Promoter Sequences of the Human Transforming Growth Factor-β1 Gene Responsive to Transforming Growth Factor-β1 Autoinduction*

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Two distinct regions of the transforming growth factor (TGF)-β1 promoter are responsive to autoregulation. Sequences located between nucleotides -454 to -323 and between the two major transcriptional start sites have positive regulatory activities and are induced by TGF-β1 in A-549 cells. The chloramphenicol acetyltransferase activity of the upstream human TGF-β1 promoter-chloramphenicol acetyltransferase gene is increased 8- to 10-fold by treatment of cells with TGF-β1, whereas that of the second promoter is increased approximately 3- to 4-fold. Using an S1 nuclease protection assay of chloramphenicol acetyltransferase mRNA, we found that the steady-state expression of chloramphenicol acetyltransferase mRNA also is markedly increased. Seven distinct factors present in nuclear extracts from A-549 cells interact with the sequences between -454 and -323, strongly supporting the involvement of sequence-specific transcription factors in the transcriptional autoactivation of the human TGF-β1 gene.

Transforming growth factor-β (TGF-β) is a 25,000-dalton homodimeric molecule which belongs to a family of structurally related multifunctional regulatory peptides (for reviews, see Refs. 1-3). The nucleotide sequences and the deduced amino acid sequences of human, murine, porcine, bovine, simian, and chicken (4-9) TGF-β1 mRNAs demonstrate that the mature polypeptide of TGF-β1 is conserved 100% across these species with the exception of a single amino acid substitution in the murine peptide. This extensive amino acid conservation is a characteristic of TGF-β's types 2 and 3 as well, strongly suggesting that the TGF-β's must play a vital role in normal cell physiology.

Several growth factors have been demonstrated to autoregulate expression of their mRNAs, among them PDGF (10), TGF-α (11), and TGF-β1 (12). These data imply the existence of an autocrine loop that may serve to amplify response to the growth factor under certain conditions. Such autoregulation may also serve to sustain growth factor action in vivo. As a first step in exploring the molecular basis for the autoregulation of TGF-β1 gene expression, we characterized the promoter region of the human TGF-β1 gene and identified sequences responsible for both promotion and inhibition of transcription by assaying the chloramphenicol acetyltransferase (CAT) activity of TGF-β1 promoter-CAT chimeric genes (13). Two promoter regions were identified: one located 5' to the most upstream transcriptional start site, and another located between the two major transcriptional initiation sites. In this report, we have localized the specific regions permissive for the optimal autoregulation of TGF-β1 gene transcription to both a 130-bp fragment located between -453 and -323 bp upstream of the 5'-most start site and a fragment containing the second promoter.

MATERIALS AND METHODS

Cells and Culture Conditions—Cells were grown in a humidified incubator (5% CO₂, 95% air) at 37 °C in the presence of antibiotics (50 units/ml penicillin, 50 µg/ml streptomycin). Human lung adenocarcinoma (A-549) cells were grown in Dulbecco's modified Eagle's medium with high glucose supplemented with 5% fetal bovine serum. Other cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum. All transfections were repeated at least three times.

DNA Constructs—The construction of a series of the human TGF-β1 gene promoter-CAT chimeric genes has been described elsewhere (13). To clone the fragment of the human TGF-β1 gene containing sequences between -453 and -323, pFBG104 (13) was first digested with BstEII, followed by treatment with T4 polymerase. The DNA was subsequently digested with HindIII, and the 130-bp fragment was isolated and cloned into the HindIII site of pGEM 4 (Promega Biotechnology).

Growth Factors and Reagents—TGF-β1 and -β2 from porcine platelets were obtained from R & D Systems (Minneapolis, MN); human PDGF was a gift of Dr. Thomas Deuel (Washington University, St. Louis, MO); basic fibroblast growth factor was obtained from Sigma; and epidermal growth factor (EGF) was purified to homogeneity from mouse submaxillary glands.

