demonstrate that the autonomous growth of KB cells is the result of an established autocrine circuit between the EGF-R and TGF-α, in which EGF-R overexpression mediates TGF-α mRNA induction. We also show that TGF-α induction in KB cells is mediated by a regulation acting predominantly at the post-transcriptional level and only marginally at the transcriptional level.

**EXPERIMENTAL PROCEDURES**

**Materials**—Murine EGF from salivary glands was from Promega; human recombinant TGF-α was from Boehringer Mannheim. Actinomycin D, aprotinin, leupeptin, phenylmethylsulfonyl fluoride, bovine serum albumin, and TES were from Sigma. GammaBind G Sepharose 4B was from Pharmacia Biotech Inc. Monoclonal IgG2b antibody against phosphotyrosine was from Upstate Biotechnologies Inc. Polyclonal anti-EGF-R serum (antibody 2913) was described previously (20). G418 and tissue culture reagents were from Life Technologies, Inc. 

**Cell Cultures**—KB, EGF-R antisense, and mock-transfected cells, described previously (1), were maintained in Dulbecco’s modified Eagle’s medium (DMEM), glutamine, penicillin, and streptomycin containing 10% NCS, with the addition of 4,18 (500 μg/ml) for the transfected cells. AS 5-7, AS 3-19, and AS FL-30 clones were

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"This work was supported in part by grants from the Associazione Italiana per la Ricerca sul Cancro and CNR (ACRO-CNR) (to L. B.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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§ Supported by an H. S. Raffaele fellowship.

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1 The abbreviations used are: EGF-R, epidermal growth factor receptor(s); TGF-α or β, transforming growth factor α or β; TES, N-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid; DMEM, Dulbecco’s modified Eagle’s medium; bp, base pairs; kb, kilobase(s); GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HB-EGF, heparin-binding EGF-like growth factor.
transfected with EGF-R antisense encoding cDNA corresponding to the first 890 bp of the 5' end (including 150 bp of the 5'-untranslated region), 560 bp of the 3' end (including 200 bp of 3'-untranslated region), and the full-length (4.2 kb) cDNA, respectively, as described previously (1). Mock cells were transfected with a control plasmid encoding the full-length β-globin antisense cDNA (pact β-globin), as described previously (1). For growth rate determination, cells were plated at 4 × 10^4 cells/60-mm dish in regular medium with 10% NCS. Eight h later, cells were washed and placed in defined medium (1) with or without EGF (20 ng/ml) or 10% NCS. Medium was replaced every 2nd day and duplicate dishes counted as indicated. For growth rate determination in KB-conditioned medium, cells were plated in regular medium and kept 4 days in defined medium; EGF (20 ng/ml) or conditioned medium from KB cells (half volume) was then added. Medium was replaced every 2nd day, and duplicate dishes were counted as indicated. For TGF-α mRNA half-life determination, cells were plated at 1 × 10^6 cells/100 mm dish in regular medium. Actinomycin D was added to the medium 48 h later, with or without pretreatment for 8 h with TGF-α (10 ng/ml); total RNA was extracted at the indicated times.

RNA Preparation and Northern Blotting—Total RNA was purified from cells by extraction with guanidine isothiocyanate followed by precipitation with LiCl (14); poly(A)^+ RNA was selected by oligo(dT). Five μg of poly(A)^+ RNA or 15 μg of total RNA was fractionated on 1% denaturing agarose gel and transferred to a Hybond-N filter (Amersham). TGF-α signal intensity was normalized to the intensity of the constitutive expressed GAPDH transcript after densitometric scanning of the autoradiogram. Data are expressed as percentage relative to the parental KB cells.

