Aberrant Regulation of Transforming Growth Factor-α during the Establishment of Growth Arrest and Quiescence of Growth Factor Independent Cells*

Gillian M. Howell‡‡‡, Lisa E. Humphrey‡‡‡, Rana A. Awwad‡‡‡**, Degeng Wang‡‡‡, Alan Koterba‡, Basker Periyasamy‡, Junhua Yang‡, Wenhui Li‡, James K. V. Willson‡‡, Barry L. Ziober‡‡, Kevin Coleman, Joan Carboni, Mark Lynch, and Michael G. Brattain‡¶

From the ¶Department of Biochemistry and Molecular Biology, Medical College of Ohio, Toledo, Ohio 43699-0008, the Department of Molecular Genetics, Oncology Drug Discovery, Bristol Myers Squibb, Princeton, New Jersey 08543-4000, the ‡Department of Medicine, Case Western Reserve University/Ireland Cancer Center, Case Western Reserve University, Cleveland, Ohio 44106, and the §§Department of Stomatology, University of California, San Francisco, California 94143

Autocrine transforming growth factor α (TGFα) is an important positive growth effector in malignant cells and plays a significant role in generating the growth factor-independent phenotype associated with malignant progression. However, the molecular mechanisms by which TGFα confers a growth advantage in progression is poorly understood. The highly tumorigenic cell line HCT116 up-regulates TGFα mRNA expression during growth arrest, whereas the poorly tumorigenic growth factor-dependent FET cell line down-regulates TGFα mRNA expression as it becomes quiescent. We have identified a 25-bp sequence at −201 to −225 within the TGFα promoter which mediates the differential regulation of TGFα expression during quiescence establishment in these two cell lines. This same sequence confers TGFα promoter responsiveness to exogenous growth factor or autocrine TGFα. The abberant upregulation of TGFα mRNA in quiescent HCT116 cells may allow them to return to the dividing state under more stringent conditions (nutrient replenishment alone) than quiescent FET cells (requires nutrients and growth factors). Antisense TGFα approaches showed that the dysregulated TGFα expression in quiescent HCT116 cells is a function of the strong TGFα autocrine loop (not inhibited by blocking antibodies) in these cells.

Malignant progression is often associated with the loss of dependence on exogenous growth factors for growth control. The production and utilization of endogenous autocrine growth factors underlies the development of autonomous growth (1–3). Transforming growth factor α (TGFα) is a positive autocrine growth factor in colon carcinoma (4–7) and contributes to malignant progression of colonic tumors (8). TGFα is a member of the epidermal growth factor (EGF) family, and like EGF, exerts its effects via interaction with the epidermal growth factor receptor (EGFR) (9, 10). It is produced as a transmembrane precursor which is cleaved to a 50-amino acid active peptide (11, 12). However, larger forms are secreted from transformed cells (12, 13) and the transmembrane form also appears to be active (14–16). TGFα was subsequently shown to be an autocrine growth factor in normal cells (17) but transformed cell lines appear to produce greater amounts of the peptide than their normal counterparts (18, 19).

Previous studies in this laboratory have identified two distinct phenotypes within our colon carcinoma cell lines which differ with respect to tumorigenicity, growth factor dependence, and the utilization of TGFα (4, 5, 20). Well differentiated, poorly tumorigenic FET cells express both TGFα and its cognate receptor EGFR and are growth inhibited by antibodies to EGFR. However, FET cells require exogenous EGF as well as transferrin and insulin for both optimal growth and DNA synthesis. They are, therefore, growth factor-dependent. This cell line expresses a classical TGFα autocrine loop in which mature, proteolytically released ligand interacts with EGFR on the cell surface. In contrast, HCT116 cells are poorly differentiated and highly tumorigenic. While this cell line expresses both TGFα and EGFR, it is not growth inhibited by blocking antibodies to the EGFR and is growth factor independent as it does not require any exogenous growth factors for DNA synthesis and growth (21, 22). However, transfection of a TGFα antisense expressing vector results in dependence upon exogenous EGF for growth (20). Therefore, the HCT116 cell line exhibits a highly active TGFα autocrine loop which is sequestered from inhibition by extracellular blocking agents. Both of these cell lines are typical members of previously documented groups of colon carcinoma cell lines which have been subclassified on the basis of these growth regulatory phenotypes (5).

Further differences in TGFα regulation between the FET and HCT116 cell lines become apparent when the cells are rendered quiescent (4, 5, 22). Both cell lines express equivalent levels of TGFα mRNA during logarithmic growth (22). However, the FET cell line down-regulates TGFα mRNA as it enters quiescence (22). It requires both nutrients and growth factors for subsequent release through the cell cycle (4, 5, 22). In contrast, HCT116 cells up-regulate TGFα mRNA as they become quiescent, and nutrient replenishment alone is sufficient for release from quiescence further emphasizing the growth factor independence of these cells (4, 5, 22). The underlying mechanism by which endogenous TGFα confers growth factor independence, and so growth advantage, to the malignant phe-

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§ These authors contributed equally to this work.