RNA Extraction and S1 Nuclease Analysis of CAT mRNA—Following experimental treatment of cells transfected with TGF-β1 gene promoter-CAT chimeric plasmids, RNA was isolated according to the guanidine isothiocyanate/cesium chloride procedure by the method of Chirgwin et al. (14). S1 nuclease was performed on total cell RNA using a single-stranded DNA probe uniformly labeled with [32P]dCTP (3000 Ci/mmol) during synthesis from a single-stranded M13 phage, M13 CAT, constructed by insertion of a EcoRI-ZeoRI fragment from pM13CAT2 (15). After synthesis of the [32P]dCTP-labeled probe, a 458-nucleotide single-stranded segment containing 256 bp of CAT coding sequences was liberated by HindIII digestion and purified on a 6% polyacrylamide-urea gel. S1 nuclease digestion was performed (16) and the products were analyzed by densitometry in a 5% polyacrylamide sequencing gel containing 8 M urea, followed by autoradiography.

Nuclear Extracts and Gel Mobility Shift Assay—Nuclear extracts were made from A-549 cells essentially as described by Dignam et al. (16), final protein concentrations were typically 1-2 mg/ml. Gel mobility shift assays were performed as previously described (17). Specifically, plasmid DNA (pFBG104), linearized with BstEII and end-labeled by T4 polynucleotide kinase, was cleaved with HindIII, and the appropriate fragments were isolated by electrophoresion after electrophoresion through a 5% polyacrylamide gel. The DNA-protein binding reaction was performed in 2%–5% incubations: nuclear extracts

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§ The abbreviations used are: TGF, transforming growth factor; PDGF, platelet-derived growth factor; bp, base pair; CAT, chloramphenicol acetyltransferase; EGF, epidermal growth factor.

7041
(1–3 μl) were incubated with 1 μg of poly(dI:dC) (Pharmacia LKB Biotechnology Inc.) in binding buffer (10 mM Tris-HCl, pH 7.5, 50 mM NaCl, 5% glycerol, 5.0 mM MgCl₂, 1.0 mM dithiothreitol, and 1.0 mM EDTA) for 10 min on ice before addition of approximately 0.1 ng of 32P-labeled probe (<10,000 cpm) after which the incubation was continued for an additional 15 min. Competitor DNAs were added at the preincubation step. Protein-DNA complexes were electrophoresed in 6% polyacrylamide gels in 0.25× TBE (1× TBE = 90 mM Tris-HCl, 90 mM boric acid, 1 mM EDTA, pH 8.3). The gels were dried and radioactivity was detected by exposure to Kodak XAR-5 film with an intensifying screen at −70 °C.

RESULTS

Localization of Regions for TGF-β1 Autoregulation—A restriction endonuclease map of the 5′ end of the human TGF-β1 gene and the structures of the TGF-β1 promoter-CAT chimeric plasmids used in this study are shown in Fig. 1; the arrow denotes the first major transcriptional start site. The recombinant plasmids were transfected into A-549 cells and the cells were then incubated in the absence or presence of TGF-β1 (5 ng/ml). All transfections were transient expression experiments in which TGF-β1 was added 24 h after transfection. After a predetermined interval, the cells were harvested and CAT activity was measured. These cells showed increased levels of CAT activity after TGF-β1 treatment (Fig. 2 and Table I). The mean results in A-549 cells of several experiments with each plasmid are summarized on the right of Fig. 1, where the level of CAT enzymatic activity is expressed as a percentage of the level of phTG5 in the absence of added TGF-β1. Plasmid phTG5, which contains 453-bp of the upstream TGF-β1 promoter, consistently produced the highest level of total CAT activity (Figs. 1, 2, and Table I) (13). This level of CAT activity was stimulated approximately 8- to 10-fold when cells transfected with phTG5 were incubated with TGF-β1, indicating that the sequences permissive for TGF-β1 autoregulation are located in that region. The activity of plasmids phTG1, 2, 3, 4, 6, and 7 was stimulated approximately 1- to 4-fold by TGF-β1 treatment, but the levels of CAT activity were only 5–10% of those seen with phTG5. These results with phTG1, 2, 3, and 4, as well as previously published results (13), suggest that sequences upstream from −453 contain a negative regulatory region which suppresses the activity of sequences in phTG5.

