Differential Dependence of the Tumorigenicity of Chemically Transformed Rat Liver Epithelial Cells on Autocrine Production of Transforming Growth Factor α

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Abstract

The tumorigenic phenotype in rat liver epithelial cells overexpressing c-myc may depend on a transforming growth factor (TGF-α)/epidermal growth factor receptor autocrine loop (L. W. Lee et al., Cancer Res., 51: 5238–5244, 1991). In the present study, we have used constitutive sense and antisense TGF-α expression vectors to modulate TGF-α production in carcinogen-transformed clonal derivatives of a rat liver epithelial cell line, WB-F344, that variably express c-myc, endogenous TGF-α, and tumorigenicity. Transgene-mediated TGF-α protein production was elevated 2- to 9-fold in derivatives of a low c-myc-expressing transformed cell line, GN4, and 35-fold in a derivative of a high c-myc-expressing cell line, GN6. Although the GN4- and GN6-derived cell lines expressed functional EGF receptor and steady-state c-myc mRNA levels that were comparable to their respective parental cell lines, increased TGF-α expression did not increase the tumorigenicity of the derivatives relative to the parental cell lines. Similarly, in vitro growth characteristics of the GN4- and GN6-derived cell lines were not markedly altered by increased autocrine TGF-α production. Additionally, GN4, GN6, and their derivatives were, for the most part, unresponsive to exogenously applied TGF-α in vitro. In contrast, antisense TGF-α RNA expression significantly suppressed endogenous TGF-α production in a high c-myc-expressing, high TGF-α-expressing, highly tumorigenic clonal line, GP9; this suppression resulted in lowered steady-state c-myc levels and attenuated in vitro growth. Antisense-mediated suppression of all of these in vitro phenotypes in GP9 was reversed by exogenous TGF-α. The latency of tumor formation by the antisense derivative of cell line GP9 was significantly lengthened (3-fold) relative to the time required for tumor formation by its parental cell line. These results demonstrate that a TGF-α/epidermal growth factor receptor autocrine loop may be necessary for exaggerated in vitro and in vivo growth of some transformed rat liver epithelial cells (e.g., GP9); however, the autocrine loop is not generally sufficient to support tumorigenicity, even in transformed clonal lines expressing elevated levels of c-myc.

Introduction

The multifactorial, multistage nature of carcinogenesis has long been recognized (1–3); however, with a very few exceptions (4), the molecular mechanisms that establish the phenotypic characteristics associated with autonomously proliferating cells are poorly understood. Interactions among endogenous regulators of cell growth and differentiation that are genetically determined and exogenous factors, such as peptide and non-peptide hormones, nutrients, tissue matrix, pericellular microenvironment, and exposure to tumor viruses or xenobiotics, ultimately determine the tumorigenicity of a cell. The diversity of regulatory pathways that control cell development and function complicates the study of cancer cell biology; a daunting array of genes may directly or indirectly contribute to oncogenesis (5–8). Determining the specific mechanism(s) that give rise to a particular cancer type thus requires identification of the necessary and sufficient oncogenic phenotypes and their underlying genotypes.

Using clonal analysis of multiple carcinogen-transformed, variably tumorigenic derivatives of a rat liver epithelial cell line, WB-F344 (9), we have demonstrated that many phenotypes that are thought to be causally linked to hepatocarcinogenesis in vivo experimental models do not cosegregate with tumorigenicity (10, 11). Overexpression of mRNA from c-myc and, to a lesser extent, c-ras genes correlates modestly with tumorigenicity among the clonal lines of transformed WB cells (12). Among clones that overexpress c-myc at levels ≥5-fold the level expressed by the nontumorigenic, untransformed parental WB cell line, tumorigenicity cosegregates strongly with de novo expression of transforming growth factor TGF-α (12). TGF-α is thought to play a key role in normal liver growth, in regeneration of the liver in response to loss of parenchyma by necrosis or resection, and in hepatocarcinogenesis (13–17). Untransformed WB cells do not produce detectable TGF-α, while the majority of the carcinogen-transformed WB cell lines express TGF-α at varying levels (12, 18). The effects of TGF-α are mediated via the EGF receptor (19, 20), which is abundantly expressed on the
surface of WB-F344 cells (11, 21, 22) and is expressed at varying levels in most of the clonal lines derived from WB cells (11, 12). Thus, the neoplastic phenotype may be driven by a TGF-α/EGF receptor autocrine loop in tumorigenic WB cell clones that express elevated steady-state levels of c-myc and have acquired de novo expression of TGF-α (12).

The objective of the present study was to determine if TGF-α expression is essential for the tumorigenicity of the putatively autocrine-stimulated WB cell clones GN4, GN6, and GP9 (11). Expression vectors producing sense or antisense rat TGF-α transcripts were used to augment (GN4 and GN6) or attenuate (GP9) TGF-α expression. Our results demonstrate that, although TGF-α expression sustains tumorigenicity in at least one of the clones studied (GP9), TGF-α expression is not universally responsible for tumorigenicity in the transformed WB cell lines examined, even in a background of high c-myc expression.

Results

Generation of Stable TGF-α Sense/Antisense Transformants and Expression of Transgene and Endogenous TGF-α mRNAs. Cotransfection of either the TGF-α expression vector (pTGFα17-PGK; Fig. 1) or the antisense expression vector (pTGFα18-PGK; Fig. 1) together with the neomycin resistance selection plasmid, pSV2neo, yielded typically ∼1 × 10⁶ total neomycin (G-418)-resistant colonies/µg of pSV2neo plasmid for each of the parental cell lines used in the present study (WB, GN4, GN6, and GP9). Neomycin-resistant colonies from each transfection were cloned and analyzed by Southern blotting of genomic DNA to identify authentic TGF-α sense and antisense transgene-containing clones for further characterization (Fig. 2). Only a single GP9 clone co-incorporating the antisense expression plasmid and pSV2neo was obtained from >20 G-418-resistant colonies screened, suggesting strong selective pressure against incorporation of the antisense gene. The frequency of coinorporation of the sense TGF-α expression plasmid and pSV2neo in WB, GN4, and GN6 cells was >50% of colonies screened (data not shown). Table 1 lists the origin and designation of the various derivative clones used throughout the remainder of these studies. The data presented in Table 1 describing the parental cell lines are summarized according to Lee et al. (12).

