Regulation of Transforming Growth Factor \( \alpha \) Messenger RNA Expression in a Chemically Transformed Rat Hepatic Epithelial Cell Line by Phorbol Ester and Hormones

Victoria W. Raymond, David C. Lee, Joe W. Grisham, and H. Shelton Earp

Lineberger Cancer Research Center, Cell Biology Program [V. W. R., D. C. L., J. W. G., H. S. E.], Departments of Microbiology and Immunology [D. C. L.], Pathology [J. W. G.], Medicine [H. S. E.], and Pharmacology, [H. S. E.], The University of North Carolina, Chapel Hill, North Carolina 27599

ABSTRACT

Transforming growth factor \( \alpha \) (TGF-\( \alpha \)) is produced by many transformed cells, but little is known about the regulation of its expression. We examined TGF-\( \alpha \) mRNA levels in a set of cloned neoplastic cell lines derived by chemical transformation of a normal rat liver epithelial cell. The untransformed parental cell line, WB-344, did not express a detectable level of TGF-\( \alpha \) mRNA, whereas GP6ac, a transformed line capable of autonomic growth in soft agar, expressed TGF-\( \alpha \). When GP6ac cells were treated with agents thought to regulate protein kinase C activity, e.g., the tumor promoter, 12-O-tetradecanoylphorbol-13-acetate (TPA), TGF-\( \alpha \) mRNA levels increased by 8- to 11-fold. The induction of TGF-\( \alpha \) mRNA was detectable at 2 h, was maximal at 8-12 h, and declined by 24 h. Angiotensin, bradykinin, epinephrine, and epidermal growth factor also increased TGF-\( \alpha \) mRNA by 2- to 5-fold. In contrast, parental WB cells neither expressed TGF-\( \alpha \) mRNA, nor responded to TPA. TPA also increased TGF-\( \alpha \) mRNA in GP6ac cells but the effect was less prolonged; maximal levels were seen at 4 h after TPA exposure and returned to control levels by 12 h.

TPA increased TGF-\( \alpha \) mRNA in GP6ac cells, in part, by increasing transcription of the TGF-\( \alpha \) gene as measured by run-on transcription rates in isolated nuclei. In addition, the induction of TGF-\( \alpha \) by TPA was blocked by concurrent incubation with agents that inhibit protein synthesis. However, if TPA was present for at least 2 h, subsequent addition of cycloheximide enhanced the effect of TPA. This indicates that the induction of TGF-\( \alpha \) in GP6ac cells is comprised of at least two phases demarcated by the requirement for protein synthesis. The time course of induction and the sensitivity to inhibition of protein synthesis distinguish the effect of TPA on TGF-\( \alpha \) mRNA from that of other genes regulated by TPA, e.g., c-myc and c-fos. These data also suggest that chemical transformation of rat liver epithelial cells leads to expression of TGF-\( \alpha \) mRNA, and that once expressed, TGF-\( \alpha \) mRNA can be modulated in a protein kinase C-dependent manner.

INTRODUCTION

TGF-\( \alpha \) is a mitogenic polypeptide which shares structural and functional homology with EGF (1). TGF-\( \alpha \), which binds to the EGF receptor and activates the receptor's intrinsic tyrosine kinase activity (2-4), was originally detected in the culture medium of retrovirally transformed fibroblasts (5, 6). Sequence analysis of cloned cDNAs encoding both human (7) and rat (8) TGF-\( \alpha \) indicates that the mature 50-amino acid polypeptide is cleaved from a transmembrane precursor of either 160 (human) or 159 (rat) amino acids by the action of an elastase-like enzyme. TGF-\( \alpha \) indicates that the mature 50-amino acid polypeptide is cleaved from a transmembrane precursor of either 160 (human) or 159 (rat) amino acids, and that once expressed, TGF-\( \alpha \) mRNA can be modulated in a protein kinase C-dependent manner. TGF-\( \alpha \) is extensively studied in neoplastic cells. Expression of TGF-\( \alpha \) has been demonstrated in many solid tumors, as well as in retrovirally or chemically transformed cultured cells (14). Apparently, certain transforming events lead to both the initial and sustained expression of TGF-\( \alpha \) mRNA and its peptide product. Evidence suggests that secretion of TGF-\( \alpha \) by cells bearing EGF receptors leads to autocrine stimulation of tumor cell growth in vitro and, presumably, in vivo (15, 16). Induction of TGF-\( \alpha \) mRNA accumulation in cultured normal keratinocytes by EGF (13), and in rat mammary tumors by estrogen (17), suggests that expression can be regulated by extracellular signals. However, little is known about the intracellular signals that control expression of TGF-\( \alpha \) mRNA or the mechanisms by which the expression is increased.