Recent experiments have demonstrated that TGF-β1 can directly enhance the transcription level of the mouse α (2) collagen gene and that this effect is mediated by NF-1 binding sites (18). Since the human TGF-β1 gene contains a sequence similar to the consensus sequence of NF-1 at nucleotides −260 to −240, it has been suggested that NF-1 may also be involved in autoregulation of TGF-β1 gene transcription (12). However, plasmid phTG6, which contains the sequences related to NF-1 showed only a low level of expression, even after a 3.5-fold induction by TGF-β1.

A time course experiment showed that the increase in CAT activity of phTG5 is slow during the early time points but then increases steadily beginning at about 18 h after the onset of TGF-β1 treatment (Fig. 3). The precise interpretation of this time course is difficult, since we do not know how much time is needed for the transfected DNA to reach the nucleus and become actively transcribed.

Effects of Other Growth Factors on the Activity of the Human TGF-β1 Promoter—A previous report (12) showed that treatment of normal rat kidney cells with EGF and PDGF also increased the TGF-β1 mRNA levels, and that the combined effects of EGF and TGF-β1 on TGF-β1 mRNA expression were greater than those of either effector alone. To examine whether these other growth factors also regulate TGF-β1 promoter activity, A-549 cells transfected with the plasmid phTG5 were treated with various growth factors. As shown in Fig. 4, EGF, PDGF, and basic fibroblast growth factor were unable to stimulate CAT gene expression, but TGF-β2 elicited a 4- to 6-fold stimulation of CAT gene expression in these cells. The combined effects of these growth factors with TGF-β1 showed little additional effect on CAT activity. Previously, it had been observed that TGF-β1 mRNA levels in normal rat kidney cells treated for 24 h with EGF are unaffected by the addition of cycloheximide, whereas the levels in cells treated with TGF-β1 are severely reduced (12). Our data support this observation and suggest that the effect of TGF-β1 on the regulation of its gene expression might occur at a transcriptional level through nuclear factors, while EGF or other growth factors might contribute to stabilization of mRNA.

Autostimulation of Transcripts from the First Promoter as Well as the Second Promoter of the TGF-β1 Gene—Previous investigations identified a second promoter region in the TGF-β1 gene located between the two transcriptional start sites at +1 and +271 (13). To determine whether this second promoter region was sensitive to autoregulation, A-549 cells were transfected with another plasmid, pHG15, containing the second promoter region (positions +95 to +727). These cells were then incubated in the presence or absence of TGF-β1. Extracts examined at 48 h contained a high basal level of CAT activity (Fig. 5A) in the absence of TGF-β1. In the presence of TGF-β1, the activity of pHG15 was increased 3- to 4-fold. To establish that the increase in CAT activity after

![Fig. 1. TGF-β1 stimulation of human TGF-β1 promoter-CAT fusion genes.](image-url)

Representative experiments to determine CAT activity in extracts of transiently transfected A-549 cells are shown. The fusion genes which were transfected are represented schematically at the left of the figure. Transfected cells were incubated in the absence or presence of 5 ng/ml of TGF-β1. TGF-β1 was added 24 h after transfection; CAT activity was assayed 24 h later. The data are expressed as a percentage of the mean activity obtained with phTG5 (−453 to +11) in the absence of added TGF-β1. Restriction sites are indicated as follows: B, BamHI; P, PstI; X, XbaI; G, HgiAl; H, HindIII; Bg, BstEI; Ba, BamHI; S, SstII.)
FIG. 2. Effects of TGF-β1 on the human TGF-β1 promoter-CAT genes in A-549 cells. A-549 cells were transiently transfected with human TGF-β1-CAT fusion genes as described in the legend to Fig. 1. Transfected cells were treated with serum-free media alone (○), with media containing 5% fetal bovine serum (●), or with media containing 5 ng/ml TGF-β1 (□). The relative chloramphenicol acetylation generated by each plasmid was determined by averaging three to five separate experiments.