All of the clones containing the TGF-α expression vector pTGFα17-PGK produced transgene mRNA of the expected size, 0.7 kb (Fig. 3). The levels of expression of mRNA corresponded qualitatively with the transgene copy number determined by Southern analysis; the clone derivatives possessing the greatest number of copies of the expression plasmids GN4T.1 and GN6T.2 expressed the highest level of mRNA. Additional probe-reactive transcripts of various sizes were also observed, but these were not further characterized. The transgene RNA transcript expected from the antisense TGF-α construct pTGFα18-PGK was not detectable by Northern analysis of poly(A⁺) mRNA prepared from either WB, GN4, or GP9 clones. The inability to detect anti-sense transcripts in poly(A⁺) and total RNA samples by Northern analysis is not an uncommon observation (23–25). A more sensitive RNase protection assay used to probe total RNA for the presence of the antisense transcript was also uninformative (data not shown).

The endogenous TGF-α mRNA of 4.5 kb was consistently detected in GP9, GP9Tas.7, and GN4T.6 but not in the other cell lines (Fig. 3). The lack of consistent detection of the endogenous TGF-α transcript in parental cell lines GN4 and GN6, which have been shown previously to express TGF-α mRNA (12) and produced TGF-α protein in the present studies (see below), is most likely due to experimental variability in probe-specific activity and/or hybridization efficiency. The steady-state levels of the endogenous 4.5-kb TGF-α transcript in the antisense clone GP9Tas.7 was, on average, 2.3-fold lower than in GP9 (n = 3). Among parental cell line/derivative groups, α-actin expression was consistent, indicating that within a parental cell line/derivative group, comparable amounts of poly(A⁺) mRNA were analyzed (Fig. 3).

TGF-α Protein Production in Clones WB, GN4, GN6, and GP9 and Derivative Cell Lines. Cell lysates and conditioned media prepared from confluent cell cultures were analyzed for TGF-α protein using a RIA that detects the fully processed 50-amino acid rat TGF-α polypeptide and incompletely processed pro-forms of rat TGF-α but does not cross-react with rodent or human EGF or other related proteins (15). Cell lysates from most of the cell lines con-
Table 1 Phenotypic characteristics of the clonal cell lines into which the TGF-α sense and antisense expression constructs were transfected and nomenclature of the derivative cell lines was generated

<table>
<thead>
<tr>
<th>Parental cell lines</th>
<th>Tumorogenicity (%)</th>
<th>c-myc</th>
<th>TGF-α</th>
<th>EGFR protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>WB</td>
<td>0.0</td>
<td>1.00</td>
<td>1.00</td>
<td>1.00</td>
</tr>
<tr>
<td>GN4</td>
<td>5.3</td>
<td>3.05 ± 0.62</td>
<td>0.9 ± 0.4</td>
<td>1.44 ± 0.08</td>
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<tr>
<td>GN6</td>
<td>35.6</td>
<td>9.46 ± 3.71</td>
<td>8.2 ± 3.0</td>
<td>1.25 ± 0.11</td>
</tr>
<tr>
<td>GP9</td>
<td>65.7</td>
<td>18.06 ± 8.63</td>
<td>19.3 ± 6.7</td>
<td>0.69 ± 0.12</td>
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</tbody>
</table>

<table>
<thead>
<tr>
<th>Derivative cell lines</th>
<th>Expression construct</th>
<th>Copy no.</th>
</tr>
</thead>
<tbody>
<tr>
<td>WBT.3</td>
<td>Sense</td>
<td>2</td>
</tr>
<tr>
<td>WBTas.5</td>
<td>Antisense</td>
<td>1</td>
</tr>
<tr>
<td>GN4T.1</td>
<td>Sense</td>
<td>≥4</td>
</tr>
<tr>
<td>GN4T.6</td>
<td>Sense</td>
<td>≥1</td>
</tr>
<tr>
<td>GN6T.2</td>
<td>Sense</td>
<td>≥6</td>
</tr>
<tr>
<td>GP9Tas.7</td>
<td>Antisense</td>
<td>1</td>
</tr>
</tbody>
</table>

*a All of the data presented in this table for the parental clonal cell lines are from Ref. 12.
*b Percentage of treated animals that developed tumors.
*c Levels of expression of mRNA, relative to expression of the mRNA in WB cells.
*d EGFR protein expression relative to expression in WB cells.
*e The sense and antisense expression constructs are pTGFα17-PGK and pTGFα18-PGK, respectively (described in "Materials and Methods," and Fig. 1).
*f The estimated number of copies of the transgene integrated into the genome of each clonal line.

Fig. 3. Northern analysis of PGK-1-driven TGF-α transgene mRNA expression (0.7 kb, arrow), endogenous TGF-α mRNA expression (4.5 kb, *), and α-actin expression in chemically transformed rat liver epithelial cells and their transfected derivatives. Poly(A*) mRNA (15 μg) was separated on formaldehyde/agarose gels, transferred to nylon membranes, and probed sequentially with 32P-labeled TGF-α and α-actin probes, as described in "Materials and Methods." The blot pictured (representative of four separate blots) was stripped between probed with boiling water/0.01% SDS.

Table 2 Quantitation by RIA of the synthesis of TGF-α protein by WB, GN4, GN6, GP9, and derivative cell lines

<table>
<thead>
<tr>
<th>Cell line</th>
<th>TGF-α protein</th>
</tr>
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<tbody>
<tr>
<td>WB</td>
<td>41, 46</td>
</tr>
<tr>
<td>WBT.3</td>
<td>ND</td>
</tr>
<tr>
<td>WBTas.5</td>
<td>49, ND</td>
</tr>
<tr>
<td>GN4</td>
<td>77, ND</td>
</tr>
<tr>
<td>GN4T.1</td>
<td>609, 791</td>
</tr>
<tr>
<td>GN4T.6</td>
<td>138, 166</td>
</tr>
<tr>
<td>GN6</td>
<td>25, 37</td>
</tr>
<tr>
<td>GN6T.2</td>
<td>1484, 840</td>
</tr>
<tr>
<td>GP9</td>
<td>180, 190</td>
</tr>
<tr>
<td>GP9Tas.7</td>
<td>91, ND</td>
</tr>
</tbody>
</table>

*a Values represent picograms of TGF-α/ng total lysate protein; replicate determinations for each clone. ND, below the limit of detection of the RIA, which was 20 to 50 pg TGF-α/ml of sample. TGF-α levels in cell lysate from WBT.3 were below the limit of detection in all assays.

