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# The Role of Transforming Growth Factor $\alpha$ in Determining Growth Factor Independence[1]

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## ABSTRACT

**Growth factor independence is a hallmark of malignancy that is attributed to the development of autocrine growth factor loops in cancer cells. However, growth factor-dependent normal cells also exhibit autocrine activity, thus raising the issue of how endogenously produced activity in cancer cells differs in a manner that leads to growth factor independence. We have examined this issue by comparing growth factor-independent HCT116 human colon carcinoma cells with a growth factor-dependent subcompartment of malignant cells designated HCT116b that was isolated from the same patient tumor. Therefore, the development of the growth factor-independent phenotype represents clonal progression within the tumor *in vivo*. The growth factor independence of HCT116 cells was shown to be dependent on autocrine transforming growth factor (TGF)-$\alpha$ activity, yet the isoparental HCT116b subcompartment showed similar levels of TGF-$\alpha$ expression as HCT116 when cells were in exponential growth. When both cell lines were growth arrested by nutrient deprivation, HCT116b cells required nutrient replenishment and growth factors for reinitiation of DNA synthesis, whereas HCT116 cells required only nutrient replenishment. In contrast to growth factor-dependent HCT116b cells, the HCT116 cells showed up-regulation of TGF-$\alpha$ expression during growth arrest as a result of enhanced transcription. This increased TGF-$\alpha$ expression in quiescent HCT116 cells was associated with constitutive epidermal growth factor receptor (EGFR) activation in the growth-arrested state, whereas growth-arrested HCT116b cells did not show EGFR activation. TGF-$\alpha$ antisense transfection of HCT116 cells showed that EGFR activation was due to increased TGF-$\alpha$ expression. Pretreatment of growth-arrested HCT116 cells with AG1478, a selective inhibitor of EGFR tyrosine kinase activity, blocked the reinitiation of DNA synthesis, demonstrating that growth factor independence was due to the increased TGF-$\alpha$ expression and EGFR activation of these cells in growth arrest relative to growth factor-dependent HCT116b cells. Importantly, the level of EGFR activation in growth-arrested HCT116 cells was only slightly higher than that of exponential cells, indicating that it was inappropriate EGFR activation in growth arrest rather than the amplitude of activation that generated growth factor independence.**

## INTRODUCTION

TGF-$\alpha$[3] is a member of the EGF ligand family that binds to and activates the EGFR (1). TGF-$\alpha$ is an important mediator of oncogenesis and malignant progression. Overexpression of TGF-$\alpha$ in transgenic mice leads to hyperplasia as well as malignancy in some tissues (2–4). Moreover, TGF-$\alpha$ acts as a strong collaborator in promoting carcinogenesis by other oncogenes (5–7) as well as chemical carcinogens (8, 9), and ectopic expression of TGF-$\alpha$ leads to the malignant progression of human tumors in athymic nude mice (10, 11).

TGF-$\alpha$ is known to bind only to the EGFR among the EGFR family members (1). Interestingly, some EGFR family members have oncogenic activity when they are constitutively activated by mutations and/or overexpressed. For example, *HER2/Neu* is constitutively activated by a point mutation in a significant proportion of breast cancers, whereas overexpression of this oncogene occurs in yet another subset (12–14). EGFR overexpression has also been implicated in the malignant progression of human tumors (15, 16). Truncation of the EGFR extracellular domain leads to its constitutive activation in human glial tumors (17–19). Interestingly, phosphorylation of this truncated receptor is restricted with respect to the amplitude of phosphorylation induced by EGF in the wild-type receptor (19). Thus, mechanisms leading to overexpression or inappropriate expression of TGF-$\alpha$ could generate malignant progression through constitutive EGFR activation.

TGF-$\alpha$ is best known as an autocrine stimulatory growth factor. The autocrine hypothesis was originally formulated to explain the growth advantage of tumor cells over their normal counterparts (20, 21). Briefly, the autocrine hypothesis suggested that tumor cells were less fastidious than normal cells in their growth factor requirements because they produced their own growth factors that would bind to cognate receptors on the tumor cell surface, resulting in the autostimulation of DNA synthesis and proliferation. However, several years after the formulation of this hypothesis, it became apparent that normal cells also displayed growth factor autocrine activity, including TGF-$\alpha$ autocrine activity (22). This raises the issue as to how autocrine TGF-$\alpha$ imparts a growth advantage to malignant cells that is not shared by normal cells. An important aspect of malignant cell progression is the acquisition of an increasingly growth factor-independent phenotype, whereas normal cells always require an exogenous source of growth factor to promote DNA synthesis (23, 24). Consequently, we reasoned that the elucidation of the mechanism underlying growth factor independence in autocrine TGF-$\alpha$-dependent malignant cells would provide substantial insight into the basis for a growth advantage imparted to malignant cells that is not shared by normal cells.

To determine the basis for autocrine TGF-$\alpha$-mediated growth factor independence in malignant cells, we used a cell line designated HCT116 that is fully independent of exogenous growth factors for reinitiation of DNA synthesis from a growth-arrested state and is typical of other highly progressed growth factor-independent human colon carcinoma cell lines (25, 26). Expression of TGF-$\alpha$ and regulation of growth in HCT116 cells were compared with those of growth factor-dependent cells (designated HCT116b) that were isolated from the same primary tumor (25). The isolation of both growth factor-dependent and -independent subsets of malignant cells from the same patient provided the model an important advantage in that these isoclonal-derived cells could be compared for the determination of the

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[3] The abbreviations used are: TGF, transforming growth factor; EGF, epidermal growth factor; EGFR, epidermal growth factor receptor; SM, supplemental McCoy's medium; TIE, transferrin, insulin, and EGF; CAT, chloramphenicol acetyltransferase; Neo, neomycin; cdk, cyclin-dependent kinase.

mechanistic basis for autocrine TGF-α-mediated growth factor independence. Moreover, as indicated above, the two subcompartments of cells were isolated from the original primary colon carcinoma (25), thus ensuring that this model system is physiologically relevant.