DNA Probes and Hybridizations—To detect the TGF-α transcript α 32P-labeled RNA probe was transcribed by T7 RNA polymerase from a HindIII-linearized pGEM-3 TGF-α containing the entire coding region and 388 bp of the 3'-untranslated region of human TGF-α cDNA (kindly provided by Dr. Ira Pastan, NIH, Bethesda) (32). To detect GAPDH and c-myc, specific cDNA encoding the entire coding region of the rat GAPDH (15) or the second and third exon of the human c-myc gene (16) was nick-translated. Similarly, to detect betacellulin, EGF, and heparin-binding EGF-like growth factor (HB-EGF), specific cDNAs encoding the entire coding region of the human betacellulin precursor (kindly provided by Dr. D. Salomon, NIH) (17), human EGF (ATCC), and human HB-EGF (kindly provided by Dr. Klagsbrun, Children's Hospital, Boston) (18) were also nick-translated. Prehybridizations were performed at 59 °C for the RNA probe (TGF-α) and 42 °C for nick-translated probes (c-myc, GAPDH, betacellulin, EGF, HB-EGF), respectively, in 50% formamide, 6×SSPE, 5×Denhardt’s reagent, 0.5% SDS, 100 μg/ml sheared salmon sperm DNA (Talent), and 50 μg/ml tRNA. After a 2-h incubation the hybridization proceeded for 20 h at 59 °C for the riboprobe or 42 °C for cDNA probes in 50% formamide, 0.5% SDS (RNA probe) and at 68 °C in 1×SSPE (GAPDH, betacellulin, EGF, HB-EGF) or 0.1×SSPE (c-myc), 0.5% SDS (nick-translated probes) (22).

Cell Lysates, Immunoprecipitation, and Western Blot Analysis—Cells grown to about 80% confluence in 100-mm dishes with regular medium were washed twice with phosphate-buffered saline. The cells were incubated without or with 100 ng/ml EGF for 2 min at 37 °C, washed three times with ice-cold Ca^2+/Mg^2+–free phosphate-buffered saline and solubilized in lysis buffer (50 mM HEPES, pH 7.5, 150 mM NaCl, 10% glycerol, 1% Triton X-100, 1.5 mM MgCl_2, 5 mM EGTA) containing freshly added protease and phosphatase inhibitors (4 mM phenylmethylsulfonyl fluoride, 1% aprotinin, 10 μg/ml leupeptin, 10 μM sodium...
orthovanadate, 20 mM sodium pyrophosphate) for 15 min at 4 °C. The lysates were clarified by centrifugation at 10,000 × g for 15 min at 4 °C (19). Total proteins were measured by Bio-Rad protein assay.

For immunoprecipitation experiments total cellular lysates (4 mg of total protein) were incubated with polyclonal antibody against EGF-R (antibody 2913) (20) for 4 h at 4 °C, and immune complexes were collected by binding to GammaBind G Sepharose 4B. Immune complexes were washed five times with an ice-cold buffer (HNTG) containing 20 mM HEPES, pH 7.5, 100 mM NaCl, 10% glycerol, 0.1% Triton X-100; immunoprecipitates solubilized in 1 × Laemmli buffer were boiled and run on a 7.5% SDS-polyacrylamide gel.

For immunoblots, proteins were transferred to nitrocellulose filters (Schleicher & Schuell). Membranes were then blocked for 2 h at room temperature in 5% bovine serum albumin-TBST buffer (20 mM Tris-HCl, pH 7.5, 150 mM NaCl). Blots were incubated for 2 h with monoclonal antibody against phosphotyrosine, washed in TBST (20 mM Tris-HCl, pH 7.5, 150 mM NaCl), and incubated with 125I-protein (1 Ci/mmol, 20 mCi/ml) and monoclonal antiphosphotyrosine antibody. The arrow indicates the EGF-R.

Exposure of the autoradiogram was for 5 days.

**Table 1**

| Clone    | EGF-R No./cell | % | TGF-α mRNA |
|----------|----------------|---|------------|
| KB       | 200,000        | 100| 100        |
| AS 5-7   | 30,000         | 15 | 14         |
| AS FL-30 | 35,000         | 18 | 18         |
| AS FL-13 | ND*            | 21 | 23         |
| AS 3-19  | 100,000        | 50 | 55         |
| Control  | ND*            | 100| 90         |

* Results are obtained by Scatchard analysis of 125I-EGF binding to intact KB and antisense transfected cells.

Results, obtained by Northern blot analysis and densitometric scanning of the autoradiograms, are the average of two to three independent experiments.

Results obtained by Northern blot analysis and densitometric scanning of the autoradiograms are the average of at least three independent experiments.

**ND**, not determined.

**FIG. 2.** EGF-R autophosphorylation in parental KB and antisense cells. KB (lanes 1 and 2) and AS FL-30 cells (lanes 3 and 4) were incubated with (lanes 2–4) and without (lanes 1–3) 100 ng/ml EGF for 2 min at 37 °C and lysates prepared. Lysates were immunoprecipitated with polyclonal anti-EGF-R (antibody 2913) run on 7.5% SDS-polyacrylamide gel, transferred to nitrocellulose filter, and reacted with a monoclonal antiphosphotyrosine antibody. The arrow indicates the EGF-R. Exposure of the autoradiogram was for 5 days.