The levels of expression of TGF-α protein among the clonal derivatives varied widely, reflecting qualitatively both transgene mRNA expression and transgene dosage. Clones GN4T.1 and GN4T.6 expressed 9- and 2-fold more TGF-α polypeptide, respectively, than did clone GN4. GN6T.2, the clonal derivative expressing the highest levels of transgene-generated mRNA, produced about 35-fold more TGF-α than did its parental clone, GN6. The highest expressers of the TGF-α transgene mRNA, GN4T.1 and GN6T.2, produced significantly more TGF-α protein than did clonal line GP9, the most tumorigenic parental cell line and highest producer of endogenous TGF-α protein (Table 2). Notably, the antisense clone GP9Tas.7 produced <40% as much TGF-α protein as its parent cell line, GP9, reflecting the lower steady-state level of expression of the endogenous 4.5-kb TGF-α mRNA in GP9Tas.7 relative to GP9 (Fig. 3).

In initial experiments, soluble forms of TGF-α were not detected in conditioned media from any of the clones (data not shown), suggesting minimal proteolytic cleavage of the pro-TGF-α protein to soluble forms or rapid reuptake of TGF-α protein by the cells. The lack of detectable TGF-α protein in medium conditioned by clonal lines GN4 and GN6 agrees with previous studies conducted with these two clonal lines (18) and is a common characteristic of many tumor cells and cell lines (26–28). In order to determine if the lack of detectable TGF-α in the culture medium was due to rapid reuptake via the EGF receptor, we conditioned media from the clones expressing the highest levels of TGF-α pro-peptide, GN4T.1, GN4T.6, and GP9, in the absence and presence of an excess (100 ng/ml) of mouse EGF competitively to inhibit reuptake of TGF-α and concentrated the conditioned media using a C18 Sep-pak prior to analysis by RIA. Soluble TGF-α was detected at very low levels (3 to 5 pg/ml) in concentrated medium from three clones expressing the highest levels of pro-peptide. Inclusion of EGF in the culture medium markedly elevated the levels of TGF-α detected; GN4T.1, GN6T.2 and GP9 secreted 128, 293, and 63 pg TGF-α/ml of media, respectively. The relative levels of secretion of soluble forms of TGF-α by the three clones reflected pro-peptide expression (GN6T.2>GN4T.1>GP9). It thus appears that the cell lines examined process the TGF-α pro-peptide to a limited extent and that the soluble forms generated are internalized via the EGF receptor.
Despite expressing the TGF-α transgene mRNA, clone WBT.3 did not produce detectable levels of TGF-α protein; clones WB and WBTas.5 were also negative for TGF-α protein expression. Clonal lines WB, WBT.3, and WBTas.5 were further characterized, but there were no significant differences among these three clones for any of the phenotypes examined. Additional (negative) data on these clones are not presented.

Expression of c-myc mRNA in Clones GN4,GN6, and GP9 and Their Derivative Cell Lines. The relative steady-state levels of c-myc mRNA in the parental cell lines GN4, GN6, and GP9 (Fig. 4A) were consistent with our previously published results: GP9 > GN6 > GN4 (12). Clone GN4T.1 consistently expressed higher levels of c-myc than did its parental cell line, GN4, suggesting that, in this high TGF-α-expressing clonal derivative, c-myc transcription may be regulated in part by TGF-α activation of the EGFR receptor. The steady-state level of c-myc mRNA in GN6T.2 was comparable to the level of c-myc expressed in its parental cell line GN6, despite a 35-fold elevation of TGF-α production, suggesting that c-myc regulation is dissociated from TGF-α activation of the EGFR receptor in this derivative cell line (Fig. 4A).

The steady-state level of c-myc mRNA was lower in poly(A*) mRNA from GP9Tas.7 cells by 5- to 8-fold relative to the levels of c-myc transcripts in poly(A*) mRNA from clonal line GP9 (Fig. 4A). c-myc expression in untreated GP9Tas.7 cells was below the limit of detection in total RNA samples (n = 5), whereas untreated GP9 cells express quantifiable levels of c-myc mRNA (Fig. 4B). Continuous treatment of cultures of GP9Tas.7 cells with TGF-α increased steady-state c-myc mRNA to levels equivalent to those in untreated GP9 cells (ratio of c-myc levels in treated GP9Tas.7 cells versus untreated GP9 = 1.01 ± 0.17; n = 6). TGF-α also doubled the steady-state c-myc levels in GP9 cells (ratio of treated to untreated GP9 cells = 2.12 ± 0.22; n = 6). Thus, the parental cell line GP9, its antisense derivative cell line GP9Tas.7, and possibly clone GN4, appear to alter c-myc expression in response to TGF-α, while the elevated steady-state expression of c-myc in GN6 and its derivative appears to be unaffected by TGF-α.

Characterization of Expression and Function of the EGF Receptor in Clones GN4, GN6, and GP9 and Their Derivative Cell Lines. All of the clones examined in the present studies expressed immunoprecipitable EGFR receptor (Fig. 5). GP9 displayed lower steady-state levels of expression of EGF receptor than did GN4 and GN6 cells, as we have demonstrated previously (12). The antisense derivative of GP9, GP9Tas.7, expressed significantly higher levels of immunoprecipitable EGF receptor protein than did GP9 (greater than 3.9 ± 1.30-fold the steady state level of EGF receptor protein expressed in GP9, n = 3). The reciprocal relationship of TGF-α to EGFR protein expression in GP9/GP9Tas.7 confirms previous results (18) which suggest that elevated expression of TGF-α down-regulates EGFR receptor in GP9, as has been seen in other systems in vitro (17, 29-31) and in transgenic mice in vivo (32). In contrast, elevated expression of TGF-α did not significantly alter the level of EGF receptor protein in GN4- or GN6-derived cell lines relative to expression of EGF receptor in their corresponding parental cell lines. The mechanistic basis for the difference in response of GP9/GP9Tas.7 versus the other transfectants that express elevated TGF-α is not clear but may be due to differences in intracellular trafficking or recycling of the TGF-α-bound EGFR receptor (33).
Steady-state background phosphorylation of the EGF receptor in the absence of added EGF was not detected in any of the parental or derivative clones, including GP9, GN4T.1, and GN6T.2, the highest producers of TGF-α protein (Fig. 6). Although increased steady-state EGF receptor autophosphorylation has been observed in NIH3T3 cells vastly co-overexpressing TGF-α and EGF receptor (>10⁶ EGF receptors/cell; Ref. 34), increased background EGF receptor autophosphorylation is not commonly detected in TGF-α-expressing cells possessing more physiological levels of EGF receptor. The EGF receptor synthesized by all of the clonal lines was functional, as autophosphorylation of the EGF receptor on tyrosine residues was stimulated in response to added exogenous EGF (Fig. 6). In addition to autophosphorylated EGF receptor, we detected a substrate of M, 100,000 that was phosphorylated on tyrosine in response to EGF only in GN6, GP9, and their derivatives (Fig. 6). As with the EGF receptor, no phosphorylation of the M, 100,000 substrate was detected in the absence of added exogenous EGF. The identity of the M, 100,000 substrate is not known, and it was not further characterized.