¶ To whom correspondence should be addressed. Tel.: 210-567-5706; Fax: 210-567-3447; E-mail: howellg@uthscsa.edu.

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The abbreviations used are: TGFα, transforming growth factor α; EGF, epidermal growth factor; EGFR, epidermal growth factor receptor; CAT, chloramphenicol acetyltransferase; NEO, neomycin; tk, thymidine kinase; CMV, cytomegalovirus.
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...type appears to be via enhanced movement of non-dividing, quiescent cells back into the cell cycle due to inappropriate expression rather than TGFα mRNA overexpression in logarithmic phase growth. In support of this hypothesis, overexpression of TGFα in poorly tumorigenic colon carcinoma cells by stable transfection of a constitutively active sense TGFα expression vector generates a more tumorigenic phenotype, which shows enhanced clonal initiation and a decreased lag phase rather than an altered exponential growth phase doubling time (8). Therefore, the increased expression of TGFα in quiescent HCT116 cells appears to “prime” them for re-entry into the cell cycle in a less favorable, growth factor-deficient milieu than FET cells, thereby imparting them with a growth advantage in limiting growth conditions.

To understand the mechanisms regulating TGFα expression in both the growth factor-dependent and growth factor-independent state of progression, we have studied TGFα promoter activity in quiescent FET and HCT116 cell lines. We hypothesized that if TGFα autocrine function plays a role in regulating TGFα promoter activity, then the changes in endogenous TGFα expression during the acquisition of quiescence should be reflected by similar changes in TGFα promoter activity, particularly as cells are not exposed to any exogenous growth factors during the establishment of quiescence. We demonstrate that regulation of transcriptional activation of the TGFα promoter in both HCT116 and FET cells is an integral part of the differential expression of TGFα in the HCT116 and FET cell lines during growth arrest and quiescence. Moreover, this transcriptional regulation maps to a unique 25-bp element which also confers EGF/TGFα autoregulation on the TGFα promoter. HCT116 cells with repressed TGFα expression following TGFα antisense expression become growth factor-dependent (20). We now show TGFα expression is not up-regulated during the transition from exponential growth to quiescence in the TGFα antisense transfected cells, further implicating TGFα autocrine function in the regulation of TGFα expression.

MATERIALS AND METHODS

Cell Culture—The FET and HCT116 cell lines are routinely maintained in a defined serum-free medium containing insulin (20 µg/ml, Sigma) transferrin (4 µg/ml, Sigma), and EGF (10 ng/ml, R + D Systems) as described previously (4, 21). To render the cell lines quiescent, cells were grown to confluence and then changed to McCoy’s medium without growth factors. The FET cells were deprived of further nutrients and growth factors for 6 days and the HCT116 cells for 5 days as described previously (22). A normal human leukocyte genomic library (CLONTECH) was screened by plaque hybridization using a probe consisting of a portion of the 5’-region of the TGFα coding sequence (+104 to +129). A 2.8-kilobase PstI-SauB1I fragment containing the TGFα promoter along with the first exon of TGFα was isolated (23, 24). Following Bal31 exonuclease digestion to nucleotide −4 (to remove the TGFα coding sequence), the resulting fragment was cloned upstream of the bacterial chloramphenicol acetyltransferase (CAT) gene in the vector pGCMAT, to give the largest construct designated p2813CAT. By use of appropriate restriction sites or polymerase chain reaction, various deletion fragments of the TGFα promoter were generated. These constructs are designated by the total number of base pairs of the TGFα promoter which they contain, e.g. p370-CAT, p201-CAT.

Cloning of the TGFα Promoter—A normal human leukocyte genomic library (CLONTECH) was screened by plaque hybridization using a probe consisting of a portion of the 5’-region of the TGFα coding sequence (+104 to +129). A 2.8-kilobase PstI-SauB1I fragment containing the TGFα promoter along with the first exon of TGFα was isolated (23, 24). Following Bal31 exonuclease digestion to nucleotide −4 (to remove the TGFα coding sequence), the resulting fragment was cloned upstream of the bacterial chloramphenicol acetyltransferase (CAT) gene in the vector pGCMAT, to give the largest construct designated p2813-CAT. By use of appropriate restriction sites or polymerase chain reaction, various deletion fragments of the TGFα promoter were generated. These constructs are designated by the total number of base pairs of the TGFα promoter which they contain, e.g. p370-CAT, p201-CAT.

Heterologous Constructs—Heterologous constructs containing the regions of interest of the TGFα promoter were also generated. Double-stranded oligonucleotides spanning the sequences were synthesized with BamHI “sticky ends” and cloned just upstream of the heterologous thymidine kinase promoter in the pBlCAT2 vector.