TABLE I

Effect of TGF-β1 on CAT expression

A-549 cells were transfected with the indicated plasmid together with 3 μg of β-galactosidase expression vector to monitor for transfection efficiency. Transfected cells were incubated in the presence or absence of 5 ng/ml of TGF-β1. Each value is the average of duplicate determinations.

| Plasmid transfected into cells | −TGF-β1 | +TGF-β1 |
|--------------------------------|---------|---------|
| Experiment 1                   |         |         |
| phTG1                          | 0.3     | 0.3     |
| phTG2                          | 4.3     | 10.1    |
| phTG3                          | 1.0     | 5.8     |
| phTG4                          | 1.8     | 5.6     |
| phTG5                          | 10.1    | 101.2   |
| phTG6                          | 4.4     | 21.3    |
| phTG7                          | 0.8     | 3.7     |
| Experiment 2                   |         |         |
| phTG1                          | 0.5     | 0.5     |
| phTG2                          | 5.8     | 13.2    |
| phTG3                          | 2.0     | 9.1     |
| phTG4                          | 2.3     | 8.6     |
| phTG5                          | 19.3    | 135.4   |
| phTG6                          | 7.8     | 28.9    |
| phTG7                          | 7.2     | 22.4    |
| Experiment 3                   |         |         |
| phTG1                          | 0.4     | 0.4     |
| phTG2                          | 4.8     | 11.5    |
| phTG3                          | 1.9     | 8.1     |
| phTG4                          | 2.2     | 6.4     |
| phTG5                          | 16.4    | 124.3   |
| phTG6                          | 8.6     | 16.1    |
| phTG7                          | 5.3     | 31.2    |

FIG. 3. Time course of the effect of TGF-β1 on the activity of the human TGF-β1 promoter. A-549 cells were transfected with 10 μg of plasmid, p̂TG5. TGF-β1 was added 4 h after the glycerol shock (time 0 of the time course). ○, cells treated with TGF-β1 (5 ng/ml); ●, untreated cells. CAT activities are expressed as counts/min of acetylated chloramphenicol.

FIG. 4. Effects of growth factors on the human TGF-β1-CAT fusion gene expression. CAT assays were performed on cell extracts from A-549 cells treated 24 h after transfection of plasmid, pH TG5, in the presence or absence of the indicated growth factor. The data are expressed as a percentage of the mean activity obtained after treatment with TGF-β1. FGF, fibroblast growth factor (10 ng/ml); EGF, 10 ng/ml; PDGF, 10 ng/ml; FCS, fetal calf serum.

lanes 3 and 8) of TGF-β1. The Yeast tRNA treated with S1 nuclease is shown in Fig. 5B, lanes 1 and 6. As a positive control, RNA extracted from the cells transfected with a HTLV-I LTR construct (19) was used (Fig. 5B, lane 5). Following treatment with TGF-β1, the CAT mRNA level was elevated 5- to 6-fold in cells transfected with either phTG5 or phTG15, even though the basal level of CAT mRNA in cells transfected with phTG5 was 4 times lower than in cells transfected with phTG15.

In Vivo Competition Assay—Having determined that the plasmid phTG5 contains sequences permissive for autoregulation by TGF-β1, we investigated whether the induction could be abolished by co-transfection of an excess of the same fragment. The 130-bp fragment (−453 to −323) was subcloned into the pGEM4 vector (plasmid pGEM H-B) and co-transfected with phTG5 into cells which were then incubated in the presence or absence of TGF-β1 (Fig. 6). At a 5:1 ratio of competitor to template, there was a >75% reduction of the activity of phTG5. This result confirms our localization of sequences permissive for TGF-β1 autoregulation to this 130-bp fragment and suggests that transacting transcription factors which bind to this region could mediate, in part, the autoregulation of TGF-β1 gene expression.