Elevated TGF-\( \alpha \) levels have been detected in the urine of patients with a variety of tumors (18, 19) e.g., a recent study demonstrated that patients with hepatocellular carcinoma excrete elevated amounts of TGF-\( \alpha \). In fact, increased levels of TGF-\( \alpha \) excretion were as sensitive a predictor of the presence of hepatocellular carcinomas as the more traditional marker, serum \( \alpha \)-fetoprotein (20). Since little is known about the regulation of TGF-\( \alpha \) expression, we examined TGF-\( \alpha \) mRNA in several chemically transformed rat liver epithelial cell lines that had been derived by \( N \)-methyl-\( N' \)-nitro-\( N' \)-nitrosoguanidine treatment of a normal rat liver epithelial cell line, WB-F344 (21, 22). One line that had been cloned from the original neoplastic population, GP6, produced tumors in 96% of the neonatal syngeneic rats into which it was transplanted (23, 24). Another line, GP6TB, was derived from one of the GP6 tumors, and produced new tumors after implantation with a much shorter latency than GP6 (mean latency 1 month versus 6.9 months for GP6 cells) (25). Previous studies showed that GP6TB secreted higher levels of TGF-\( \alpha \) into the media than GP6 (26). A third line, GP6ac, was derived by passing GP6 cells in soft agar (22) until a line that did not require added EGF for anchorage-independent growth emerged (27). In addition to examining the level of TGF-\( \alpha \) mRNA in the normal and transformed cell lines, we studied whether tumor promoters that act via protein kinase C, or other hormones that have been shown to increase protein kinase C activity in liver cells, regulate TGF-\( \alpha \) mRNA levels.

MATERIALS AND METHODS

Materials. TPA, epinephrine, and the protein synthesis inhibitors cycloheximide and puromycin were obtained from Sigma. Angiotensin II and bradykinin were obtained from Calbiochem. EGF was purified from mouse salivary glands by the method of Savage and Cohen (28).

Cell Culture. WB-F344, GP6, GP6ac, and GP6TB cells were grown in 100-mm dishes in Richter's modified Eagle's medium supplemented with 10 mM 4-(2-hydroxyethyl)1-piperazineethanesulfonic acid, glucose, insulin, and 10% fetal calf serum in a humidified 5% \( \text{CO}_2 \) atmosphere.

RNA Analysis. Total RNA was extracted from treated or control cells by lysis in 4 M guanidine isothiocyanate followed by centrifugation through cesium chloride (29). Total (20 \( \mu \)g) or oligo(dT)-selected...
TGF-α mRNA levels were undetectable in the normal WB cells, and were only detectable in GP6 cells when poly(A)⁺ RNA was probed (data not shown). In contrast both GP6ac cells (selected for spontaneous soft agar growth), and GP6TB (derived from a GP6-induced tumor), exhibited an abundant 4.5-kilobase TGF-α mRNA detectable in blots of total RNA. The levels of TGF-α mRNA in GP6TB cells were 7-fold greater than in GP6ac cells (Fig. 1). The induction of TGF-α mRNA was observed with TPA concentrations as low as 2.5 nM, and was maximal with concentrations between 25 and 100 nM (data not shown). To test whether TGF-α mRNA induction could be affected by other agents known to activate phospholipase C in WB cells (31), we treated GP6ac cells with EGF (100 ng/ml), angiotensin II (1 μM), epinephrine (10 μM), or bradykinin (10 μM) for 4 or 8 h. Treatment with EGF and the various hormones increased the level of TGF-α mRNA by 2- to 5-fold at 4 and 8 h (Fig. 2) but did not significantly change α-actin mRNA levels. In contrast TGF-α mRNA was not detected in WB cells even after treatment with TPA (data not shown).