Comparison of HCT116b with HCT116 cells showed that growth-arrested HCT116b cells required exogenous growth factors for reinitiation of cell cycle transit to S phase, whereas HCT116 cells were completely independent of the need for exogenous growth factors for cell cycle reentry. Growth factor independence in HCT116 cells was previously shown to require autocrine TGF-α by stable TGF-α antisense expression (24). Therefore, we hypothesized that differences in TGF-α levels or control of TGF-α expression were responsible for the different growth-regulatory phenotypes of the two isoparental tumors. Both HCT116 and HCT116b cells expressed a similar level of TGF-α in exponential phase tissue cultures. However, during the establishment of a growth-arrested state by growth factor and nutrient deprivation, HCT116 showed up-regulation of TGF-α expression, whereas HCT116b did not. The up-regulation of TGF-α in quiescent HCT116 cells was associated with increased EGFR activation in the growth-arrested state. Moreover, growth factor-independent stimulation of DNA synthesis in HCT116 cells from a growth-arrested state was blocked by AG1478, an EGFR-specific tyrosine kinase inhibitor, whereas ectopic expression of TGF-α in HCT116b cells generated a growth factor-independent phenotype.

## MATERIALS AND METHODS

**Cell Lines and Tissue Culture.** HCT116 and HCT116b sublines were isolated from a primary tissue culture of a single human colon carcinoma as described by Brattain *et al.* (25). The two cell lines were adapted to growth in serum-free medium (26) consisting of McCoy's 5A medium (Sigma) supplemented with amino acids, pyruvate, and antibiotics (designated SM) containing the growth factors transferrin (4 μg/ml; Sigma), insulin (20 μg/ml; Sigma) and EGF (5 ng/ml; Collaborative Research). SM containing these three growth factors was designated TIE. The HCT116 clones stably transfected with a TGF-α antisense cDNA have been described previously (24).

**Mitogenesis Assay.** Mitogenesis assays were performed as described previously (23). Briefly, cells were plated into 6-well plates at a density of $1.2 \times 10^5$ cells/well. The cells were grown to confluence in 2 ml/well serum-free medium. The cells were then changed to SM, and the medium remained unchanged for 5 days to induce growth arrest. On day 5, the medium was switched to SM or TIE for the indicated periods of time. The cells were treated with methyl-[$^3$H]thymidine (25 μCi; 40–60 Ci/nmol; Amersham Life Science) an hour before analysis of the samples, and the amount of tritium was estimated in DNA precipitated with 10% trichloroacetic acid.

**Immunoblot Analysis.** Immunoblotting was performed as described previously (27). Briefly, cells were lysed in lysis buffer (150 mM NaCl, 1% NP40, 1 mM phenylmethylsulfonyl fluoride, 1 mM DTT, 25 μg/ml aprotinin, 25 μg/ml soybean trypsin inhibitor, 25 μg/ml leupeptin, 50 mM sodium fluoride, 1 mM sodium vanadate, and 20 mM β-glycerophosphate) containing 0.1% SDS and then boiled for 5min to solubilize membrane protein. After centrifugation and removal of particulate matter, protein concentration in the supernatant was determined using the Bio-Rad protein assay system. Samples (150 μg of total protein) were analyzed on 7.5% SDS-PAGE gels. After transfer onto nitrocellulose membranes, the membranes were blocked at room temperature for 1 h in 5% milk/TTBS [20 mM Tris-HCl (pH 7.5), 150 mM NaCl, and 0.05% Tween 20] and then incubated with the primary antibody overnight at 4°C. Incubation with the primary antibody was continued for 1 h at room temperature before replacement with the secondary antibody. Blots were developed using the enhanced chemiluminescence system (Amersham). Activated EGFR was blotted using an antibody that is specific for the activated form (Clone 47; Transduction Laboratories).

**Histone H1 Kinase Assay.** Polyclonal antibody (1 μg) to human cdk2 (SC-163) or cyclin A (SC-248; Santa Cruz Biotechnology) was incubated with 50–100 μg of cell lysates at 4°C for 2–3 h as described previously (28). Protein A- or G-Agarose was added to the samples to precipitate the antibody-protein complexes for 2 h at 4°C. After centrifugation, the samples were washed three times with lysis buffer and then washed three times with kinase buffer [20 mM Tris-HCl (pH 7.4) containing 4 mM $MgCl_2$]. The precipitates were resuspended in 10 μl of reaction buffer [8 mM Tris-HCl (pH 7.4), 1.6 mM $MgCl_2$, 50 μM ATP (Pharmacia), 0.5 μCi of [γ-$^{32}$P]ATP (3000 Ci/nmol; Amersham), and 2 μg of histone H1 (Sigma)] and incubated at 37°C for 30 min. The reactions were boiled in 10 μl of 2× Laemmli sample buffer for 5 min. Samples were then analyzed by SDS-PAGE (10%, w/v) followed by autoradiography. The bands corresponding to phosphorylated histone H1 were scanned using a Lynx densitometer (Applied Imaging), and activity was calculated as fold induction over activity of quiescent cells (0 h).

**Promoter Assays.** Assays for CAT were conducted using cells stably transfected with TGF-α promoter/-CAT chimeras as described previously (24, 29). The promoter construct contains 370 bp of the TGF-α promoter upstream of the transcription initiation site. This region contains the previously identified TGF-α autoregulatory element located between –225 and –201, 5′-GAGTGGCGAGGAGGTGACGGTAGCCG-3′ (24, 29). At the indicated growth stages, the cells were washed three times with PBS containing 1 mM EDTA and then harvested in 1 ml of TEN buffer [40 mM Tris-HCl (pH 7.4), 1 mM EDTA, and 150 mM NaCl]. After centrifugation, the cells were resuspended in 100 μl of 250 mM Tris-HCl buffer (pH 7.0) containing 15% glycerol (v/v) and then lysed by three cycles of freeze-thawing. Protein concentration in the supernatant was determined using bicinchoninic acid reagent (Pierce). The CAT assay was performed using a final concentration of 3.6 mM acetyl-CoA.