**FIG. 3.** Conditioned medium from parental KB cells stimulates growth of antisense cells. AS 5-7 cells were plated in DMEM containing 10% NCS. After 8 h the medium was replaced with defined medium. Four days later, EGF (20 ng/ml) or KB conditioned medium (half-volume) was added. The medium was replaced every other day. Duplicate dishes of cells were counted as indicated under “Experimental Procedures.” [], defined medium; ⌡, conditioned medium; ⌢, 20 ng/ml EGF.

**FIG. 4.** Antisense cells display a decrease in TGF-α mRNA steady-state levels. Total RNA was extracted from subconfluent parental, AS FL-13, AS FL-30, AS 5-7, and mock-transfected (control) KB cells grown in DMEM containing 10% NCS. Northern blot hybridization was performed with a 32P-labeled TGF-α and GAPDH probes as described under “Experimental Procedures.”

**RESULTS**

**Growth Analysis of KB and EGF-R Antisense Cells—**Expression of EGF-R antisense RNA drastically reduces EGF-R levels in human KB cells resulting in suppression of their transformed phenotype. Even in serum-supplemented medium, growth of antisense cells is slower and their doubling time longer than parental KB cells (36 versus 22 h) (1). As shown in Fig. 1, antisense clones AS 5-7, AS FL-13, and AS FL-30 fail to grow in defined medium; this property is overcome by exogenous addition of 20 ng/ml EGF (Fig. 1). On the contrary, parental KB cells grow in defined medium independent of the presence of EGF. This result suggests that parental KB cells produce an EGF-related molecule, which together with its receptor sustains an autocrine stimulatory loop. This autocrine loop is absent in antisense cells. In this case one would expect a constitutively activated EGF-R tyrosine kinase in KB cells. Indeed, in the absence of EGF, EGF-R autophosphorylation is detected in parental KB but not in AS FL-30 cells, even with a very long exposure of the autoradiogram (Fig. 2 lanes 1 and 3).
In the presence of EGF, the EGF-R is autophosphorylated in both parental and AS FL-30 cells (Fig. 2, lanes 2 and 4). Quantitation of the autoradiogram shows that EGF-induced autophosphorylation in AS FL-30 was 18% of that in KB cells, which agrees with the lower residual EGF-R levels in the antisense clone (Table I). Similar results were obtained with antisense clones AS 5-7 and AS FL-13 (data not shown).

If KB cells produce a growth factor their conditioned medium might stimulate growth of antisense cells in the absence of exogenous EGF. AS 5-7 cells, which express 15% of residual EGF-R (Table I), were growth arrested by serum deprivation and then supplemented with EGF (20 ng/ml) or KB-conditioned medium. As shown in Fig. 3, conditioned medium from parental KB cells is able to stimulate the growth of antisense cells and to mimic EGF-dependent growth. The growth factor present in KB cell medium most likely belongs to EGF family members such as TGF-α, EGF, amphiregulin, or betacellulin. Indeed, TGF-α mRNA has been detected previously in KB cells (23).

TGF-α, EGF, Betacellulin, and HB-EGF mRNA Expression in KB and EGF-R Antisense Cells—To examine the levels of TGF-α mRNA in KB and antisense cells total RNA was extracted and analyzed by Northern blotting. As shown in Fig. 4, EGF-R antisense cells show a significant reduction of the 4.5-kb TGF-α mRNA compared with parental or mock-transfected KB cells. AS 5-7, AS FL-30, and AS FL-13 express, respectively, 15, 18, and 23% of TGF-α mRNA levels present in KB cells (Table I). Strikingly, TGF-α mRNA levels correlate well with EGF-R levels in the different clones (Table I). These findings suggest a precise correlation between EGF-R expression and TGF-α production similar to that described between residual EGF-R expression and severity of the transformed phenotype (1).