To determine whether the basal expression of TGF-α mRNA was correlated with the response to TPA, GP6, GP6ac, and GP6TB cells were incubated with 100 nM TPA for 4 h (Table 1). In this experiment basal levels of TGF-α mRNA were undetectable in GP6 cells. A faint 4.5-kilobase band was seen in the lane containing RNA from TPA-treated cells. GP6TB cells exhibited the highest basal level of TGF-α mRNA, and in

**Table 1 Expression and induction of TGF-α mRNA in several transformed cell lines**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>RELATIVE LEVEL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>1.000</td>
</tr>
<tr>
<td>TPA</td>
<td>0.507</td>
</tr>
<tr>
<td>GP6</td>
<td>1.664</td>
</tr>
<tr>
<td>GP6ac</td>
<td>12.098</td>
</tr>
<tr>
<td>GP6TB</td>
<td>20.238</td>
</tr>
</tbody>
</table>

**Fig. 1.** Northern blot analysis of TGF-α mRNA induction in GP6ac cells. Poly(A)⁺ RNA (5 μg) was isolated from control cultures of GP6ac cells or cells treated with 100 nM TPA for either 8 or 24 h. The RNA was blotted and hybridized to plasmid 3Blb, containing rat TGF-α complementary DNA sequences. A single 4.5-kilobase band is shown in each case.
these cells TPA produced a 1.4-fold induction.

To more fully characterize the regulation of TGF-α mRNA by TPA, we studied the most inducible cell line, GP6ac. An RNase protection analysis of nucleic acid extracts from TPA-treated cells indicated that TGF-α mRNA levels were increased by twofold at 2 h, reached maximal levels at 8–12 h, then declined by 24 h (Fig. 3, top). Since we have earlier shown that TPA induced EGF receptor mRNA in WB cells (31), we compared the inductions of TGF-α and EGF receptor mRNAs in GP6ac cells. Northern analysis of total RNA (Fig. 3, bottom) showed a time course for TPA induction of TGF-α mRNA that is comparable to that shown in Fig. 3, top. TPA also induced EGF receptor mRNA in these cells, but the response was more rapid. Densitometric quantitation of the blots showed that the maximum increase in EGF receptor mRNA levels (5-fold) occurred at 4 h, and returned to control values by 12 h. In contrast, levels of α-actin mRNA levels did not change by more than 1.5-fold over this same time course.

To determine whether increased transcription is responsible for the TPA-mediated induction of TGF-α mRNA in GP6ac cells, we performed nuclear run-on analyses. GP6ac cells were treated with TPA for the indicated times; nuclei were prepared and run-ons performed as described in “Materials and Methods.” Densitometric measurement of the autoradiograms indicated that TGF-α transcripts increased 2.6- to 3.4-fold 4 h after exposure to TPA, then declined to control levels by 6 h (Table 2). Levels of actin gene transcription remained unchanged over this same time period (not shown).

The role of protein synthesis in the induction of TGF-α mRNA was investigated. Cumulative results from five experiments showed that whereas cycloheximide (10 µg/ml) alone had little effect on expression of TGF-α mRNA, when the protein synthesis inhibitor was added together with TPA, the induction was completely abolished (Fig. 4). Puromycin (10 µg/ml) gave similar results (data not shown). To determine whether the protein synthesis requirement affects the initiation or maintenance of the induction of TGF-α mRNA, cycloheximide was added to cells at 30 min, 1 h, 2 h, 3 h, 4 h, or 5 h after the addition of TPA. The incubation was then continued for 5.5, 5, 4, 3, 2, and 1 h. In this experiment, cycloheximide produced a 6.0-fold increase in TGF-α mRNA levels 6 h after TPA treatment.