In Vitro Growth Characteristics. Enhanced expression of TGF-α was associated with slight increases in both the saturation density and doubling time of clones GN4T.1 and GN4T.6 relative to the parental clone GN4 (Table 3). Although saturation densities and doubling times were concordant with the relative expression of TGF-α among GN4 and its derivative clones (GN4T.1, GN4T.6, GN4), the differences in the in vitro growth parameters were not statistically significant (ANOVA, P > 0.05; Table 3). Clone GN6T.2 grew to a significantly lower saturation density than did GN6 (P < 0.05), while maintaining a comparable doubling time (Table 3). The antisense derivative of GP9, GP9Tas.7, grew to only 30% the saturation density of its parental cell line (P < 0.05; Table 3), although the doubling times of the two clones were similar. Addition of exogenous, recombinant human TGF-α to the culture medium (8.0 nM) did not alter the saturation densities of parental cell lines GN4 and GN6, while the growth of the highest expressing derivative clones, GN4T.1 and GN6T.2, was significantly suppressed (Fig. 7). The decrease in saturation density of GN6T.2 in response to TGF-α was accompanied by marked morphological changes that were absent in GN6 cells treated with exogenous TGF-α; greatly increased cytoplasmic/nuclear ratios, cell flattening, and appearance of binucleated cells (data not shown). In contrast to the lack of effect or suppressive effects of exogenous TGF-α on GN4, GN6, and their derivatives, exogenous TGF-α significantly elevated the saturation density of GP9 cells and promoted the growth of GP9Tas.7 cells to a saturation density comparable to that of GP9 (Fig. 7).

The efficiencies of colony formation in semisolid agar of all of the cell lines examined in the present study, including the tumorigenic GP9 line, were low (<0.1%) and the colonies were generally small (<25 cells). Enhanced autocrine expression of TGF-α in derivatives of GN4 and GN6 suppressed somewhat the formation of colonies in semisolid agar relative to colony formation by the parental lines (Table 3). The ability of GP9Tas.7 cells to form soft agar colonies was also attenuated relative to its parental cell line, GP9 (Table 3). Incorporation of recombinant human TGF-α into the soft agar assays had little effect on the growth of clonal lines GN4T.1, GN6, and GN6T.2 but markedly enhanced the colony-forming efficiency of GP9, GP9Tas.7 (61- and 57-fold increases, respectively) and, somewhat surprisingly, parental line GN4 (25-fold increase; Table 3). Given the marked difference in tumorigenicity between clonal lines GN4 (0-5%) and GP9 (>80%; see below), the phenotype of TGF-α-stimulated soft agar growth is clearly dissociated from tumorigenicity in our system.

Inhibition of Growth by Anti-Rat TGF-α Antiserum. The effects of normal sheep serum (nonimmune; NSS) and AbaTGF, a rat-specific TGF-α neutralizing antiserum9 (15), on the growth of GP9 and GP9Tas.7 in vitro are illustrated in Fig. 8. Continuous culture in the presence of 2 nm synthetic rat TGF-α significantly elevated the saturation density of GP9Tas.7 cells in the presence of control NSS; substitution of AbaTGF for NSS completely neutralized the effects of the exogenous TGF-α. Notably, in the absence of added rat TGF-α, AbaTGF reduced significantly the saturation density of GP9Tas.7 cells by about 16% relative to GP9Tas.7 cells cultured in the presence of NSS alone (P < 0.05). None of the treatments (2 nm rat TGFr, NSS, AbaTGF, or combinations thereof) altered the saturation density of clone GP9 (Fig. 8), suggesting that there is a threshold of TGF-α expression which supports some of the in vitro growth phenotypes.

Tumorigenicity. The parental GN4 clone was not tumorigenic in the present study, whereas in previous studies, it was weakly tumorigenic, generating tumors in 5% of injected animals (12). GP9 cells and GN6 cells generated tumors with incidences and latencies comparable to our previous observations (Table 4; Ref. 12); stable incorporation of the neomycin-resistance plasmid alone did not alter the tumorigenicity of the parental cell lines (data not shown). Unexpectedly, clone GN6T.2 did not produce tumors, and only one of the TGF-α-expressing GN4 derivative cell lines, GN4T.1, was tumorigenic at a low frequency (5%; Table 4). The cumulative tumor incidence for both GN4 derivative clonal lines was about 3%. The GP9Tas.7 antisense derivative cell line was somewhat less tumorigenic than was its parent, 70% tumor incidence for

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1 W. Russell, unpublished observations.
Table 3  In vitro growth characteristics of GN4, GN6, GP9, and their clonal derivatives incorporating the TGF-α sense and antisense expression constructs