**RNase Protection Assay.** Cells were washed three times with PBS containing 1 mM EDTA and lysed at the indicated growth stages in 8 ml of 5 M guanidine isothiocyanate. Total RNA was isolated by centrifugation through a cesium trifluoroacetic acid density gradient (30). Total RNA (40 μg) was hybridized to a TGF-α riboprobe that was synthesized by SP6-RNA polymerase (24) in the presence of [$^{32}$P]UTP (3000 Ci/nmol; Amersham). Actin probe was also used to normalize for equal sample loading.

**Northern Analysis.** Electrophoresis of total RNA (isolated as described above) was performed in a 1.2% agarose formaldehyde-containing gel using a phosphate buffer system and transferred to a Nytran nylon membrane (Schleicher and Schuell). The membrane was hybridized with a 930-bp TGF-α cDNA probe labeled with [$^{32}$P]dCTP using a Multiprime Labeling Kit (Amersham). Northern blots were rehybridized with a 1.16 kb *Pst*I fragment of glyceraldehyde-3-phosphate dehydrogenase to quantify loading.

**Electrophoretic Mobility Shift Assays.** Assays were conducted as described previously (24). Exponentially growing and quiescent cells were harvested in PBS containing 1 mM EDTA, containing the protease and phosphatase inhibitors described in the immunoblot lysis buffer described above. After hypotonic lysis (31), nuclear proteins were extracted with potassium chloride and dialyzed and concentrated (32). Protein concentrations were determined using the bicinchoninic acid system (Pierce). Nuclear extracts were incubated at 30°C for 20 min with $^{32}$P-labeled TGF-α autoregulatory element (−225 to −201) in the presence of 25 μg/ml nonspecific competitor poly(deoxyinosinic-deoxycytidylic acid). The oligonucleotide probe was labeled with [α-$^{32}$P]dGTP (3000 Ci/mmol; Amersham) by Klenow fragment. The reaction mixtures were electrophoresed on a 4% polyacrylamide gel at 150 V for 1.5 h at 4°C. The gel was visualized by autoradiography.

The competition assay was performed in the same manner, except that unlabeled probe containing the TGF-α autoregulatory sequence was incubated with the nuclear extracts for 15 min at 30°C before adding the labeled probe.

**Tumorigenicity.** Parental HCT116 cells, mock-transfected parental HCT116 cells (cells containing the Neo expression vector without insert), and TGF-α antisense-transfected HCT116 cells isolated from logarithmic growing cultures were injected s.c. behind the anterior forelimb of 5–6-week-old Balb/C athymic female mice. Growth curves for xenografts were determined by externally measuring tumors in two dimensions using a caliper. Volume ($V$) was determined by the equation $V = (1 \times W) \times 0.5$, where $L$ is length of the tumor, and $W$ is the width of the tumor. For nucleic acid analysis, tumors were removed from anesthetized animals, frozen in liquid nitrogen (sections were taken for histology at this step), pulverized, and solubilized in guanidinium isothiocyanate.

## RESULTS

**HCT116b Cells Require Exogenous Growth Factors for Optimal Release from Growth Arrest.** Mitogenesis assays were conducted on growth-arrested cells released with SM or TIE. Fig. 1*A* indicates that maximal DNA synthesis was stimulated in HCT116b only when exogenous growth factors (TIE) were added. On the other hand, replenishment of nutrients without growth factor supplementation was sufficient to stimulate maximal DNA synthesis in HCT116 cells (Fig. 1*B*). In previous studies we have shown that HCT116 cells are growth factor independent due to utilization of a strong autocrine TGF-α loop during quiescence (24). We hypothesized that HCT116b cells are growth factor dependent because of the lack of TGF-α expression during the establishment of quiescence. Ectopic expression of TGF-α in HCT116b should release the cells from this requirement because the constitutive promoter driving TGF-α expression would allow for continued TGF-α expression in growth arrest. To test this hypothesis, a stable TGF-α transfectant of HCT116b, designated HCT116b-αS, was subjected to mitogenesis assay as described for the HCT116b and HCT116 cell lines above. As shown in Fig. 1*C*, nutrient replenishment was sufficient to induce DNA synthesis in the TGF-α-transfected HCT116b-αS cells. As in the case of HCT116 cells, the addition of exogenous growth factors in SM to HCT116b-αS did not affect the level of DNA synthesis. The cdk2 enzyme activity in association with cyclin E and cyclin A is necessary for S-phase entry and transit. Therefore, stimulation of cdk2 enzyme activity after stimulation of growth-arrested cells to reenter the cell cycle should reflect the difference in growth factor requirements between HCT116 and HCT116b cells. The histone H1 kinase activity of cdk2 was measured at 2, 18, and 24 h after stimulation of the HCT116 or HCT116b cells with either growth factors and nutrients (TIE) or nutrients alone (SM). Fig. 2 shows that cdk2 activity was stimulated during S-phase entry (18 h) and transit (24 h) when exogenous growth factors were added to HCT116b cells (12-fold induction at 24 h), but not by nutrient stimulation alone (only a 2-fold change in cdk2 activity at 24 h). In contrast, when HCT116 cells were released with nutrients alone, the level of cdk2 activity increased to 16-fold at 24 h. The addition of exogenous growth factors to the medium did not result in any significant increase over the nutrient-mediated level of cdk2 histone H1 kinase activity. As in the case of the mitogenesis assay, the requirement for exogenous growth factors to stimulate cdk2 hisone H1 activity in HCT116b was abrogated by the constitutive TGF-α expression in the HCT116b-αS transfectant.