Table 2 Effect of TPA on transcription of the TGF-α gene

<table>
<thead>
<tr>
<th>Exp.</th>
<th>Control</th>
<th>2-h TPA</th>
<th>4-h TPA</th>
<th>6-h TPA</th>
</tr>
</thead>
<tbody>
<tr>
<td>TGF-α</td>
<td>1.0</td>
<td>1.4</td>
<td>2.5</td>
<td>0.9</td>
</tr>
<tr>
<td>Actin</td>
<td>1.0</td>
<td>0.9</td>
<td>0.9</td>
<td>0.8</td>
</tr>
</tbody>
</table>

EFFECT OF CYCLOHEXIMIDE ON TGF-α mRNA INDUCTION

![Figure 4](cancerres.aacrjournals.org/article-fig4.png)

Fig. 4. Inhibition of TGF-α induction by cycloheximide. Confluent cultures of GP6ac cells were incubated with TPA (100 nM), cycloheximide (CH) (10 µg/ml), or both for 6 h. Total RNA was extracted and analyzed by Northern blot hybridization as described. Relative levels of the 4.5-kilobase TGF-α mRNA were quantitated using an LKB laser densitometer with peak integration. Untreated control densitometric areas were assigned a relative value of 1.0. Bar heights, mean fold increase in five experiments; error bars, standard errors.
a modest increase in TGF-α mRNA accumulation. However, when cycloheximide was present during the initial 1–2 h of TPA incubation, the induction of TGF-α mRNA was completely blocked (Fig. 5). When TPA was present for at least 2 h before the protein synthesis inhibitor was added, TGF-α mRNA was induced to a level greater than that found with TPA alone. At the later times, cycloheximide produced a 2- to 4-fold increase over that seen with TPA alone. The highest level of TGF-α mRNA was observed at the latest addition of cycloheximide when cells had been exposed to TPA for 5 h prior to the 1 h cycloheximide treatment. In contrast, the level of actin mRNA varied less than twofold when the blot shown in Fig. 5 was reprobed (not shown).

DISCUSSION

Assessment of the production of autocrine growth factors by transformed cells derived from the parental WB line previously showed that some of the cell lines secreted a factor which competed with 125I-EGF in receptor binding assays (26). Moreover, previous studies using WB cells showed that TPA as well as hormones and growth factors which stimulate the accumulation of inositol triphosphates (InsP3), increased EGF receptor mRNA levels (31). We therefore assessed TGF-α and EGF receptor mRNA levels in a set of transformed cells and their normal parental cell line before and after presumed direct or indirect activation of protein kinase C. The normal parental WB cell line did not express detectable TGF-α mRNA nor could it be induced to produce TGF-α by TPA. Neoplastic clones that grow well in soft agar without added EGF, or which rapidly formed tumors in rats, expressed detectable TGF-α mRNA and responded to TPA with induction of even higher levels. We treated GP6ac cells, the most responsive cell line, with angiotensin II, epinephrine, or bradykinin. Treatment with each hormone increased the level of TGF-α mRNA at both 4 and 8 h. In addition, treatment of GP6ac cells with EGF (which also increases accumulation of InsP3 in WB cells) increased TGF-α mRNA by as much as 4.5-fold at 8 h.

Of the agents studied, TPA is the most effective inducer of TGF-α mRNA. The long duration of the TPA response may result, in part, from the slow metabolism of TPA, and activation of protein kinase C by TPA is of much longer duration than that of the other agents. Hormones produce a transient activation of PKC, whereas TPA translocates protein kinase C to the membrane and retains it in an active conformation until it is proteolytically degraded (36, 37).