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Saturation densitya</th>
<th>Doubling timeb</th>
<th>Soft agar colony formation</th>
<th>Fold inductiond</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>−TGF-α</td>
<td>+TGF-α</td>
</tr>
<tr>
<td>GN4</td>
<td>264757 ± 23525 (9)</td>
<td>13.9</td>
<td>11 ± 1 (12)</td>
<td>271 ± 7 (3)</td>
</tr>
<tr>
<td>GN4.T.1</td>
<td>340776 ± 24756 (9)</td>
<td>16.4</td>
<td>5 ± 1 (12)</td>
<td>13 ± 1 (3)</td>
</tr>
<tr>
<td>GN4.T.6</td>
<td>328781 ± 41508 (9)</td>
<td>16.0</td>
<td>4 ± 1 (10)</td>
<td>ND</td>
</tr>
<tr>
<td>GN6</td>
<td>207990 ± 11800 (21)</td>
<td>16.3</td>
<td>7 ± 1 (12)</td>
<td>28 ± 4 (3)</td>
</tr>
<tr>
<td>GN6.T.2</td>
<td>151824 ± 8601 (18)</td>
<td>17.4</td>
<td>0.2 ± 0.1 (12)</td>
<td>2 ± 0.3 (3)</td>
</tr>
<tr>
<td>GP9</td>
<td>323962 ± 17244 (9)</td>
<td>18.1</td>
<td>14 ± 2 (14)</td>
<td>853 ± 39 (5)</td>
</tr>
<tr>
<td>GP9Tas.7</td>
<td>103029 ± 9965 (12)</td>
<td>17.4</td>
<td>0.3 ± 0.2 (15)</td>
<td>17 ± 1 (5)</td>
</tr>
</tbody>
</table>

a Cells per cm²; cells were grown in IMEMZO medium ("Materials and Methods").
b Time for one population doubling, in hours.
c Human recombinant TGF-α ("Materials and Methods"). 8 mM final concentration in both the soft agar and the IMEMZO medium. Assays without TGF-α received standard IMEMZO medium.
d Ratio of the mean number of colonies formed in the presence of added TGF-α to the mean number of colonies in assays with no added TGF-α.

* Values are mean ± SEM of (n) culture dishes.

† Significant differences in variances exist among clones in this group (i.e., parental cell line and corresponding derivatives) by ANOVA, P < 0.05.

‡ Significantly different from the corresponding untreated soft agar growth assay by Student’s t test, P < 0.05.

ND, not determined.

GP9Tas.7 versus 80% for GP9, but the latency of tumor formation for GP9Tas.7 cells was significantly lengthened (more than 3-fold) over that of GP9 (P < 0.05; Table 4).

Discussion

Through the use of clonal analysis, we have demonstrated previously that de novo expression of TGF-α coupled with an elevated background expression of c-myc cosegregates with tumorigenicity among clonal lines of chemically transformed WB cells (12). The goal of the present study was to determine if the correlative relationships between elevated c-myc expression, de novo expression of TGF-α, and tumorigenicity in our model transformation system reflected truly causative interactions. In order to test for causation, we generated specific phenotypes of TGF-α expression in a background of either low or elevated c-myc expression and examined the relationship of the composite phenotypes to both tumorigenicity and in vitro transformation-associated phenotypes. Using sense and antisense TGF-α expression vectors, we successfully created the gene expression profiles we wished to examine: (a) a low c-myc-expressing, high TGF-α-expressing transfectant cell line, GN4T.1; (b) a high c-myc-expressing, high TGF-α-expressing transfectant cell line, GN6T.2; and (c) a transfectant cell line, GP9Tas.7, with attenuated TGF-α expression against a background of high c-myc expression, which was generated from a high c-myc-expressing, high TGF-α-expressing, highly tumorigenic clonal cell line. All of the cell lines examined in the present studies maintained functional EGF receptor; thus, all of the clones studied appear to have functional ligand and receptor components of signal transduction necessary for autocrine stimulation by TGF-α.

Surprisingly, the GN4 and GN6 derivative cell lines expressing elevated TGF-α were no more tumorigenic than...
Table 4  Tumorigenicity and latency of tumor formation of clones GN4, GN6, GP9, and derivative cell lines injected into syngeneic (F-344) rats

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Tumorigenicitya</th>
<th>Latencyb</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Frequency</td>
<td>Percentage</td>
</tr>
<tr>
<td>GN4</td>
<td>0/19</td>
<td>0</td>
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<tr>
<td>GN4T.1</td>
<td>1/21</td>
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<td>GN4T.6</td>
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<tr>
<td>GN6T.2</td>
<td>0/15</td>
<td>0</td>
</tr>
<tr>
<td>GP9</td>
<td>4/5c</td>
<td>80</td>
</tr>
<tr>
<td>GP9Tas.7</td>
<td>19/27</td>
<td>70</td>
</tr>
</tbody>
</table>

a Tumorigenicity is expressed as the number of treated animals developing tumors (frequency) and as the percentage of treated animals developing tumors.

b Time (weeks) elapsed until tumors of 1-cm size developed. The values in parentheses indicate the range of latencies of tumor formation for a given clonal line.

c All of the animals that received injections of GP9 cells were sacrificed at 13.5 weeks due to the rapid growth of the s.c. tumors. The single animal that was tumor free at 13.5 weeks had received an i.p. injection of GP9 cells; it is possible that tumors might eventually have developed in this animal as well.

were their parental clonal cell lines, even in the face of up to a 35-fold increased expression of TGF-α polypeptide. If the level of c-myc expression does indeed constrain the fully neoplastic phenotype, which is predicted by our previous clonal analysis (12), the lack of tumorigenicity of the relatively low c-myc-expressing GN4 derivative cell lines expressing elevated TGF-α is not unexpected. Indeed, overexpression of TGF-α in a nontransformed rat liver epithelial cell line, RL6-413, does not induce tumorigenicity (35). However, that GN6T.2 cells were not tumorigenic was remarkable, considering that the steady-state level of c-myc expression in this clone was comparable to the level expressed in the highly tumorigenic clonal line, GP9. Based on these results, we conclude that coincident, elevated expression of c-myc and TGF-α in rat liver epithelial cells expressing functional EGFR receptor is not sufficient for unregulated autocrine growth stimulation and tumorigenicity. Similar conclusions have been arrived at by several groups through the study of transgenic mice that overexpress TGF-α alone, c-myc alone, or both genes concurrently. Mice transgenic for either TGF-α (36, 37) or c-myc (38, 39) develop hepatic tumors with low multiplicity (average number of tumors/liver) after long latencies. Transgenic mice overexpressing both TGF-α and c-myc (38, 40), and TGF-α transgenic mice treated with chemical carcinogens or tumor promoters (41) develop hepatic tumors with increased multiplicity much more rapidly than do transgenic mice bearing one transgene. Despite the increased tumor multiplicity and decreased latency in double transgenic mice and chemically treated TGF-α transgenic mice, the number of liver tumors that form is still extremely low relative to the number of cells in the liver that are putatively predisposed to tumor formation by expression of the transgene(s) (theoretically, all of the parenchymal cells in the liver). Analysis of breast tumor development in c-myc transgenic mice, as well as in c-myc/mutant c-ras double transgenic mice, yields similar results (reviewed in Ref. 4). These findings indicate that additional genetic changes must accompany transgene expression in order for a cell to develop the fully neoplastic phenotype in these transgenic systems. Furthermore, these observations suggest that TGF-α functions as a tumor promoter/progression agent and not a tumor initiator (36–38, 40, 41). The acquisition of secondary genetic changes, such as somatic mutations, that combine to determine the fully neoplastic phenotype is presumed to be a stochastic process, the frequency of which is increased with increasing population doublings induced by TGF-α under permissive conditions (42–44). Our current results using transformed rat liver epithelial cells support these conclusions.