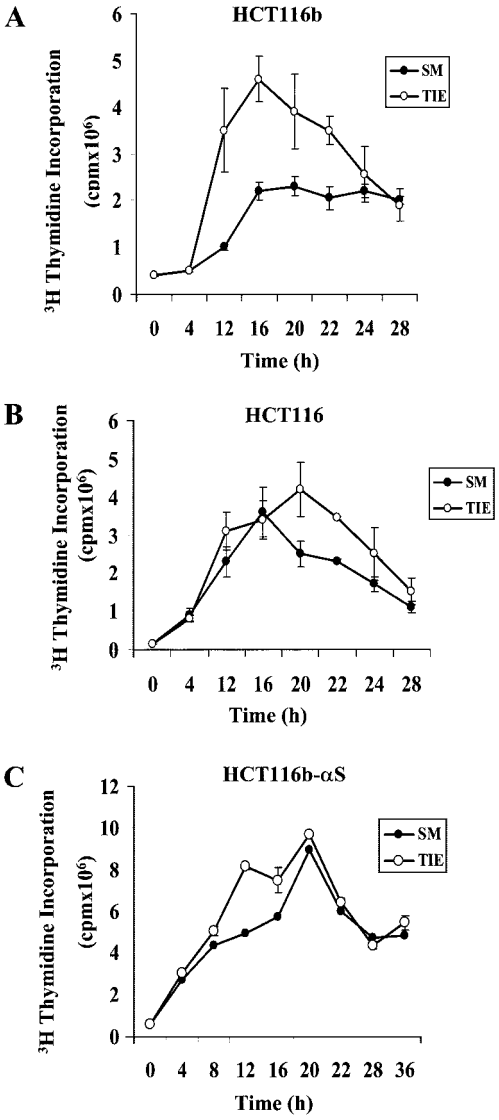


Fig. 1. The effect of nutrients and exogenous growth factors on DNA synthesis. *A*, HCT116b; *B*, HCT116; *C*, HCT116b-αS. Confluent cells were growth arrested by maintaining them in SM for 5 days. The medium was then changed to SM (●) or TIE (○). [$^3$H]Thymidine incorporation was determined in trichloroacetic acid-precipitated DNA at the indicated time points. Values are plotted as mean ± SE ($n$ = 3) of a representative experiment.

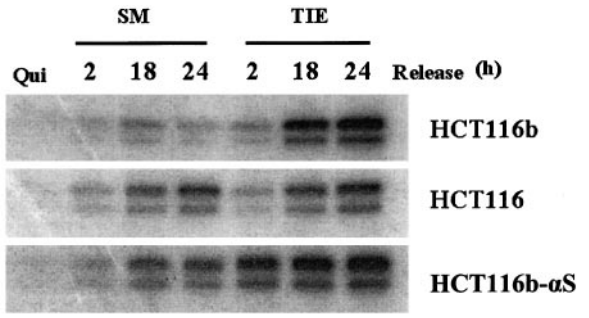


Fig. 2. The effect of nutrients and exogenous growth factors on cdk2 histone H1 kinase activity in HCT116b, HCT116, and TGF-α sense-transfected HCT116b (HCT116b-αS) cells. Growth-arrested cells were released with SM or TIE for the indicated periods of time. Cell lysates were incubated with a polyclonal antibody for cdk2. Histone H1 kinase assay was performed on the immunoprecipitates.

**TGF-α mRNA Is Up-Regulated in Quiescent HCT116 Cells.** The growth factor independence of the HCT116 subline is linked to strong TGF-α autocrine activity (24). To determine the mechanism by which TGF-α autocrine activity contributes to the malignant progression of HCT116 cells, we compared the steady-state levels of TGF-α mRNA in HCT116b and HCT116 cells at different growth stages. Using RNase protection assays, total RNA from exponential and growth-arrested HCT116 and HCT116b cells was probed with a TGF-α probe as described in "Materials and Methods." Fig. 3 shows that TGF-α mRNA levels in exponential cells were essentially the same in both cell types. However, TGF-α levels in HCT116 cells showed a 2.5-fold increase with induction of growth arrest, whereas those of HCT116b cells decreased (an approximately 30–40% decrease). These results suggested that regulation of TGF-α mRNA expression in HCT116 cells is disrupted, and this disruption is manifested in growth-arrested cells. If maintenance of TGF-α expression in quiescence is the key mechanism accounting for growth factor independence in the HCT 116 cells, then addition of TGF-α alone to the basal SM should substitute for the growth factor combination TIE in reinitiating DNA synthesis in the growth factor-dependent HCT116b cells. This was the case, as shown in Fig. 4.

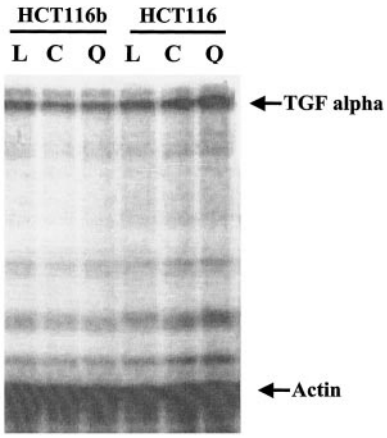


Fig. 3. TGF-α mRNA expression in the HCT116b and HCT116 sublines. Total mRNA from exponentially growing cells (*L*), confluent cells (*C*), and growth-arrested cells (*Q*) was isolated and analyzed for TGF-α expression using RNase protection assay. An actin probe was used to normalize for loading.

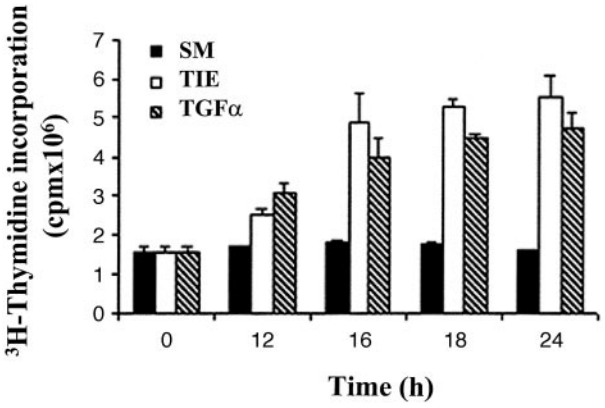


Fig. 4. Effect of TGF-α alone on mitogenesis in the growth factor-dependent HCT116b cells. HCT116b cells were starved into quiescence and then released with either TIE or SM containing TGF-α alone.