The results with GP6ac cells, including both the time course and the initial requirement for protein synthesis, clearly differentiate the pattern of induction of TGF-α mRNA from the pattern of induction of other TPA responsive genes, e.g., c-myc and c-fos (38–40). The induction of myc and fos by TPA is rapid and may result from alteration of protein binding to specific DNA elements flanking the gene sequences (41, 42). It is not blocked by cycloheximide. Posttranslational modification such as phosphorylation of DNA-binding proteins has been postulated to explain the rapid alteration of protein interactions with the DNA enhancer elements (43). EGF receptor mRNA levels are altered by TPA in a time course whose duration is intermediate between the rapid response of fos and myc and the prolonged response of TGF-α mRNA.

The induction of TGF-α mRNA in GP6ac cells apparently requires synthesis of a protein that directly or indirectly increases the rate of TGF-α gene transcription, as cycloheximide added to cells at the same time as TPA inhibits mRNA accumulation. Later stages of the induction, and particularly the prolonged TGF-α mRNA accumulation over 8–24 h, may be due to mRNA stabilization. This mechanism is suggested by the fact that inhibition of protein synthesis following initiation of the induction process results in significantly increased accumulation of TGF-α mRNA. The fact that cycloheximide alone has little effect on TGF-α mRNA indicates that stabilization alone is not wholly responsible for the induction, and some other TPA-dependent event must precede the stabilization.

The data show that the untransformed liver epithelial cells (WB) do not express TGF-α mRNA, nor do they secrete material which binds to the EGF receptor (26). The data also suggest that transformation of WB cells makes them sensitive to TPA-dependent regulation of TGF-α gene expression. While we have no direct proof that TGF-α expression is essential for a malignant phenotype, the expression of TGF-α is correlated with a more aggressively tumorigenic phenotype in this family of chemically transformed cells. The initial clone, GP6, did cause tumors when implanted into neonatal rats, but a long latency was observed (24). When GP6TB cells (cloned from a disaggregated GP6 tumor) were examined, a high level of basal TGF-α mRNA expression was observed and TGF-α-like material was detected in the culture media (26). Implantation of GP6TB cells into neonatal rats produced tumors with a much shorter latency period (25). This may suggest that selection during formation of the original tumor resulted in a tumor cell line with a significant growth advantage due to high level expression of TGF-α.

The cell lines GP6ac and GP6TB also exhibited easily detectable induction of TGF-α mRNA by agents that may stimulate phosphoinositide breakdown and generate diacylglycerol or by agents that directly activate protein kinase C. EGF and certain hormones increase TGF-α mRNA levels in GP6ac cells, and stimulate phospholipase C activity in many cells of epithelial origin, particularly hepatocytes (31). This observation suggests that activation of protein kinase C in susceptible cells may be one pathway by which TGF-α mRNA expression can be induced. In the case of neoplastic liver cells the expression of TGF-α mRNA can be regulated by multiple hormones present in the normal circulation. A transformed cell exhibiting low level TGF-α expression could be stimulated to increase TGF-α mRNA levels by a number of hormones in the portal circulation utilizing their normal signaling pathways. The normal signaling transduction could result in increased TGF-α production in a cell whose TGF-α gene regulation has been altered by some event in transformation. Studies are in progress to determine whether the induction of TGF-α mRNA in GP6ac cells by TPA or hormones results in secretion of elevated TGF-α protein.
However, the demonstration that a tumor promoter can increase TGF-α mRNA is an important first step in understanding the regulation of the gene for this autocrine growth stimulant.

ACKNOWLEDGMENTS

We thank Dr. Lester W. Lee and Joanna Harris for important contributions and Dr. Jenny Ting for her timely assistance.

REFERENCES

Regulation of Transforming Growth Factor $\alpha$ Messenger RNA Expression in a Chemically Transformed Rat Hepatic Epithelial Cell Line by Phorbol Ester and Hormones


Updated version

Access the most recent version of this article at:

http://cancerres.aacrjournals.org/content/49/13/3608

E-mail alerts

Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions

To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions

To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.