Our results with clonal lines GP9 and GP9Tas.7 show that, although not sufficient, a TGF-α/EGFR autocrine loop may in some cases be necessary to support tumorigenicity in vivo, in addition to modulating transformation-associated growth characteristics in vitro. The phenotype of the antisense clone GP9Tas.7 is consistent with functional quenching of endogenous TGF-α production by antisense RNA-mediated interference with translation and/or stimulation of degradation of the endogenous TGF-α mRNA (45). Endogenous 4.5-kb TGF-α mRNA levels and TGF-α polypeptide expression are both reduced by greater than 60% in GP9Tas.7 as compared with its parental cell line, GP9. The attenuated in vitro growth characteristics, reduced steady-state c-myc mRNA levels, and increased steady-state EGFR protein levels in GP9Tas.7 relative to GP9 are all consistent with the reduction in TGF-α expression observed in GP9Tas.7. The ability of TGF-α neutralizing antibody AbTGF to further decrease the saturation density of GP9Tas.7 in monolayer culture provides additional evidence that autocrine production of TGF-α supports the growth of GP9/GP9Tas.7. In agreement with the changes in in vitro phenotype, the tumorigenicity of clonal line GP9Tas.7 is attenuated as compared with the tumorigenicity of GP9 cells, and tumor latency is lengthened more than 3-fold. A recent report by Laird et al. (46) details analogous findings using antisense TGF-α minigene expression constructs and a transformed rat liver epithelial cell line, LE25sp1. Antisense-mediated reduction of TGF-α expression to about 30% of control values (comparable to our results with GP9Tas.7) increased tumor latency for LE25sp1 derivative cell lines by 2–3-fold (46). Our finding that both GP9Tas.7 and GP9 respond to exposure to exogenous TGF-α by increasing steady-state c-myc mRNA levels, saturation density, and colony-forming efficiency in soft agar suggests that TGF-α directly controls these phenotypes in both cell lines.

Two potential mechanisms can account for the differential responsiveness of the cell lines we have described to the mitogenic effects of TGF-α: (a) clones GN4, GN6, and their respective derivative cell lines may have independently lost signal transduction components that are required for mitogenic responsiveness to TGF-α, while GP9 and GP9Tas.7 have retained an ability to proliferate in response to autocrine or paracrine TGF-α production; or (b) GN4T.1 and GN6T.2 (and, by extension, their parental clones) have maintained the integrity of a growth regulatory mechanism that normally restricts proliferation in response to TGF-α to highly defined circumstances, e.g., postnatal liver growth or regeneration of liver mass after resection or chemical-induced necrosis, but this regulatory mechanism has been disabled in GP9 and GP9Tas.7. The latter explanation, i.e., TGF-α-dependent proliferation is determined by maintenance or loss of a normal, growth-suppressing regulatory pathway, is supported by several observations. EGFR and TGF-α do not stimulate cellular proliferation in quiescent rat liver (14, 16, 47), and TGF-α enhances DNA replication...
in transgenic mice only during the normal period of postnatal growth of the liver (37). Furthermore, liver hyperplasia induced by transgene expression in TGF-α transgenic mice ceases by the end of postnatal liver development and remains stable thereafter (48). After postnatal liver development is completed, individual hepatocytes in TGF-α transgenic liver can express extremely high levels of TGF-α with no apparent deleterious effects or morphological anomalies (36). Thus, we hypothesize that the tight control over mitogenesis in response to TGF-α that exists in normal liver cells remains functional in many transgenic liver cells and in some of our carcinogen-transformed rat liver epithelial cell clones (e.g., GN4, GN6, and their derivatives). Abrogation of this control mechanism permits TGF-α-stimulated clonal expansion and tumor promotion/progression.

Although the exact nature of the putative growth-regulatory mechanism is not known, some recent studies suggest a complex relationship between ligand/receptor interactions, cellular microenvironment, and growth control. Shum et al. (49) have demonstrated that the TGF-α propeptide associates with two proteins, one M, 86,000 and the other M, 106,000; the M, 86,000 protein requires the C-terminal 31 amino acids of the TGF-α propeptide for binding (49). The TGF-α/protein complex possesses kinase activities towards tyrosine, threonine, and serine, suggesting that juxta-Grk interactions of TGF-α propeptide with EGFR on adjacent cells (50, 51) or interactions of the propeptide with the truncated, soluble extracellular domain of EGFR (52) could result in “reverse” or bidirectional signaling (49). Thus, soluble TGF-α species and the propeptide could mediate differential signaling. Differential responsiveness of rat liver epithelial cells to soluble versus membrane-associated TGF-α has been described recently (35), and the growth inhibition and morphological changes we observed in monolayers of GN6T.2 cells treated with soluble, exogenous TGF-α illustrate differential effects of the two forms of TGF-α.

