MOLECULAR AND CELLULAR BIOLOGY, Dec. 1989, p. 5508–5515
0270-7306/89/125508-08$02.00/0

Vol. 9, No. 12

Complex Regulation of Transforming Growth Factor β1, β2, and β3 mRNA Expression in Mouse Fibroblasts and Keratinocytes by Transforming Growth Factors β1 and β2

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Received 25 July 1989/Accepted 15 September 1989

Regulation of transforming growth factor β1 (TGFβ1), TGFβ2, and TGFβ3 mRNAs in murine fibroblasts and keratinocytes by TGFβ1 and TGFβ2 was studied. In quiescent AKR-2B fibroblasts, in which TGFβ induces delayed stimulation of DNA synthesis, TGFβ1 autoregulation of TGFβ1 expression was observed as early as 1 h, with maximal induction (25-fold) after 6 to 12 h. Increased expression of TGFβ1 mRNA was accompanied by increased TGFβ protein production into conditioned medium of AKR-2B cells. Neither TGFβ2 nor TGFβ3 mRNA, however, was significantly induced, but both were apparently down regulated at later times by TGFβ1. Protein synthesis was not required for autoinduction of TGFβ1 mRNA in AKR-2B cells. Nuclear run-on analyses and dactinomycin experiments indicated that autoregulation of TGFβ1 expression is complex, involving both increased transcription and message stabilization. In contrast to TGFβ1, TGFβ2 treatment of quiescent AKR-2B cells increased expression of TGFβ1, TGFβ2, and TGFβ3 mRNAs, but with different kinetics. Autoinduction of TGFβ2 mRNA occurred rapidly with maximal induction at 1 to 3 h, enhanced TGFβ3 mRNA levels were observed after 3 h, and increased expression of TGFβ1 occurred later, with maximal mRNA levels obtained after 12 to 24 h. Nuclear run-on analyses indicated that TGFβ2 regulation of TGFβ2 and TGFβ3 mRNA levels is transcriptional, while TGFβ2 induction of TGFβ1 expression most likely involves both transcriptional and posttranscriptional controls. In BALB/MK mouse keratinocytes, minimal autoinduction of TGFβ1 occurred at only the 12- and 24-h time points and protein synthesis was required for this autoinduction. The results of this study provide an example in which TGFβ1 and TGFβ2 elicit different responses and demonstrate that expressions of TGFβ1, TGFβ2, and TGFβ3 are regulated differently. The physiological relevance of TGFβ1 autoinduction in the context of wound healing is discussed.

Transforming growth factor β (TGFβ) was originally identified by its ability to induce anchorage-independent growth alone in murine AKR-2B fibroblasts (37) or in combination with epidermal growth factor (EGF) in normal rat kidney cells (47). TGFβ has now been purified from a variety of sources and cloned, and its complete nucleotide sequence is known (13). Several TGFβ-like molecules have recently been identified; the more distantly related ones include Müllerian inhibiting substance (6), activins and inhibins (34), and some developmentally regulated genes, such as the drosophila decapentaplegic gene complex (42) and the xenopus VG-1 gene (59). TGFβ was also shown to be closely related to an inhibitor produced by BSC-1 cells (56). This molecule, which has been cloned and sequenced (18, 32), has been identified as TGFβ2 (or polyergin) to distinguish it from the previously described TGFβ1. For the most part, the biological activities of TGFβ1 and TGFβ2 appear to be similar; however, TGFβ1 may play a greater role in certain situations, such as hematopoiesis (41) and inhibition of endothelial cell proliferation (23). In contrast, TGFβ2 has been implicated as the principal mediator of mesoderm induction (48). Another TGFβ-related gene, TGFβ3, has recently been cloned and sequenced (14, 21, 54; D. A. Miller, A. Lee, E. Y. Chen, H. L. Moses, and R. Derynck, Mol. Endocrinol., in press). Very little is known about TGFβ3 in terms of cellular distribution, protein production, and binding to cell surface receptors. Further, cloning of TGFβ4 from a chicken chondrocyte cDNA library has recently been reported (22).

TGFβ1, which is expressed by most cultured cell lines (13), is released as an inactive 110-kilodalton precursor and is activated by extremes of pH or by certain proteases (28, 31). Most cell types have membrane receptors that bind both TGFβ1 and TGFβ2 with high affinity. Three cell surface TGFβ-binding proteins have been identified by affinity cross-linking methods (35). These structurally distinct glycoproteins have been classified as type I (about 65 kilodaltons), type II (about 80 to 95 kilodaltons), and type III (approximately 280 kilodaltons) receptors. Recent reports (5, 50) suggest that the type I receptor is involved in mediating TGFβ biological actions. Putative second-messenger systems involved in transducing the TGFβ1 signal have yet to be identified; however, G-protein-dependent and -independent pathways have recently been implicated in mediating TGFβ1 actions in AKR-2B cells (P. H. Howe, C. C. Bascom, M. R. Cunningham, and E. B. Leof, Cancer Res., in press).