EGF mRNA expression, however, is not detected in KB or antisense cells even after a long exposure of the autoradiogram (Fig. 5, lanes 1 and 2). The 4.9-kb EGF transcript is, however, present in mouse salivary gland, used as a positive control (Fig. 5A, lane 3). In addition, the 3-kb and a longer transcript of betacellulin mRNA are only present in antisense-expressing cells but are not detected in parental KB cells (Fig. 5B, lanes 1 and 2). The same two transcripts are observed in the mouse liver poly(A)+ RNA used as a positive control (data not shown). Similarly, the 2.5-kb HB-EGF mRNA is expressed in antisense-expressing cells but only at low levels in parental KB cells (Fig. 5C, lanes 1 and 2). Both betacellulin and HB-EGF mRNA are present in a much lower amount compared with TGF-α.

Regulation of Steady-state TGF-α mRNA—The production of TGF-α by KB cells might be sustained by an autostimulatory mechanism involving the EGF-R (24–26). Conversely, the reduction of TGF-α mRNA levels in EGF-R antisense clones may be a consequence of reduced EGF-R expression, which interrupts the autocrine loop. To test this possibility we studied the
effects of exogenous TGF-α on its own synthesis. As shown in the Northern blot of Fig. 6 (panel A), the addition of exogenous TGF-α to AS FL-30 cells results in increased steady-state expression of TGF-α mRNA. TGF-α stimulates production of its own mRNA within 4 h after the addition, peaking after 8 h (3-fold over basal expression) and decreasing thereafter (panel B). Similarly, exogenous EGF is able to increase TGF-α mRNA levels further in parental KB cells by about 3–4-fold (data not shown). Exogenous EGF is able to stimulate TGF-α mRNA levels as well (data not shown).

To determine the level at which exogenous TGF-α increases the steady-state expression of TGF-α, the half-life of TGF-α was studied. Total RNA was extracted from AS FL-30 and parental cells treated for different times with the RNA synthesis inhibitor actinomycin D and analyzed by Northern blotting. As shown in Fig. 7, parental KB cells display a prolonged stability of TGF-α mRNA compared with the EGF-R antisense cells within a 2-h frame. Later time points show that TGF-α transcript in KB and mock-transfected cells was stable for up to 6–8 h (data not shown). In the antisense clone, the half-life of the TGF-α transcript was approximately 40–60 min, similar to the c-myc transcript, whose regulation depends on a significant degree on rapid mRNA decay. The similarly rapid c-myc mRNA decay in KB, antisense (Fig. 7), and mock-transfected cells (data not shown) demonstrates the specificity of the differential decay of TGF-α.

To investigate the mechanism underlying TGF-α mRNA stimulation by TGF-α, we have measured the effect of exogenous TGF-α on the half-life of the TGF-α transcript. The addition of TGF-α (10 ng/ml) to the AS FL-30 cells 8 h before the actinomycin D treatment results in a marked stabilization of TGF-α mRNA but has no effect on c-myc rapid decay (Fig. 8, panels A and B). This result supports the possibility of a TGF-α/EGF-R autocrine loop in parental KB cells acting through a post-transcriptional mechanism. TGF-α stimulation of its own mRNA is also independent of protein synthesis, as determined by cycloheximide experiments (data not shown).

Nuclear run-on has also been performed to determine the TGF-α rate of transcription in KB and antisense cells. Quantitation of the data shown in Fig. 9, derived from three independent experiments, shows a less than 2-fold difference in the transcriptional rate of TGF-α RNA in KB versus antisense cells. A similar result is obtained when the amount of nascent TGF-α RNA was compared in mock-transfected and antisense cells (data not shown). Taken together, these results therefore suggest that TGF-α synthesis in KB cells is under a regulatory mechanism acting predominantly through a stabilization of the TGF-α transcript.
DISCUSSION