**Differential TGF-α Autocrine Expression Is Associated with Differential TGF-α Promoter Activity and Changes in the Binding Activity of Nuclear Proteins to the TGF-α Autoregulatory Element.** TGF-α promoter activity was measured in HCT116b and HCT116 cells at different growth stages to determine whether there was a transcriptional basis for their differences in TGF-α mRNA expression. Cells were stably transfected with a human TGF-α promoter CAT reporter construct containing the TGF-α autoregulatory element as described in "Materials and Methods" (24). The CAT activity of this construct was measured in HCT116b and HCT116 cell lysates as a function of the establishment of growth arrest. TGF-α promoter activity increased in HCT116 as the cells were rendered quiescent, whereas promoter activity in HCT116b cells did not (Fig. 5). Although equal protein amounts were used in the assay, the basal activity of the promoter construct is higher in HCT116b than in HCT116. Nonetheless, the results show that HCT116 up-regulates TGF-α promoter activity in growth arrest.

Previous work from our laboratory (24) has shown that TGF-α mediates its own expression by modulating the activity of DNA-binding protein complexes to the previously described autoregulatory *cis*-element within the TGF-α promoter. If binding of these proteins regulated autocrine TGF-α expression, then it would be expected that quiescent HCT116 cells would show higher levels of protein binding to this same *cis*-element than HCT116b cells. Therefore, gel shift assays were conducted to investigate the regulation of the proteins that bind to this autoregulatory *cis*-element of the TGF-α promoter. Fig. 6*A* shows a gel retardation assay in which the TGF-α autoregulatory element, designated oligonucleotide 3/4 (−225 to –201 bp relative to the ATG codon) was used as a probe.

The gel shift of nuclear extracts from both HCT116 and HCT116b shows a pattern of five bands similar to that reported in another colon carcinoma cell line, FET (29). As in the FET gel shift, band 5 is not

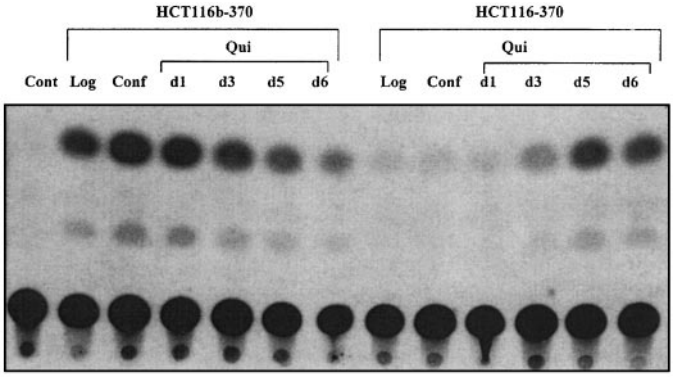


Fig. 5. TGF-α promoter activity in the HCT116b and HCT116 sublines. Cell lines (HCT116b-370 and HCT116–370) that are stably transfected with TGF-α promoter-CAT construct containing 370 bp of the TGF-α promoter upstream of the transcription initiation site were harvested for CAT assays at exponential phase (*Log*), confluence (*Conf*), and growth arrest (*Qui*). Growth arrest was induced by starving the cells in SM for 1 day (*d1*), 3 days (*d3*), 5 days (*d5*), and 6 days (*d6*).

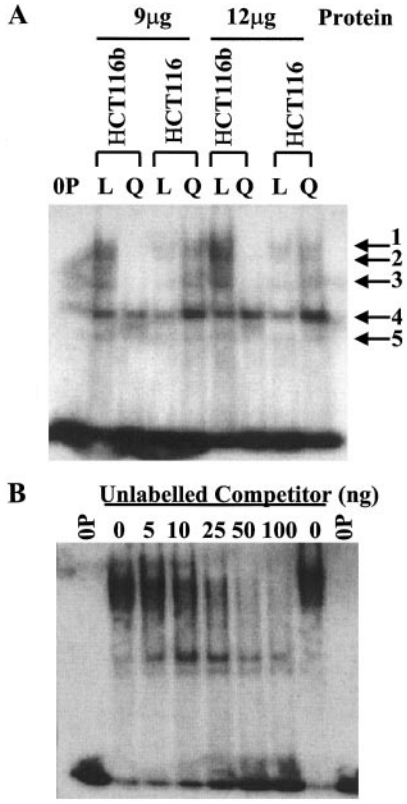


Fig. 6. Regulation of binding of DNA-binding proteins to the TGF-α autoregulatory *cis*-element. *A*, nuclear extracts from exponentially growing cells (*L*) and growth-arrested cells (*Q*) of HCT116b and HCT116 sublines were run against labeled probe corresponding to the TGF-α autoregulatory element (−225 to −201); 9 and 12 μg of total protein from the nuclear extracts were used. *0P* represents the probe alone without any added protein. *B*, 12 μg of exponentially growing HCT116b cells were run against the labeled TGF-α autoregulatory element in the absence or presence of increasing amounts of competing unlabeled TGF-α autoregulatory element. *0P* represents the labeled probe without any added protein.

competed by excess cold competitor oligonucleotide 3/4 (Fig. 6*B*) and is therefore nonspecific. In the previous study with the FET cells, we used deletion constructs of oligonucleotide 3/4 as probe. Only bands 1–3 showed sequence-specific binding with the deletion probes. Band 4 was present in all deletion constructs and therefore probably represents a nonspecific DNA binding protein. Significantly, bands 1–3, which we have identified as the most important DNA-binding proteins binding to oligonucleotide 3/4, show differential regulation in the two cell lines described here and reflect the TGF-α mRNA expression in these two sublines. In the growth factor-dependent HCT116b cell line, the binding of the putative transcription factors represented by bands 1–3 is down-regulated as these cells are rendered quiescent. However, in the HCT116 cell line with the growth factor-independent phenotype, the binding of bands 1–3 is increased during the establishment of quiescence. This binding pattern also correlates with the changes in TGF-α promoter activity in the two cell lines during quiescence establishment. The enhanced protein binding to the TGF-α autoregulatory element in nuclear extracts of exponentially growing HCT116b cells compared with quiescent HCT116 cells probably reflects the higher level of TGF-α promoter activity in this subcompartment of cells. Interestingly, the binding of nuclear proteins to this element is higher in exponentially growing HCT116b cells than HCT116 cells. This corresponds to the higher activity of the p370-CAT construct in logarithmic phase HCT116b cells compared with the HCT116 cells noted above.