Isolation of Stable Transfectants—FET or HCT116 cells (70–80% confluent) were harvested by trypsinization, washed once in serum-free medium, and resuspended at 2 × 10^5 and 2 × 10^6 cells per 800 µl of serum-free medium, respectively, and transferred to a 0.4-cm electroporation cuvette. Cells were electroporated at 250 volts and 960 microfarads in a Bio-Rad gene pulser with 30 µg of vector and 5 µg of an SV-40 Neomycin (NEO) plasmid and plated at 1:20 to 1:50 dilutions. At 2 days following plating, cells were switched to medium containing 600 µg/ml active Geneticin (G418; Life Technologies, Inc.) to allow selection for NEO resistant clones. These clones were expanded and tested for CAT expression. The development of the TGFα antisense clone (clone U) has been described previously (20). Briefly, HCT116 cells were transfected with a vector containing 900 bp of TGFα cDNA in the antitumour orientation relative to the cytomegalovirus (CMV) promoter. The plasmid also contained a NEO selection marker to allow cloning in Geneticin.

To study TGFα promoter activity during the establishment of growth arrest and quiescence in the TGFα antisense RNA expressing clone U and the revertant phenotype clone UX, it was necessary to generate stable transfectants containing TGFα promoter constructs using a selection protocol. Therefore, both clones were transfected with the TGFα deletion constructs p370-, p343-, and p247-CAT which contain the EGF-responsive element and the p201-CAT construct which does not. These constructs were co-transfected with the pREP4 vector (Invitrogen) which allowed selection with the antibiotic hygromycin B (Calbiochem; 50 µg/ml). Hygromycin-resistant cells were difficult to isolate, but we were able to obtain stable transfectants of clone U containing p370-CAT and p201-CAT and stable transfectants of clone UX containing p343-CAT and p201-CAT.

Transient Transfection Studies—For the transient transfection studies in FET cells, the TGFα promoter-CAT constructs (30 µg) were also transfected by electroporation into FET cells maintained in serum-free medium minus EGF. A Rous-Sarcoma virus-driven β-galactosidase vector was co-transfected to allow normalization for transfection efficiency. Control and EGF-treated cells (6 h) were harvested at 30–48 h following transfection for CAT assay.

CAT Assays—Following 3 washes in phosphate-buffered saline, cells were harvested in 1 ml of TEN buffer (40 mM Tris, pH 7.4, 1 mM EDTA, and 150 mM NaCl). After centrifugation, cells were resuspended in 100 µl of 250 mM Tris·HCl, pH 7.8, containing 15% glycerol (v/v) and lysed by three cycles of freeze-thawing. Protein concentration in the cleared lysates was determined by microassay using BCA reagent (Pierce). The β-galactosidase activity was determined by following the Promega protocol. The CAT assay was performed essentially as described (25) but with the acetyl-CoA present at a concentration of 3.6 mM in the final reaction.

RNA Preparation and RNase Protection Assay—Total RNA was isolated using a cesium trifluoroacetic acid gradient (Pharmacia; Ref. 26). High specific activity, double-stranded oligonucleotide labeled with [32P]dGTP by klenow fragment (Pharmacia) was incubated with nuclear extracts as has been described previously (29). Following incubation on ice, the reaction mixture was loaded onto a 4% polyacrylamide gel in 0.25 × TBE (25 mM Tris, 25 mM boric acid, 0.5 mM EDTA) and run at 150–250 V for 90 min at 4 °C (30).

RESULTS

Growth factor-independent HCT116 cells showed different regulation of TGFα mRNA from FET cells during the establishment of quiescence. HCT116 cells up-regulated TGFα mRNA during the transition from exponential phase to the quiescent state, whereas FET cells down-regulated TGFα mRNA expression. The development of the TGFα promoter-CAT constructs (30 µg) were also transfected by electroporation into FET cells maintained in serum-free medium minus EGF. A Rous-Sarcoma virus-driven β-galactosidase vector was co-transfected to allow normalization for transfection efficiency. Control and EGF-treated cells (6 h) were harvested at 30–48 h following transfection for CAT assay. The CAT assay was performed essentially as described (25) but with the acetyl-CoA present at a concentration of 3.6 mM in the final reaction.

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the acquisition of quiescence. To begin to understand the mechanism underlying this differential regulation, we next studied whether the alterations in TGFα mRNA expression were transcriptionally regulated. The HCT116 and FET cell lines were each stably transfected with a TGFα promoter-reporter construct, p2813-CAT and several clones were isolated. Fig. 2 shows a typical result with clones designated HCT116-8 and Fet 3. The cells were grown in serum-free medium and harvested during exponential phase, at confluence, and at various times after removal of growth factors for induction of quiescence to assay for CAT activity as a function of growth state. The CAT activity of the p2813-CAT construct in the HCT116-8 clone progressively increases from exponential phase to quiescence (starve day 6; approximately 10–15-fold increase), whereas the CAT activity in FET-3 is decreased approximately 50% by starve day 6 (quiescence). Similar results were obtained with different clones of the FET and HCT116 cells confirming that these results are not an integration site effect. Therefore, it appears that transcriptional regulation of the TGFα promoter does indeed play a role in determining the level of TGFα expression as cells are rendered quiescent. However, in the case of HCT116, the increase in promoter activity is greater than the overall increase in the level of TGFα mRNA expression. This high level of CAT activity may reflect accumulation of the relatively stable CAT protein over the course of the assay. Alternatively, post-transcriptional mechanisms may also be involved in the regulation of the TGFα mRNA in addition to transcription in the quiescent HCT116 cells.