We have found recently that the tumorigenicity of a tumor cell line, GN6TF, which was isolated from a primary tumor produced by GN6 cells injected s.c. into Fischer 344 rats, is susceptible to site-specific suppression in vivo (53). Unlike GN6, cell line GN6TF is highly tumorigenic at s.c. transplantation sites (100% incidence, with a latency of about 28 days). When injected directly into the livers of young syngeneic Fischer 344 rats, the tumorigenicity of GN6TF cells is completely suppressed, and the cells incorporate into the hepatic plates and differentiate into functional hepatocytes (53). With the exception of its extreme s.c. tumorigenicity, GN6TF is in many ways similar to both its parent, GN6, and to the high TGF-α-producing derivative of GN6 generated in the present studies, GN6T.2. Like GN6, GN6TF expresses EGFR and does not secrete appreciable amounts of TGF-α (18). The growth of GN6TF in semi-solid agar is unaffected by EGF, which is similar to the phenotype of GN6T.2. Proliferation of GN6TF cells in monolayer culture (measured as [3H]thymidine incorporation into DNA) is actually suppressed by EGF (18); GN6T.2 exhibited a similar TGF-α-induced attenuation of proliferation in monolayer culture, relative to GN6, and this effect was enhanced by application of exogenous TGF-α. Considering the site-specific suppressibility of the tumorigenicity of GN6TF in the context of the present studies, it thus appears that clonal line GN6 possesses several primary controls over proliferation that have been differentially retained in the derivative cell lines GN6TF and GN6T.2. One control mechanism present in both GN6TF and GN6T.2 cells keeps TGF-α-induced cell proliferation in check. A second mechanism, present in cell line GN6T.2 but not in GN6TF, restricts tumor growth at s.c. injection sites (perhaps as a consequence of overexpression of TGF-α propeptide). A third control present in GN6TF (and probably GN6T.2) maintains the ability to respond to extracellular signals within the liver that are not present at s.c. transplantation sites such that cell cycle progression is kept under control.

Collectively, the results of the present study clearly demonstrate that c-myc expression, several in vitro phenotypes, and the tumorigenicity of some neoplastically transformed liver epithelial cells may be controlled to a significant extent by autocrine production of TGF-α. However, elevated c-myc and TGF-α expression per se is insufficient to confer the phenotype of tumorigenicity. Additional lesions in growth regulatory pathways, such as loss of mechanism(s) that restrict mitogenesis in response to TGF-α to particular developmental or physiological contexts, must occur to permit cells to proliferate uncontrollably in response to TGF-α.

Materials and Methods

Plasmid Construction. The expression plasmids used in the present study use a 504-bp constitutively active promoter sequence from the mouse PGK-1 gene (originally described and distributed by Dr. Michael McBurney, University of Ottawa, Ottawa, Canada); kindly provided by Dr. Oliver Smithies (University of North Carolina, Chapel Hill, NC) as a subclone from the plasmid pKJ-1 (54) into the EcoRI/PstI sites of pBluescript II KS(−) (Stratagene, La Jolla, CA). A 715-base pair XmaI fragment encompassing the entire rat pro-TGF-α coding sequence (55) was excised from the plasmid p3B1b (Ref. 56; kindly provided by Dr. David Lee, University of North Carolina, Chapel Hill, NC) and inserted in both sense (pTGFa17) and antisense (pTGFa18) orientations into an XmaI site upstream of the polyadenylation signal from the human growth hormone (hGH) gene (57) in the plasmid pEV142 (Ref. 58; generously provided by Dr. Lawrence Mathews, Salk Institute, La Jolla, CA). The TGF-α inserts plus the hGH polyadenylation signal were then copied as cassettes from pTGFa17 and pTGFa18 by PCR using primers flanking the 3′-end of the hGH polyadenylation signal and the 5′-end of the TGF-α inserts: 5′-cagagactagtGTCACACGACTATAAGAGC-3′; 3′-flanking primer, 5′-gagacgccgaccGGA CATCTAGTCGACAAAAATGTAG-3′. Lower-case letters designate linker sequences. The underlined sequence in the 5′-flanking primer is a SpeI restriction site that was not used in construction of the plasmids; the underlined sequence in the 3′-flanking primer is a NotI restriction site used to clone directionally the TGF-α/hGH gene segment. The PCR products were blunt ended with bacteriophage T4 DNA polymerase, restricted with NotI, and directionally cloned into the Sma/NotI polylinker sites downstream of the PGK-1 promoter in pBluescript II KS(−), producing the expression vectors pTGFa17-PGK (sense) and pTGFa18-PGK (antisense; Fig. 1). The PCR subcloning used to generate pTGFa17-PGK resulted in deletion of nt. 8 through nt. 86 of the cDNA; this deletion did not affect generation or processing of the pro-peptide (see “Results”), which is initiated at
nt. 145 (55). DNA was sequenced at the UNC-CH Automated DNA Sequencing Facility on a model 373A DNA Sequencer (Applied Biosystems) using the Taq Dye Deoxy Terminator sequencing kit (Applied Biosystems).

Cell Culture. WB-F344 cells and the clonal, transformed variants GN4, GN6, and GP9 have been extensively characterized (reviewed in Refs. 11 and 12). The parental WB, GN4, GN6, and GP9 cell lines and derivatives of these cell lines produced by transfection of the TGF-α sense or antisense RNA expression vectors described above were cultured routinely in Richter’s improved MEM with zinc option (Irvine Scientific, Santa Ana, CA) supplemented with 4.0 mg insulin/liter, 10% characterized fetal bovine serum (HyClone Laboratories, Logan, UT), 20 μM HEPES, 2.6 mM sodium bicarbonate, and 40 μg gentamicin sulfate/ml (hereafter referred to as IMEMZO medium), as described previously (12). Human recombinant TGF-α (Intergen Co., Purchase, NY) or rat synthetic TGF-α (Peninsula Laboratories, Belmont, CA) were included in the culture medium in some experiments, as detailed in the text and table and figure legends. Growth of the various cell lines in semisolid agar was assayed as described previously (60) using 1 x 10⁴ cells of each cell line/culture; human recombinant TGF-α (8 nM) was added to some cultures, as noted. Assays of in vitro cell growth kinetics, population doubling times, and saturation density of the various parental and derivative cell lines were initiated by seeding 2 x 10⁴ cells of each clone into 60-mm plastic tissue culture dishes (three or more dishes/clone/time point; replicate experiments). Cell numbers were determined at 24-h intervals for up to 2 weeks by electronically counting trypanized cells with a Coulter counter (Coulter Electronics). Alterations in saturation density induced by various combinations of synthetic rat TGF-α, NSS, and anti-rat TGF-α neutralizing serum (AbaTGF) were determined by seeding 2 x 10⁴ cells in 35-mm plastic tissue culture dishes and culturing the cells to confluence (10–12 days) in the presence of the various additions, as detailed in the text and legend to Fig. 8. AbaTGF is the rat-specific antibody that was developed for the RIA (Ref. 15; see below). Cell numbers were determined by Coulter counter.