**EGFR Activation Is Up-Regulated and Required for Growth Factor-independent DNA Synthesis in HCT116 Cells.** The continued expression of TGF-α during the establishment of quiescence suggested that the constitutive expression of this EGFR ligand would result in constitutive activation of the EGFR, a known transforming event as indicated above. Moreover, constitutive EGFR activation in growth-arrested cells could provide a mechanism that would abrogate the need for exogenous growth factors for cell cycle reentry. Therefore, EGFR expression and activation were determined in HCT116 cells. Western blot analysis for activated EGFR protein indicated continued activation of the EGFR signaling pathway in growth arrest (Fig. 7*A, left panel*). Insulin-like growth factor I receptor protein was used as a control to show equal loading of protein from exponential and growth-arrested cells.

Whereas EGFR activation in growth-arrested HCT116 cells is correlated with constitutive TGF-α expression, this does not demonstrate that TGF-α is directly responsible for this activation. To provide a direct link for TGF-α-mediated EGFR activation, we used HCT116 cells stably transfected with a TGF-α antisense cDNA. These TGF-α antisense transfectants have been extensively characterized and show an approximately 80% reduction in TGF-α mRNA and protein expression (24). If EGFR activation is due to continued TGF-α expression in growth-arrested HCT116 cells, then loss of TGF-α expression in TGF-α antisense-transfected cells should result in loss of EGFR activation. The *right panel* in Fig. 7*A* shows that TGF-α antisense transfectants rendered quiescent by nutrient deprivation lack EGFR activation, thus directly linking continued TGF-α expression in quiescent HCT116 cells to constitutive EGFR activation. Further evidence for a direct link between EGFR activation and TGF-α expression is shown in Fig. 7*B*. In HCT116b cells, the level of EGFR activation is markedly decreased in quiescent compared with logarithmic phase cells (compare activated EGFR *versus* total EGFR), whereas the constitutive expression of TGF-α in the HCT116b-αS cells abrogated this decrease. Actin was used to show equal loading.

The autocrine TGF-α-mediated constitutive activation of EGFR in HCT116 cells raised the issue of whether activation of the receptor was required for growth factor-independent reentry into the cell cycle. AG1478 is a tyrphostin with high selectivity for inhibition of EGFR tyrosine kinase activity (33). When quiescent HCT116 cells were exposed to AG1478 for 15 min and then treated with nutrients in the presence or absence of AG1478 to allow reinitiation of cell cycle transit, DNA synthesis was blocked in a concentration-dependent manner (Fig. 8*A*), as was the activation of the EGFR (Fig. 8*B*). However, AG1478 had little effect on total EGFR.

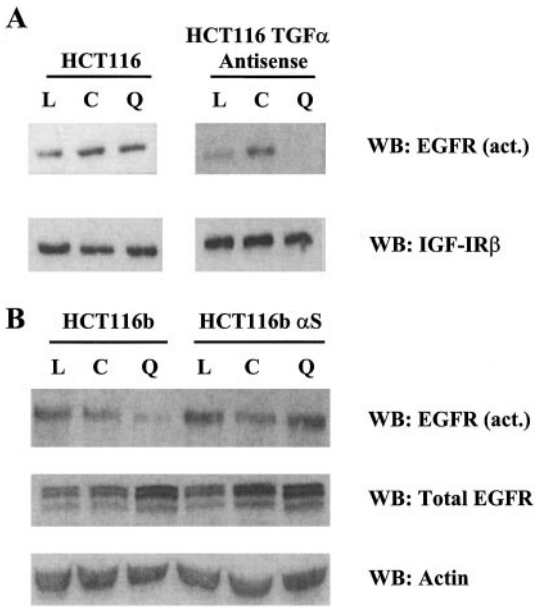


Fig. 7. Analysis of EGFR activation in growth-arrested HCT116 cells and TGF-α antisense-transfected HCT116 cells. Western blot analysis of cell membrane proteins was performed as described in "Materials and Methods." Protein loading was normalized using another cell surface tyrosine kinase receptor, the insulin-like growth factor I receptor, which is not altered as a function of growth state. *A*, HCT116 cells and HCT116 cells in which TGF-α expression is repressed by stable expression of a TGF-α antisense cDNA. *B*, HCT116b cells and TGF-α sense-transfected HCT116b (HCT116b-αS) cells.

**Effect of Repression of Autocrine TGF-α on HCT116 Tumorigenicity in Athymic Mice.** In previous work (10), we showed that ectopic expression of TGF-α in growth factor-dependent cells resulted in malignant progression as reflected by increased tumorigenicity and more rapid kinetics of tumor formation in athymic nude mice. Whereas dependence of growth on autocrine TGF-α has been shown using TGF-α antisense approaches (34, 35), it has not yet been demonstrated that repression of autocrine TGF-α modulates malignancy of colon carcinoma cells *in vivo*. Others have shown that the unattenuated signaling from a truncated *EGFR* is critical to the tumorigenic properties of cells expressing this receptor (19).

To determine whether the constitutive EGFR activation that is dependent on TGF-α was critical to the tumorigenic properties of the HCT116 cells, stable TGF-α antisense transfectants were compared with control HCT116 cells for the ability to form tumors in athymic nude mice. Previous work had shown that HCT116 cells formed rapidly growing tumors in 100% of animals injected with $10^6$ cells (24). Tumorigenicity of several TGF-α antisense clones was tested at inocula of $5 \times 10^6$ and $10^6$ cells. At $5 \times 10^6$ cells, tumor formation was obtained from all HCT116 control cell-injected animals as well as all TGF-α antisense transfectant-injected animals. However, tumor growth kinetics were substantially slower for the TGF-α antisense transfectants because the number of days required for the tumors to reach a size of 500 $mm^3$ was 2-fold longer (typical mean of $9.3 \pm 1.2$ days and overall range of 3–12 days for the control cells compared with typical mean of $20.2 \pm 1.3$ days for clone E and overall range of 16–32 days for all of the TGF-α antisense-transfected clones). Fig. 9 shows a typical tumor growth curve for xenografts generated from $5 \times 10^6$ control HCT116 cells and TGF-α antisense transfectants.