To determine how much 5′-flanking sequence of the TGFα promoter was required for regulating TGFα transcription during the acquisition of quiescence, additional stable clones were made. Stable clones of both FET and HCT116 cells transfected with the TGFα promoter deletion constructs p370-CAT and p201-CAT were isolated and several clones with CAT activity identified. It is necessary to use stable clones rather than transient transfections for these experiments because of the length of time to complete the experiment (13–14 days) and because any media changes in transient transfection protocols would disrupt the establishment of quiescence. These clones were then made quiescent and the CAT activity was assayed during the transition from exponential phase to quiescence. Typical results are shown in Figs. 3 and 4. The p370-CAT construct showed increasing CAT activity as HCT116 cells were rendered quiescent (Fig. 3A). By day 5 of quiescent induction, the CAT activity was increased 7.4-fold compared with log phase. Thus the activity of the p370-CAT construct, like the full-length p2813-CAT construct, paralleled the changes in the level of endogenous TGFα mRNA during the transition from exponential growth to quiescence in HCT116 cells. In contrast, the transcriptional activity of the TGFα deletion construct p201-CAT, which is very low, did not change during growth from exponential phase to quiescence (Fig. 3B). In Fig. 3C,
results from several different clones are presented. These results indicate that the major region of the TGFα promoter involved in up-regulation of TGFα expression during the acquisition of quiescence in HCT116 cells lies between −370 and −201 of the TGFα 5′-flanking DNA sequence.

We next investigated whether this same region was involved in the apparent down-regulation of TGFα expression in FET cells rendered quiescent. Fig. 4A shows a typical experiment in which the CAT activity of the p370-CAT construct was measured in an FET clone from exponential phase to quiescence. The CAT activity showed a 3–4-fold decrease as the cells became quiescent. In contrast, the p201-CAT TGFα promoter deletion construct again had a low basal level of transcription which did not change as the FET cells were rendered quiescent from the confluent state (Fig. 4B). This construct also showed no change in CAT expression during growth from exponential phase to quiescence. Fig. 4C shows data from several different clones represented graphically. Therefore, the same region which mediates transcriptional activation of the TGFα promoter in quiescent HCT116 cells shows decreased regulatory activity in quiescent FET cells.

The finding that the region of the TGFα promoter regulating TGFα expression during quiescence lies between −370 and −201 was significant because we had co-localized EGF responsiveness to the same region of the TGFα promoter. Using the FET cell line, we examined the EGF responsiveness of various deletion constructs of the TGFα promoter in transient transfection assays. The constructs p370-, p343-, p247-, and p201-CAT were co-transfected with β-galactosidase into FET cells adapted to continuous growth in serum-free medium minus EGF. At 24–48 h after plating, the cells were treated with EGF.
for 6 h. The results are summarized graphically in Fig. 5A as mean ± S.E. from several experiments. The constructs p370-, p343-, and p247-CAT all show a significantly increased level of CAT activity after EGF treatment (p < 0.05). However, the p201-CAT construct shows no such increase in CAT activity in response to EGF. This experiment localized the region of the TGFα promoter responsive to EGFR activation to between −201 and −247 of the promoter sequence. The sequence of this region is shown in Fig. 5B. This region does not contain any known consensus sequences. Importantly, this region shows no homology to known EGF-response elements which suggests that the element represents a unique cis-element. Therefore, studies were carried out to further define the EGF-responsive DNA sequence within the TGFα promoter. Oligo pair 1/2 spanned −246 to −220 (designated pBL-1/2-tk-CAT) and oligos 3/4 spanned −225 to −199 of the TGFα promoter (pBL-3/4-tk-CAT). A control oligo pair, 5/6, spanned the region −202 to −176 (pBL-5/6-tk-CAT). As shown in Fig. 5C, only the pBL-3/4-tk-CAT construct showed significantly increased CAT activity (p < 0.05) upon treatment of FET cells with exogenous EGF. This localized the EGF-responsive region of the TGFα promoter to the region −225 to −200.

Stable clones of FET and HCT116 were isolated containing either the 25-bp EGF-response element cloned just upstream of the heterologous thymidine kinase promoter in a CAT vector (the vector pBL-3/4-CAT) or the control thymidine kinase promoter-CAT vector (pBLCAT2). Typical data of the activity of these constructs (mean ± S.E.; n = 3) in each of the two cell lines is presented graphically in Fig. 6. The CAT activity of the pBL-3/4-CAT construct in HCT116 cells increases to approximately 4.5–6-fold the exponential phase activity in quiescence. In contrast, the activity of this construct in the FET cell line exhibits approximately a 40% decrease during the establishment of quiescence. The activity of the control pBLCAT2 construct remains relatively constant during the establishment of quiescence in both cell lines (Fig. 6). Therefore, we conclude that the 25-bp region of the TGFα promoter which contains the EGF-responsive cis-element is also involved in the autoregulation of TGFα expression during the establishment of quiescence.