Identification of Nuclear Proteins Bound to the Sequences
**Human TGF-β1 Autoregulation**

**Fig. 5.** TGF-β1 increases the steady-state expression of the two promoter regions of the human TGF-β1 promoter-CAT fusion genes (phTG5 and phTG15). A, regulation of phTG5 and phTG15 by TGF-β1 and -β2 in A-549 cells. Plasmids were transfected into A-549 cells and incubated in the presence or absence of TGF-β1 or TGF-β2 (5 μg/ml). The CAT activities generated by these constructs were measured. ACM, chloramphenicol acetylated; CM, chloramphenicol. B, S1 nuclease analyses of CAT mRNA synthesized in A-549 cells in the presence or absence of TGF-β1 after transfection with phTG5 or phTG15. The upper panel shows the schematic diagram of both plasmids and the single strand probe. Samples (60 μg) of total RNA were subjected to S1 nuclease protection analysis for CAT mRNA as described under "Materials and Methods." Lanes: 1 and 6, yeast tRNA; 2 and 7, total RNA from A-549 cells before transfection; 3 and 4, total RNA from A-549 cells transfected with phTG5; 3 and 8, no TGF-β1 treatment; 4 and 9, TGF-β1 treated after transfection; 5, as a positive control, CAT mRNA of HTLV I-LTR CAT (19); M, molecular weight markers. The size of CAT-specific fragment protected from S1 nuclease digestion is 256 nucleotides.

**Discussion**

In this report, we have identified two distinct regions of the TGF-β1 promoter that are particularly responsive to autinduction. Previously, we reported that sequences located between nucleotides -454 to -323 have positive regulatory activity; the transcriptional capacity of the upstream TGF-β1 promoter was completely abolished by removal of this region (13). We had also demonstrated that sequences located between the two major transcriptional start sites have even greater relative promoter activity than the upstream regula-
between nucleotides -454 to -323 because these sequences with the upstream region of the TGF-β1 promoter responsible for autoregulation, suggesting the involvement of sequence-specific transcription factors in the transcriptional autoactivation of the human TGF-β1 gene. These major and minor band complexes did not appear to be cell-type specific because similar complexes were also detected following incubation with HeLa cell nuclear extracts (data not shown). This suggests that the proteins responsible for formation of the specific complexes might be common to several different cell types.

In previous experiments demonstrating autoinduction of TGF-β1 mRNA (12), it had been suggested that the autoinductive effects might be mediated by an NF-1 binding site, as had been demonstrated for induction by TGF-β1 of mouse α(2)I collagen gene expression (18). Although sequences similar to the NF-1 consensus sequence have been identified at positions -260 to -240 of the TGF-β1 promoter, the CAT activity of the plasmid containing these sequences (pHtG6) showed only a low level of expression, even after induction by TGF-β1, compared to the CAT activity for plasmid pHtG5, which contained an additional 130 bp. This 130-nucleotide fragment, as mentioned above, is essential to elicit the maximum expression of the hybrid gene. These results suggest that the mechanisms involved in the autoinduction of TGF-β1 expression and the induction of the mouse α(2)I collagen gene expression are different. Since TGF-β1 has been shown to increase expression of a number of genes for structural and matrix-associated proteins (20-22) and protease inhibitors (23-25), it remains to be seen whether there will be common or gene-specific mechanisms mediating the response to TGF-β1. Moreover, both with respect to its autoinduction and its induction of other genes, it will be important to determine if the effects of TGF-β1 are mediated through the modification of pre-existing transcription factors, or through the induction of specific transcription factors.