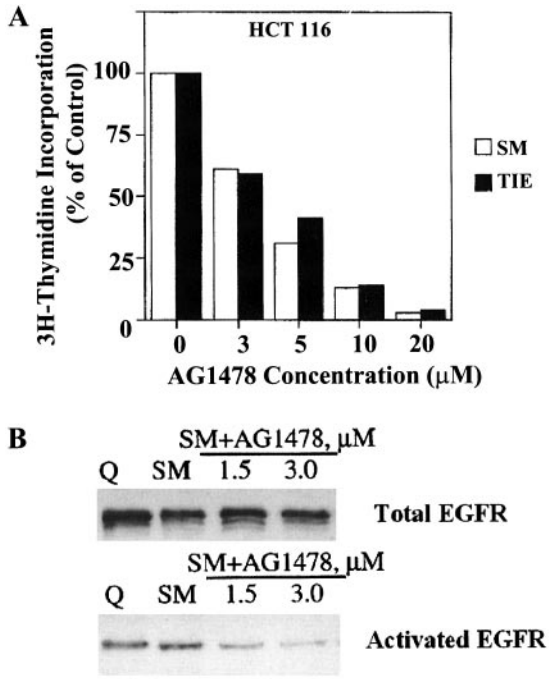


Fig. 8. The effect of AG1478 on DNA synthesis and EGFR activation. *A*, concentration-dependent inhibition of DNA synthesis by AG1478. Quiescent cells were pretreated with the indicated concentrations of AG1478 for 15 min and then released from growth arrest by SM (□) or TIE (■) containing the appropriate concentrations of AG1478. DNA synthesis was determined by [$^3$H]thymidine incorporation at 18 h as described in "Materials and Methods." *B*, inhibition of EGFR activation as a function of AG1478 concentration. Quiescent cells were pretreated with increasing concentrations of AG1478 for 15 min and then released with SM in the presence of increasing concentrations of AG1478. Cells were harvested for subsequent Western analysis.

Antisense TGF-α xenografts displayed a lag phase of approximately 2 weeks before substantial growth, whereas control cells, as noted in previous studies, did not show a lag phase. Once the TGF-α antisense-transfected cells began to grow, they showed approximately the same kinetics of xenograft growth as the control cells.

The long lag phase of the TGF-α antisense transfectants suggested the selection of a subpopulation in which antisense TGF-α expression became curtailed due to the lack of continued selection with G418 *in vivo*. Consequently, Northern blot analysis to probe for endogenous TGF-α mRNA and TGF-α antisense RNA was performed in both control cell and antisense TGF-α xenografts and the cultured cells used for the tumorigenicity studies. The TGF-α mRNA was reduced 60–80% in cultured TGF-α antisense-transfected cells relative to Neo-transfected HCT116 control cells, and the antisense TGF-α RNA levels were substantially higher than the TGF-α mRNA level (data not shown). Analysis of antisense TGF-α xenografts indicated that the TGF-α mRNA levels were considerably higher than the antisense TGF-α mRNA levels. Moreover, the TGF-α antisense RNA levels were greatly reduced relative to the cultured cells. Interestingly, TGF-α mRNA levels in TGF-α antisense xenografts were almost restored to the level observed in HCT116 cultured cells. Taken together, these results suggest that TGF-α antisense xenograft growth was associated with a population of cells in which TGF-α antisense RNA expression had been lost.

Tumorigenicity at an inoculum of $10^6$ was also tested. Neo-transfected control HCT116 cells showed tumor formation in 100% of the animals inoculated, whereas, at this lower inoculum, three of four TGF-α antisense clones showed reduced numbers of tumors. Antisense TGF-α-transfected cells that formed tumors again showed a 2-fold delay in the time required to attain a tumor size of 500 mm$^3$ [typical mean of 16.6 ± 0.5 days for Neo clone 5 and a range of 14–21 days for all Neo clones compared with a mean of 32.5 ± 2.6 days (clone 33) and an overall range of 26–41 days for all of the antisense clones]. Previous work had already shown that the HCT116b subline was poorly tumorigenic relative to HCT116 (25).

## DISCUSSION

Accumulation of genetic mutations leads to tumor formation and malignant progression (36, 37). It is of critical importance to ascertain the molecular basis of malignancy to develop effective treatments for cancer and to understand patterns of abnormal growth regulation. Heterogeneity of colon carcinoma cell lines obtained from different patients has provided models to study the pathogenic progression of the disease (25, 38). However, these models are potentially restricted in that comparisons of nonisogenic models may lead to erroneous conclusions because their biological and growth-regulatory properties may reflect individual variations. The isolation and subsequent development of primary tissue cultures of two sublines, HCT116 and HCT116b (which were directly derived from a single primary tumor), have provided us with a unique opportunity to study an isoclonal system reflecting malignant progression *in vivo*. Previous characterization of these two subcompartments had revealed that the HCT116b subline was differentiated and weakly tumorigenic in comparison with the HCT116 subline (25). HCT116 cells have been extensively studied and were shown to be independent from exogenous growth factors for DNA synthesis and growth (23, 24). This autonomous growth as well as aggressive growth at low inoculum in athymic mice is indicative of the high level of the HCT116 subcompartment's malignant progression.

We compared regulation of reentry into cell cycle transit of HCT116b with HCT116 cells to determine how TGF-α contributes to growth factor independence. Much like normal cells, the HCT116b subline was dependent on exogenous growth factors for initiation of DNA synthesis. Normal cells, insofar as is now known, do not exhibit growth factor-independent reentry into cell cycle transit. Normal keratinocytes (39, 40) as well as premalignant adenoma-derived epithelial cells (38) require activation of the EGFR by exogenous EGF family ligands at low cell densities in order for colony formation to occur, whereas autocrine TGF-α activity appears to be able to substitute for this exogenous EGF requirement during their exponential growth. However, in contrast to HCT116 cells, these nonmalignant cells require exogenous factors such as insulin even during exponential growth. This indicates that autocrine TGF-α by itself is not adequate to stimulate the reentry of these normal cells into a prolif-