We next examined whether nuclear protein binding to the TGFα autoregulatory cis-region was regulated in a different manner in the two cell lines during the acquisition of quiescence. Nuclear extracts were prepared from both exponential
marked difference between the two cell lines is in the regulation of the binding of nuclear proteins to the DNA sequence of oligonucleotide 3/4 during the acquisition of quiescence. Logarithmic phase HCT116 nuclear extracts show predominantly high molecular weight complexes 1, 2, and 3, as well as bands 4 and 5. In quiescent HCT116 nuclear extracts, the amount of binding in bands 1, 2, and 3 is markedly increased and there is the appearance of diffuse binding between bands 2 and 3. Nuclear protein binding in bands 4 and 5 shows a small increase in the quiescent state compared with logarithmic growth. In contrast, logarithmic phase FET nuclear extracts show all 3 high molecular weight complexes (bands 1 through 3), although band 1 is less intense than bands 2 and 3. Again, there is diffuse binding between bands 2 and 3. In contrast to the situation in HCT116 cells, these high molecular weight complexes disappear in quiescent FET cell nuclear extracts. The binding in band 4 decreases but the intensity of band 5 shows a slight increase between logarithmic phase and quiescent extracts.

A competition study was performed to examine the specificity of binding of the nuclear proteins to oligo 3/4. Nuclear extracts from quiescent HCT116 cells and logarithmic phase FET cells were incubated with 32P-labeled oligonucleotide sequence 3/4 in the presence of increasing amounts of unlabeled oligonucleotide 3/4 (Fig. 7B). Nuclear protein binding in the low mobility shift bands (1 through 3) are competed by the unlabeled DNA sequence equivalent to 10–100-fold excess over labeled probe and is completely abolished at 200-fold excess showing the specificity of these bands. However, bands 4 and 5 are more resistant to competition than bands 1 to 3 and remain visible even at 200-fold excess unlabeled competitor. The competition study suggests that the high molecular weight binding protein complexes in low mobility shift bands 1 to 3 are the ones of interest. It should also be noted that the bands in the quiescent HCT116 nuclear extracts are less readily competed (i.e. it takes greater amounts of cold oligonucleotide 3/4 to decrease binding) than the bands in logarithmic phase FET nuclear extracts. This may be due to the presence of higher amounts of binding protein in quiescent HCT116 cells, or the affinity of the complexes may be higher due to differential secondary modifications such as phosphorylation between the two cell lines. When the nonspecific competitor salmon sperm DNA is competed against 32P-labeled oligonucleotide 3/4, little effect on HCT116 nuclear protein binding is observed until 200 ng of nonspecific competitor (equivalent to 400-fold excess over labeled probe) is reached (data not shown).

To investigate whether the TGFα autocrine loop in HCT116 cells contributes to the up-regulation of TGFα during the establishment of quiescence, we examined TGFα mRNA expression in a clone stably expressing TGFα antisense RNA. This clone (clone U), which contains a disrupted TGFα autocrine loop, is dependent upon exogenous EGF for growth much like FET cells (20). If the TGFα autocrine loop of HCT116 is the major determinant of TGFα up-regulation during the acquisition of quiescence, disruption of this loop should abrogate the increased TGFα expression at quiescence. In Fig. 8A, it can be seen that this is the case. Clone U shows a markedly decreased ability to up-regulate TGFα mRNA during the establishment of quiescence. High TGFα expressing revertants of U were isolated and designated UX (20). Like the parental HCT116 cells, clone UX which still expresses antisense TGFα RNA and so contains a functional CMV promoter, provides evidence that the effects.

FIG. 5. Localization of the EGF-response element within the TGFα promoter in FET cells. Panel A, the EGF responsiveness of the TGFα promoter deletion constructs p370-, p343-, p247-, and p201-CAT. FET cells, maintained in serum-free medium minus EGF, were transiently transfected as described under "Materials and Methods," and harvested at 30–48 h later with or without EGF treatment (10 ng/ml) for 6 h. Asterisk (*) denotes significantly increased CAT activity (p < 0.05) upon treatment with EGF. Panel B, sequence of the EGF-responsive region of the TGFα promoter.

The design of the oligonucleotides 1/2 and 3/4 used to further delineate the EGF-responsive region are also shown. The control oligonucleotide 5/6 comprises the region −202 to −178 of the TGFα promoter which is not EGF responsive. Its sequence is GCCCTTCATATTCCGGCGGCC. Panel C, the effect of EGF on the heterologous promoter constructs. Only the heterologous construct containing −225 to −199 of the TGFα promoter (pBL-3/4-tk-CAT) showed a significant (p < 0.05; *) response to EGF. The constructs pBL-1/2-tk-CAT and pBL-5/6-tk-CAT containing −248 to −220 and −202 to −176 of the TGFα promoter, respectively, showed no response to EGF.
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![Graph A and B showing transcriptional regulation](image)

**DISCUSSION**

As reported previously, the growth factor-dependent FET cell line down-regulates endogenous TGFα mRNA expression during growth factor and nutrient deprivation leading to growth arrest and quiescence (4, 5, 22). In contrast, the poorly differentiated, growth factor-independent cell line HCT116 up-regulates TGFα expression upon entry into quiescence. In both cell lines, we established that these changes were reflected by changes in the activity of our full-length TGFα promoter construct p2813-CAT.