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REFERENCES

1. Sporn, M. B., Roberts, A. B., Wakefield, L. M., and Associan, R. K. (1986) Science 233, 532-534
2. Sporn, M. B., Roberts, A. B., Wakefield, L. M., and de Crombrugghe, B. (1987) J. Cell Biol. 105, 1039-1045
3. Massagué, J. (1987) Cell 49, 437-448
4. Derynck, R., Jarrett, J. A., Chen, E. Y., Eaton, D. H., Bell, J. R., Associan, R. K., Roberts, A. B., Sporn, M. B., and Goeddel, D. V. (1985) Nature 316, 701-706
5. Derynck, R., Jarrett, J. A., Chen, E. Y., and Goeddel, D. V. (1986) J. Biol. Chem. 261, 4377-4379
6. Derynck, R., and Rhie, L. (1987) Nucleic Acids Res. 15, 2187
7. Van Obberghen-Schilling, E., Kondisah, P., Ludwig, R. L., Sporn, M. B., and Baker, C. C. (1987) Mol. Endocrinol. 1, 693-698
8. Shibuya, K., Flowman, G. D., Rose, T. M., Twardzik, D. R., and Pincus, A. F. (1987) DNA (N.Y.) 6, 239-244
9. Jakowlew, S. B., Dillard, P. J., Sporn, M. B., and Roberts, A. B. (1988) Nucleic Acids Res. 16, 8730-8738
10. Paulsson, Y., Hammacher, A., Heldin, C.-H., and Westermark, B. (1987) Nature 328, 712-717
11. Coffey, R. J., Derynck, R., Wilcox, J. N., Bringman, T. S., and Roberts, A. B. (1988) J. Biol. Chem. 263, 7411-7416
12. Van Obberghen-Schilling, E., Roche, N. S., Flanders, K. C., Sporn, M. B., and Roberts, A. B. (1988) J. Biol. Chem. 263, 7411-7416
13. Kim, S.-J., Glick, A., Sporn, M. B., and Roberts, A. B. (1988) J. Biol. Chem. 264, 402-408
14. Chirgwin, J. M., Przybyla, A. E., MacDonald, R. J., and Rutter, W. J. (1979) Biochemistry 18, 5294-5299
15. Loeken, M. R., Khouy, G., and Brady, J. (1986) Mol. Biol. Cell. 6, 2020-2026
16. Dignam, J. D., Lebovitz, R., and Roeder, R. G. (1983) Nucleic Acids Res. 11, 1475-1489
17. Jeang, K.-T., Boros, I., Brady, J., Radonovich, M., and Khouy, G. (1988) J. Virol. 62, 4499-4509
18. Rossa, P., Karsenty, G., Roberts, A. B., Roche, N. S., Sporn, M. B., and de Crombrugghe, B. (1988) Cell 52, 405-414
19. Jeang, K.-T., Shank, P. R., and Kumar, A. (1988) Proc. Natl. Acad. Sci. U. S. A. 85, 8291-8295
20. Igozat, R. A., Endo, T., and Massagué, J. (1979) J. Biol. Chem. 264, 6443-6446
21. Roberts, A. B., Sporn, M. B., Associan, R. K., Smith, J. M., Roche, N. S., Wakefield, L. M., Heine, U. L., Liotta, L. A., Faigina, V., Kehrl, J. H., and Fauci, A. S. (1986) Proc. Natl. Acad. Sci. U. S. A. 83, 4167-4171
22. Igozat, R. A., and Massagué, J. (1986) J. Biol. Chem. 261, 4377-4345
23. Edwards, D. R., Murphy, G., Reynolds, J. J., Whitman, E. S., Doeherty, A. P., Angel, P., and Heath, J. K. (1987) EMBO J. 6, 1899-1904
24. Lund, L. R., Riccio, A., Andreassen, P. A., Nielsen, L. S., Kristensen, P., Laiho, M., Saksela, O., Blasi, P., and Dano, K. (1987) EMBO J. 6, 1261-1266
25. Laiho, M., Saksela, O., Andreassen, P. A., and Kekki-Oja, J. (1986) J. Cell Biol. 105, 2403-2410