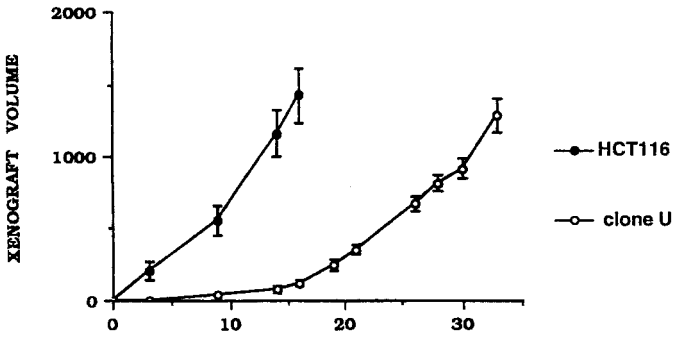


Fig. 9. Xenograft growth curves of parental HCT116 and a TGF-α antisense clone. HCT116 parental and TGF-α antisense clone U cells in logarithmic growth were injected s.c. ($5 \times 10^6$ cells) behind the anterior forelimb of athymic nude mice. Tumors were measured externally on the indicated days in two dimensions using a caliper, and volume (*V*) was determined from the following equation, where *l* is length of the tumor, and *W* is width of the tumor: $V = (l \times W) \times 0.5$. Parental HCT116, ●; TGF-α antisense transfectant clone U, ○.

erative mode during the lag phase of tissue culture that occurs at low seeding. In contrast to HCT116 cells, HCT116b cells exhibit an inadequacy for cell cycle reentry that can be overcome by exogenous EGFR activation, indicating that these cells share some of the growth restrictions of nonmalignant cells.

The loss of growth regulation in HCT116 cells was attributed to an autocrine TGF-α loop (24). The disruption of this loop by constitutive TGF-α antisense RNA expression led to a growth factor-dependent phenotype with down-regulated expression of TGF-α mRNA in growth-arrested cells (24). The present work indicates that up-regulation of TGF-α expression in the HCT116 subline allows for reentry into the cell cycle as soon as nutrients are replenished because of constitutive activation of EGFR during growth arrest. Thus, HCT116 cells are more likely to continue to divide under more stringent growth conditions with inadequate growth factor supply than the less progressed HCT116b counterpart, which requires exogenous growth factors in addition to nutrients for stimulation of DNA synthesis. Human cancers, including colorectal tumors, often show labeling indexes of less than 1% of the malignant cells (41), indicating that environmental conditions in the tumor *in vivo* are restrictive to growth. Thus, a growth factor-independent phenotype such as that observed for HCT116 would provide a potentially substantial growth advantage *in vivo*.

Characterization of TGF-α regulation has revealed that the basis for the disruption in TGF-α expression in growth-arrested HCT116 cells is altered promoter activity. In contrast to growth factor-dependent HCT116b cells, transcriptional activity of the TGF-α promoter was up-regulated in HCT116 cells during the establishment of growth arrest. Gel shift assays showed that there are several protein complexes that bind to the TGF-α autoregulatory element. Other work from our laboratory has colocalized the TGF-α autoregulatory element to an identical EGF response region within the TGF-α promoter (24). Three different binding activities were shown to interact with the TGF-α autoregulatory DNA element. The binding activity of these factors is increased over that observed for extracts from exponentially growing cells in nuclear extracts of quiescent HCT116 cells. These results suggest that the increased binding (and possibly expression) of DNA-binding proteins to the TGF-α autoregulatory element leads to up-regulation of TGF-α mRNA expression in HCT116 cells during quiescence, resulting in constitutive activation of the EGFR.

Interestingly, although the HCT116 and HCT116b cell lines were derived from the same patient tumor, these studies reveal that in addition to differential regulation of TGF-α in the quiescent state, these two cell lines may use different mechanisms to maintain of TGF-α mRNA expression in exponential growth. Although both cell lines show similar levels of TGF-α mRNA expression at this growth state, the activity of the TGF-α promoter construct p370-CAT and the binding of the nuclear proteins to the TGF-α autoregulatory element were higher in logarithmic phase HCT116b cells than HCT116 cells. The HCT116 cells show the replication error-positive phenotype and thus are DNA repair deficient (42). This results in mutation of many genes including transcriptional activators such as *p300* (43). The HCT116b cell line expresses p300.[4] It is not known whether HCT116b shows the replication error-positive phenotype. However, the lack of p300 may force HCT116 cells to compensate for the loss of certain transcriptional activation mechanisms by use of posttranscriptional mechanisms to maintain the TGF-α expression level. Alternatively, the development of the strong TGF-α autocrine loop in the HCT116 cell line (24) may be another mechanism by which this cell line compensates for altered transcriptional activity.

Several types of human tumors appear to be dependent on constitutive EGFR signaling due to a rearrangement that eliminates a DNA fragment containing exons 2–7 of the EGFR gene (17, 18, 19). The product of this in-frame deletion is an EGFR with a truncated extracellular domain (designated ΔEGFR) that does not bind EGF but can apparently assume a partially active conformation (19). The partially active conformation provides for unattenuated signaling, but at a relatively low level of autophosphorylation (19). This low level of ΔEGFR phosphorylation, estimated to be on the order of 10–15% that of EGF-mediated activation of an equivalent amount of wild-type EGFR, indicates that amplitude of EGFR activation was not a key issue in the ability of the ΔEGFR to enhance malignancy. Instead, it was the sustained generation of a threshold level of activation upon which tumorigenicity was dependent. The data presented here show that colon carcinoma cells also have a mechanism to generate a sustained EGFR signal. However, in contrast to the constitutive activation of the ΔEGFR derived from a mutation generating independence of ligand binding, the mechanism presented here depends on sustained TGF-α expression to provide constitutive EGFR activation in growth-arrested cells. Growth-arrested HCT116 cells show little or no increase of EGFR activation relative to exponential phase cells, thus implying that the timing of EGFR activation, rather than its amplitude, is critical in this newly described mechanism of aberrant growth-regulatory control. Tumorigenicity experiments in athymic nude mice indicate that the constitutive EGFR activation resulting from endogenous TGF-α expression is critical to the tumorigenicity and growth factor independence of HCT116 cells.

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