Further studies in the FET cell line led us to identify a 25-bp element consisting of -225 to -199 of the TGFα promoter which conferred EGF responsiveness to a heterologous thymidine kinase promoter. Studies with stably transfected clones of HCT116 and FET containing this construct confirmed that this region was also responsive to the altered TGFα autocrine status in quiescent cells. Moreover, putative transcription factors binding this element were similarly modulated in response to growth arrest and quiescence in the two cell lines. This 25-bp

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**Fig. 6.** Transcriptional regulation of the 25-bp EGF-response element within the TGFα promoter during growth arrest in HCT116 and FET cells. Panel A, the CAT activity of the heterologous construct pBL-3/4-tk-CAT, containing the EGF-responsive element of the TGFα promoter, during the establishment of quiescence in stably transfected HCT116 cells. The activity of the pBLCAT2 vector (containing only the thymidine kinase promoter) is shown as a control. Panel B, the CAT activity of the heterologous construct pBL-3/4-tk-CAT, during the establishment of quiescence in stably transfected FET cells. Cells were harvested during exponential phase growth (Log), at confluence (Con), and at quiescence (Qui). The CAT activity was quantitated as fold-induction over exponential phase cells (log = 1; x ± S.E.; n = 3). The activity of the parental pBLCAT2 vector (containing only the thymidine kinase promoter) is shown as a control.

The HCT116 and FET cells express similar amounts of TGFα mRNA during logarithmic phase growth (22) but show different phenotypes with respect to autocrine TGFα. The HCT116 cell line possesses a sequestered, antibody-inaccessible TGFα autocrine loop which can only be disrupted by constitutive expression of TGFα antisense RNA. The FET cell line expresses a classical autocrine TGFα loop, in which secreted TGFα interacts with its cognate EGFR on the same or adjacent cells, and is susceptible to inhibition by EGFR blocking antibodies. This led us to hypothesize that the changes in TGFα expression upon the acquisition of quiescence might reflect the degree of TGFα autocrine activity in the cell, particularly as these cells are not exposed to any exogenous growth factors and so endogenous TGFα would be expected to be a major determinant of TGFα promoter activity. Support for this hypothesis came when stable transfection of various TGFα deletion promoter constructs into the FET and HCT116 cell lines narrowed the region responsive to quiescent changes in endogenous TGFα status to between -370 and -201. We have identified the same region of the TGFα promoter as responsive to exogenous EGF using the FET cell line. Moreover, this is in agreement with previous studies which localized EGF responsiveness of the TGFα promoter to between -373 and -59 bp of the TGFα promoter but did not identify the precise location of the element (31).

Further studies in the FET cell line led us to identify a 25-bp element consisting of -225 to -199 of the TGFα promoter which conferred EGF responsiveness to a heterologous thymidine kinase promoter. Studies with stably transfected clones of HCT116 and FET containing this construct confirmed that this region was also responsive to the altered TGFα autocrine status in quiescent cells. Moreover, putative transcription factors binding this element were similarly modulated in response to growth arrest and quiescence in the two cell lines. This 25-bp

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**on TGFα promoter activity in clone U are not due to squelching of general transcription factors by the CMV promoter. Moreover, in transient transfection studies in which the CMV expression vector was co-transfected into HCT116 cells stably transfected with TGFα promoter CAT constructs, the activity of the CAT constructs was not altered by the presence of the CMV promoter (data not shown).**

We next examined whether the differential regulation of TGFα mRNA in the TGFα antisense expressing clone U was mediated at the transcriptional level. Clone U cells containing p370-CAT and clone UX cells containing p343-CAT were obtained as described under “Materials and Methods.” These different constructs both contain the EGF-response element and if this region is important in growth state regulation of these clones, these constructs should show differential regulation. These cells were grown up and harvested at various times during the establishment of quiescence. The promoter activity of the p370-CAT construct in clone U is not up-regulated during growth arrest (Fig. 8C). However, the activity of the TGFα promoter construct p343-CAT is up-regulated during the establishment of quiescence in the revertant clone UX which behaves like the parental HCT116 cell line (Fig. 8D). The p201-CAT construct stably transfected in U and UX cells, which does not contain the EGF-response element, showed no regulation in either cell line during the establishment of quiescence (data not shown). This experiment provides further evidence that autocrine TGFα function determines TGFα promoter activity in growth-arrested cells.
This autoregulatory 25-bp cis-sequence represents a significant point in the regulation of cell growth by TGFα. Previous studies in this laboratory have shown that the degree of growth factor dependence of the FET and HCT116 cell lines is reflected in their requirements for release from quiescence. The growth factor-dependent FET cell line requires both growth factors and nutrients for the subsequent release back into the cell cycle while the growth factor-independent HCT116 cell line requires only nutrients (4, 5, 22). Therefore, it appears that the increased production of TGFα in the HCT116 cell line during a period of starvation allows the cells to return to a dividing state under more stringent (growth factor deficient) conditions than the FET cell line. This increased expression of TGFα in the growth factor-independent cell line under adverse conditions may, therefore, represent a deregulated transcriptional control mechanism which serves to confer a growth advantage to this more aggressive phenotype. The growth factor-dependent phenotype of the FET cells reflects a less progressed phenotype. Like FET cells, the VACO 330 cell line, a non-malignant cell line established from a colonic, tubular adenoma, requires EGF or TGFα for growth stimulation at low plating density but not when plated at high density (32). Normal keratinocytes also require exogenous EGF for clonal initiation at low density but a high density growth proceeds in the absence of growth factor (33). This suggests that there is very little TGFα autocrine activity present in non-dividing, non-transformed cells.