Transfection and Selection of Clones. Sense and antisense TGF-α expression vectors were transfected into cells by electroporation (60). Cultures were harvested in mid- to late-log growth by brief trypsinization. Cells (1 x 10⁶) of each parental line were washed in IMEMZO medium and resuspended in 0.5 ml IMEMZO medium containing 10 μg of linearized expression plasmid, together with 0.5 μg of linearized expression plasmid pSV2neo, which confers resistance to the neomycin analogue, G-418 (61). After electroporation (300V/m, 400mF, 1.0 sec field power; pulse supply manufactured by Pds, Inc., Madison, WI), the cells were immediately replated in IMEMZO medium and allowed to recover 24 h prior to initiation of selection in G-418 (400 mg active G-418 isomer/ml; Gibco-BRL, Grand Island, NY). G-418-resistant colonies were cloned 8 to 12 days after initiation of selection; clonal populations were expanded for subsequent screening for the presence of the expression vectors and further characterization.

Nucleic Acid Extraction and Hybridization. Total cellular RNA was isolated from confluent cell cultures using the guanidinium isothiocyanate/cesium chloride method (62, 63) as described previously (12). Poly(A)+ mRNA was prepared from CsCl2-purified total RNA using oligo-DT cellulose chromatography kits (Pharmacia LKB Biotechnology, Piscataway, NJ), according to the manufacturer’s instructions. Northern blot analysis was performed as described previously (12).

High molecular weight DNA was isolated from confluent cell cultures by standard protocols (63). For Southern blot analysis, 15 μg of genomic DNA digested with EcoRI or a combination of EcoRI and SacI (detailed in the legend to Fig. 2) was fractionated on 0.8% agarose gels and transferred to Hybond-N nylon membranes (Amersham, Arlington Heights, IL) for hybridization. Prehybridization and hybridization of the nylon membranes were performed as described by Sambrook et al. (64). Both Northern and Southern blot analyses used [α-32P]dCTP-labeled probes produced by random priming (Pharmacia LKB or BRL probe labeling kits). The following plasmid inserts were used to generate probes: a 1.3-kb EcoRI/SacI insert from pTFA17-PK, encompassing both the PKG-1 promoter and TGF-α cDNA; a 1.1-kb PstI murine α-actin cDNA insert from pAM91 (65); and a 0.4-kb PstI fragment of exon 2 of the rat c-myc gene, which was subcloned from a genomic fragment contained in pDX1 (Ref. 66; provided by Dr. K. Hayashi, National Cancer Center, Tokyo, Japan). Autoradiography was performed using Kodak X-OMAT AR film as described previously (12).

Tumorigenicity Assays. Tumorigenicity assays were carried out as described previously (12) by injecting 1 x 10⁶ cells resuspended in 0.1 ml Hanks’ balanced salt solution either i.p. or into the dorsal s.c. tissue of 1-day-old Fischer 344 rats (Charles River Laboratories, Wilmington, MA). Immuno precipitation and Western Analysis of EGF Receptor Expression and Function. Cells were plated in 35-mm culture dishes (5 x 10⁵ cells/dish) and allowed to proliferate to confluence (approximately 5 days). Proteins were metabolically labeled in the confluent cell cultures with [35S] cysteine (40 μCi/dish) for 2 h at 37°C in serum-free, cysteine/cysteine-free Eagle’s MEM culture. The monolayers were washed, the cells lysed (67), and immunoprecipitation of EGF receptor using a polyclonal anti-EGF antisera (pAB 1382) was carried out as described previously (67). Western blot analysis of autophosphorylation of EGF receptor was carried out as described previously (12, 68) using PT-66, a monoclonal anti-phosphotyrosine antibody (Sigma Chemical Co., St. Louis, MO) and enhanced chemiluminescence detection (Amersham, Arlington Heights, IL).

RIA of TGF-α. Cells (1 x 10⁶) were plated onto 100-mm tissue culture dishes and grown to confluence. In initial experiments, confluent cultures were washed twice with Ca²⁺- and Mg²⁺-free Hanks’ balanced salt solution, twice with serum-free IMEMZO medium, and overlaid with 5 ml serum-free IMEMZO medium. Thirty hours later, the conditioned, serum-free medium was removed, and phenylmethylsulfonyl fluoride was added to a final concentration of 1 μg/ml. The medium was centrifuged at 10,000 x g for 30 min to remove debris and was frozen in aliquots at −80°C until assay by RIA. After removal of serum-free conditioned medium, cell monolayers were overlaid with 2 ml ice-cold lysis buffer [50 mM NaCl, 25 mM Tris-HCl (pH 8.0), 0.5% sodium deoxycholate; 0.5% NP40, 1 mg phenyl methylsulfonyl fluoride/ml, and 1 mg leupeptin/ml] per 100-mm dish. Cell lysates collected by scraping with a Teflon spatula were centrifuged to remove debris (10,000 x g for 30 min), and aliquots of the lysates were stored at −80°C until they were assayed by RIA for cell-associated TGF-α. The RIA was performed as described by Russell.
et al. (15) using a rat TGF-α-specific antibody that does not cross-react with murine or human TGF-α, EGFR, or other related peptides; development and validation of the RIA are detailed by Russell et al. (15). Values of TGF-α were normalized to the protein contents of the lysates (determined using the Bio-Rad DC protein assay; Richmond, CA). The limit of detection of TGF-α was 2 to 5 pg/tube. In a subsequent experiment, media (IMEMZO supplemented with 1% fetal bovine serum) was conditioned in the presence of 100 ng/ml mouse EGF (culture grade, Sigma); harvest and subsequent processing of the conditioned media for RIA was as described above, except the conditioned media were concentrated prior to analysis.

**Densitometric Analysis of Autoradiograms.** Comparisons of the relative levels of gene expression (Northern) or EGFR protein production (immunoprecipitations) were made by scanning autoradiograms with an LKB UltraScan XL laser densitometer (LKB Produkter; Pharmacia LKB, Bromma, Sweden). Blots were autoradiographed for various times to insure that low- and high-density bands could be quantitated within the linear range of the X-ray film. Peak integration and background corrections were made using the LKB GelScan XL software (version 2.1).

**Statistics.** One-way ANOVA was used to test for significant differences between variances among groups. Appropriate pairwise comparisons were conducted using a two-tailed Student’s t-test. All statistical calculations were made using Microstat-II software, version 2.5 (Ecosoft, Inc., Indianapolis, IN).

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**References**