In this report we used KB clones expressing EGF-R antisense RNA together with their parental cells as a genetic system in which the autocrine loop hypothesis of cell growth could be tested directly in the same cell line. We have demonstrated previously the primary role played by overexpression of EGF-R in the EGF-independent growth of KB cells (1). EGF-R antisense clones with the most reduced EGF-R expression, such as AS FL-30, AS FL-13, and AS 5-7, exhibit a totally restored serum-dependent growth and are unable to grow in the absence of serum and to form colonies in soft agar (1). In normal cells, EGF-R overexpression leads to transformation only when coexpressed with EGF or TGF-α (8, 9, 27). KB cells express TGF-α, as shown by the presence of TGF-α mRNA in these cells and by the ability of KB-conditioned medium to sustain the growth of the EGF-R antisense cells and to mimic EGF-dependent growth. In addition, we have evidence of the presence of the 24-kDa precursor in KB cell membranes, although we were unable to detect it in antisense-expressing cells (data not shown). Furthermore since EGF, betacellulin, and HB-EGF mRNA are not detectable or expressed at very low levels in parental KB cells, the growth-promoting activity detected in KB-conditioned medium is most likely due to the presence of TGF-α, although we cannot exclude the production of amphiregulin as well. Interestingly, although betacellulin and HB-EGF mRNA are expressed in antisense cells, these two growth factors do not seem to stimulate their growth, as demonstrated by the inability to grow in the absence of exogenous EGF or serum and the lack of basal EGF-R autophosphorylation. Future studies will elucidate the mechanism by which betacellulin and HB-EGF steady-state mRNA levels are increased in antisense cells.

The decrease in state-steady TGF-α mRNA levels observed in the EGF-R antisense cells and the strong correlation among EGF-R, TGF-α expression, and transformed phenotype support the possibility that in these clones the autocrine circuit that leads to KB cell growth is impaired, leading to a reversion of the transformed phenotype. A similar autocrine loop is present in A431 cells (28) where receptor activation and autophosphorylation occur by endogenously produced and secreted TGF-α (29).

The finding that the most inhibited clones express reduced levels of not only EGF-R, but also TGF-α mRNA and most likely TGF-α protein, suggests that a signal responsible for TGF-α regulation is due to the activation of the EGF-R by TGF-α. Coffey et al. (24) have reported that treatment of human keratinocytes with TGF-α stimulates expression of its own mRNA. The finding that treatment of EGF-R antisense clones with exogenous TGF-α also stimulates TGF-α mRNA expression suggests that the difference in TGF-α mRNA levels between AS FL-30 and parental KB cells may be the result of the inability of low levels of TGF-α, in the EGF-R antisense cells, to stimulate the few residual EGF-R and thus to induce its own mRNA. The TGF-α induction mechanism in KB cells occurs mostly at the post-transcriptional level because of a prolonged stability of the transcript and only minimally at the transcriptional level, since a low increased transcriptional rate is also observed.

The half-life of TGF-α mRNA is more than 8 h in parental KB and mock-transfected cells but less than 1 h in AS FL-30 cells. Our results are in agreement with the long half-life of TGF-α mRNA in another carcinoma cell line, the MDA 468 (25), which expresses about two million EGF-R/cell and with a very short half-life in normal human keratinocytes and LIM 1215 cells, with very low levels of EGF-R (24). The addition of exogenous TGF-α to the antisense clones produces TGF-α transcript stabilization. Interestingly, in KB cells, stabilization of the EGF-R mRNA by EGF was also observed, but the specific mechanism responsible for receptor transcript stabilization has not been further elucidated (30). Additional studies are now in progress to study whether the two mechanisms share similar signal
transduction pathways and to address their contribution in the uncontrolled KB cell proliferation.

The TGF-α is encoded by a 4.5-kb mRNA (23) which is the predominant transcript in cells expressing this growth factor. A less represented 1.6-kb mRNA and minor species of 3.8, 3.2, and 2.4 kb have also been described (28, 31–33). Despite the presence of several transcription start points, alternative use of distinct polyadenylation signals seems to generate this transcript variety, as shown for the 4.5- and 1.6-kb species (34). In KB cells, as in other transformed cell lines, the predominant 4.5-kb transcript is detected together with a minor 1.6-kb mRNA and two minor species of 3.8 and 2.4 kb (data not shown). The presence of 13 AUUUA sequences in the TGF-α 3′-untranslated region could account for a differential regulation of stability for the different transcripts. Additional experiments are in progress to evaluate the role played by alternative polyadenylation sites on TGF-α mRNA stability and therefore on TGF-α expression in the parental and EGF-R antisense cells.

Acknowledgments—We thank Maria Mazzotti for great help with tissue culture, Professor Francesco Blasi (DBIT, H. S. Raffaele, Milano) for helpful discussions, Dr. Ira Pastan (NIH) for TGF-α cDNA, Dr. D. S. Salomon (NIH) for the betacellulin cDNA, and Dr. Micheal Klagsbrun (Children’s Hospital, Boston) for HB-EGF cDNA.

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