Based on our findings in the FET and HCT116 cells, we propose that the type of TGFα autocrine activity determines whether or not TGFα production could be sustained in the absence of exogenous growth factors and so, in circular fashion, determine the phenotype of the cell. The growth factor-dependent FET cell line has relatively weak TGFα autocrine activity which is insufficient to sustain a high level of TGFα expression in the absence of exogenous growth factors. Therefore, during the transition to the quiescent state, TGFα expression is decreased, which in circular fashion, would necessitate the need for exogenous growth factors to aid re-entry into the cell cycle and DNA synthesis. In contrast, the growth factor-independent HCT116 cells possess a relatively strong TGFα autocrine function, which is apparently unaffected by environmental factors such that it allows the maintenance of high levels of TGFα expression in the absence of exogenous growth factors. This ability to maintain highly active TGFα autocrine function in the absence of growth factors in turn re-enforces the growth factor-independent phenotype in these cells through transcriptional activation of TGFα. In turn, this positive feedback control not only contributes to the growth factor-independent phenotype of the cells but increases the level of TGFα autocrine function as well. Further support for this model is provided by the studies with the TGFα RNA antisense expressing clone U, which was derived from the HCT116 cell line. Clone U shows dependence on exogenous growth factors for growth and no longer up-regulates TGFα mRNA during growth arrest and quiescence. Furthermore, the activity of the p370-CAT construct which contains the 25-bp autoregulatory element is no longer activated during the establishment of quiescence in this growth factor-dependent clone. However, the revertant clone UX which has switched to a high TGFα expressing, growth factor-independent phenotype despite the presence of antisense TGFα RNA (20), again up-regulates TGFα mRNA expression during growth arrest and the establishment of quiescence. The activity of the TGFα promoter construct p343-CAT, which contains the TGFα autoregulatory element, is also up-regulated in the revertant clone UX during the establishment of quiescence. Therefore, TGFα autocrine function which al-

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**Fig. 7. Gel shift assay of nuclear extracts from exponential phase and quiescent HCT116 cells and FET cells with the 25-bp EGF/TGFα response element.** Panel A, gel shift of HCT116 and FET nuclear extracts with the EGF response element. Nuclear extracts were prepared from exponential phase (Log) and quiescent (Qui) HCT116 or FET cells and used in a gel shift assay using 32P-labeled oligonucleotide 3/4 containing the 25-bp EGF-response element as probe, as described under “Materials and Methods.” A control lane of protein-free probe was also run (OP). The bands of interest are numbered and described in the text. Panel B, specificity of binding of oligo 3/4. Quiescent HCT116 nuclear extracts and exponential phase FET nuclear extracts were incubated with 32P-labeled oligonucleotide 3/4 in the presence of 5–100 ng of unlabeled oligonucleotide 3/4 equivalent to a 10–200-fold excess of unlabeled DNA compared with 32P-labeled probe 3/4. A protein-free lane was also run as a control (OP). 0 C, no competitor DNA.

Another important observation is the finding that deletion of the TGFα promoter construct to −201, which removes the EGF/growth state-responsive DNA sequence, results in a construct with very low basal promoter activity. The basal activity of the p201-CAT construct was low in several independent clones of both the HCT116 and FET cell lines, suggesting that repression of the CAT activity was not a positional integration effect. Moreover, the activity of the p201-CAT construct was also very low in previous transient transfection studies in which it was observed that the major elements controlling TGFα basal expression were localized to between −1140 and −201 (24). In our studies localizing the EGF-response element, the p247-, p343-, and p370-CAT constructs showed similar basal CAT activity which was lost in the p201-CAT construct. Therefore, the EGF/growth state responsive DNA sequence within the TGFα might also be the major determinant of basal activity.
allows for inappropriate TGFα expression in the non-dividing state may confer the growth advantage and growth factor independence of the progressed tumor cell, rather than just increased TGFα mRNA expression alone.

Although we have identified a transcriptional mechanism as the control point for the differences in TGFα regulation in the two cell types, it remains to be determined how EGFR signaling affects the transcription factors involved. The gel shift assay
shows increased binding of nuclear proteins during the acquisition of quiescence in HCT116 cells and decreased binding in growth arrested FET cells. However, it is not clear whether the binding of the transcription factors is regulated transcriptionally or post-transcriptionally through EGFR or growth state-regulated signaling peptides such as the mitogen-activated protein kinases and cyclin-dependent kinases.

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REFERENCES
1. Sporn, M. B., and Todaro, G. J. (1980) N. Engl. J. Med. 303, 878–880
2. Sporn, M. B., and Roberts, A. B. (1985) Cancer Surv. 4, 627–632
3. Sporn, M. B., and Roberts, A. B. (1985) Nature 313, 745–747
4. Mulder, K. M., and Brattain, M. G. (1989) Mol. Endocrinol. 3, 1215–1222
5. Mulder, K. M., and Brattain, M. G. (1989) The Cell and Molecular Biology of Colon Cancer, pp. 45–67, CRC Press, Boca Raton, FL
6. Sizeland, A. M., and Burgess, A. W. (1991) Mol. Cell. Biol. 11, 4005–4014
7. Sizeland, A. M., and Burgess, A. W. (1992) Mol. Biol. Cell 3, 1235–1243
8. Ziober, B. L., Willson, J. K. V., Humphrey, L. E., Childress-Fields, K., and Brattain, M. G. (1993) J. Biol. Chem. 268, 691–698
9. Derynck, R. (1992) Adv. Cancer Res. 58, 27–52
10. Todaro, G. J., Rose, T. M., Spooner, C. E., Shoyab, M., and Plowman, G. D. (1990) Semin. Cancer Biol. 1, 257–263
11. Derynck, R., Roberts, A. B., Winkler, M. E., Chen, E. Y., and Goeddel, D. V. (1984) Cell 38, 287–297
12. Derynck, R. (1988) Cell 54, 593–595
13. Watkins, I. F., Brattain, M. G., and Levine, A. E. (1988) Cancer Lett. 40, 59–70
14. Brachman, R., Lindquist, P. B., Nagashima, M., Kohr, W., Lipari, J., Napiér, M., and Derynck, R. (1989) Cell 56, 691–700
15. Wong, S. T., Winchell, L. P., McCune, B. K., Earp, H. S., Teixido, J., Massague, J., Herman, B., and Lee, D. C. (1989) Cell 56, 495–506
16. Anklesaria, P., Teixido, J., Laiho, M., Pierce, J. H., Greenberger, J. S., and Massague, J. (1990) Proc. Natl. Acad. Sci. U. S. A. 87, 3289–3293
17. Kudlow, J. E., and Bjorge, J. D. (1990) Cancer Biol. 1, 293–302
18. Derynck, R., Goeddel, D. V., Ullrich, A., Gutterman, J. U., Williams, R. D., Bringman, T. S., and Berger, W. H. (1987) Cancer Res. 47, 707–712
19. Berkowitz, E. A., Hissong, M. A., and Lee, D. C. (1996) Oncogene 12, 1991–2002
20. Howell, G. M., Ziober, B. L., Humphrey, L. E., Willson, J. K. V., Sun, L., Lynch, M., and Brattain, M. G. (1995) J. Cell. Physiol. 162, 256–265
21. Boyd, D. D., Levine, A. E., Brattain, D. E., McKnight, M. K., and Brattain, M. G. (1988) Cancer Res. 48, 2469–2474
22. Mulder, K. M. (1991) Cancer Res. 51, 2256–2262
23. Saeed, T., Cristiano, A., Lynch, M. J., Brattain, M., Kim, N., Normanno, N., Kenney, N., Ciardiello, F., and Salomon, D. S. (1991) Mol. Endocrinol. 3, 1955–1963
24. Lynch, M. J., Pelosi, L., Carboni, J. M., Merwin, J., Coleman, K., Wang, R. R. C., Lin, P. F. M., and Brattain, M. G. (1994) Cancer Res. 53, 4041–4047
25. Maniatis, T., Fritsch, E. F., and Sambrook, J. (1982) Molecular Cloning: A Laboratory Manual, pp. 202–203, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
26. Chirgwin, J. M., Przybyla, A. E., MacKendrick, R. J., and Rutter, W. J. (1979) Biochemistry 18, 5294–5299
27. Enoch, T., Zinn, K., and Maniatis, T. (1986) Mol. Cell. Biol. 6, 801–810
28. Dignam, J. D., Lebowitz, R. M., and Roeder, R. G. (1983) Nucleic Acids Res. 11, 1475–1489
29. Gorski, K., Carneiro, M., and Schibler, U. (1986) Cell 47, 767–776
30. Scheidereit, C., Heguy, A., and Roeder, R. G. (1987) Cell 51, 783–783
31. Raja, R. H., Paterson, A. J., Shin, T. H., and Kudlow, J. E. (1991) Mol. Endocrinol. 5, 514–529
32. Markowitz, S. D., Molkentin, K., Gerbic, C., Jackson, J., Stellato, T., and Willson, J. K. V. (1990) J. Clin. Invest. 86, 356–362
33. Pittelkow, M. R., Cook, P. W., Shipley, G. D., Derynck, R., and Coffey, R. J. (1993) Cell Growth & Differ. 4, 513–521