TGFβ1 has numerous biological effects on cells, depending on the cell type and the culture conditions (38). In mouse embryo-derived AKR-2B fibroblasts, TGFβ1 indirectly stimulates DNA synthesis, possibly by induction of c-*sis* mRNA and a platelet-derived growth factor-like protein (30). TGFβ1 also regulates expression of several other genes in fibroblasts; these genes include c-*myc*, c-*fos*, JE, KC, β-actin,

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procollagen type I, fibronectin, and plasminogen activator inhibitor type I (24, 29, 30). Conversely, TGFβ1 is an inhibitor of epithelial cell proliferation. Shipley et al. (51) and Coffey et al. (10) have shown that secondary cultures of human keratinocytes or BALB/c mouse keratinocytes (BALB/MK) are reversibly growth arrested by TGFβ1. Transformed cells, however, are either sensitive or refractory to the inhibitory effects of TGFβ1 (19, 51). In BALB/MK cells, TGFβ1 selectively inhibits gene expression; expression of c-*myc* and KC is inhibited, c-*fos* expression is unaffected, and β-actin expression is slightly augmented (8). In addition, TGFβ1 and TGFβ2 are equipotent in stimulation of soft agar growth of AKR-2B (clone 84A) cells and in inhibition of BALB/MK cell proliferation (3, 56).

Recently, it was demonstrated that TGFα and platelet-derived growth factor can enhance their own message levels in human keratinocytes and fibroblasts, respectively (9, 43) and that TGFβ1 induces its own message in normal and transformed cells (57). It was suggested that autoregulation of these transcripts by their respective ligands possibly provided a feedback loop to further modulate growth regulation. The present study was carried out to determine whether TGFβ1 could regulate expression of the closely related TGFβ2 and TGFβ3 genes and whether TGFβ2 would regulate expression of TGFβ genes in the same manner. The results indicate a complex pattern of auto- and heterologous regulation of TGFβ genes by TGFβ1 and TGFβ2.

MATERIALS AND METHODS

Cell culture. Mouse embryo-derived AKR-2B fibroblasts were grown in McCoy 5A medium containing 5% fetal bovine serum (Armour Pharmaceutical Co., Kankakee, Ill.). Examination of autoinduction in proliferating AKR-2B cells was performed essentially as described for BALB/MK cells. AKR-2B cells were made quiescent by continued culture in complete medium for 7 to 8 days. At that time, TGFβ1 or TGFβ2 (10 ng/ml), EGF (10 ng/ml), or TGFβ1 (10 ng/ml)-EGF (10 ng/ml) was added directly to the medium. For each experiment, acid-precipitable counts from a 1-h pulse with [^{3}H]thymidine (Dupont, NEN Research Products, Boston, Mass.) at 23 h after TGFβ1 or TGFβ2 treatment demonstrated that TGFβ1 and TGFβ2 were biologically active by inhibition of the early S phase induced by EGF (52).

BALB/MK cells were grown in minimal essential medium containing 0.05 mM calcium supplemented with 8% dialyzed fetal bovine serum (Hazelton Research Products, Inc., Lenexa, Kans.) and EGF (4 ng/ml; Collaborative Research, Inc., Waltham, Mass.). Porcine TGFβ1 and TGFβ2 were provided by R & D Systems, Inc., Minneapolis, Minn.). Autoinduction of TGFβ1 mRNA in proliferating monolayer cultures of BALB/MK cells was examined by addition of fresh complete medium containing TGFβ1 (10 ng/ml) when the cells reached 80% confluence.

RNA analysis by Northern hybridization. RNA was extracted from either BALB/MK or AKR-2B cells as described previously (8). Oligo(dT)-selected mRNA was separated by electrophoresis in 1.2% agarose–formaldehyde gels, and Northern (RNA) analysis was performed as previously described (30). Purified insert cDNAs of TGFβ1 (13), TGFβ2 (32), TGFβ3 (14), and the constitutively expressed gene 1B15 (11) were labeled by random primer extension (53). For all Northern blots, hybridization with the control 1B15 cDNA probe demonstrated equivalent loading.

Radioreceptor assay on AKR-2B conditioned medium. Quiescent AKR-2B cells were treated with TGFβ1 (10 ng/ml) for 4 h. The cells were washed five times with an excess of serum-free medium and then placed in serum-free medium for an additional 24 h. Following collection of the conditioned medium, some of the medium was acid activated as described previously (31) and then increasing concentrations of either control or acid-activated conditioned medium were used directly in the radioreceptor assay. Binding of [^{125}I]TGFβ to AKR-2B (clone 84A) cells in increasing concentrations of control and treated conditioned media was performed essentially as previously described (31).

Nuclear run-on analyses. Quiescent AKR-2B cells were treated with TGFβ1 (10 ng/ml) or TGFβ2 (10 ng/ml), and nuclei were collected at the indicated times by Nonidet P-40 lysis (17) and stored at −70°C in 50 mM Tris hydrochloride (pH 8.3)–40% glycerol–5 mM $MgCl_2$–0.1 mM EDTA. Nuclear run-on assays were performed essentially as described by Greenberg and Ziff (17) with slight modifications. Briefly, 100 μl of nuclei was added to 100 μl of reaction buffer (10 mM Tris hydrochloride [pH 8.3]; 5 mM $MgCl_2$; 300 mM KCl; 0.5 mM ATP, CTP, and GTP; 250 μCi of [^{32}P]UTP [800 Ci/mmol; Dupont, NEN]) and incubated for 30 min at 30°C. The nascent transcripts were then processed for hybridization essentially as described by Matrisian et al. (36). An equal amount of radioactivity was added to each filter and incubated for 3 days at 42°C. Filters were prepared by adding 10 μg of alkali-denatured plasmid per slot, washed with 5× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate), and UV cross-linked with a UV Stratlinker 1800 (Stratagene, La Jolla, Calif.). Three 30-min posthybridization washes were performed with 1× SSC and 0.1% sodium dodecyl sulfate at 42°C, and the filters were then exposed overnight to Kodak XAR-5 film with intensifying screens.

Dactinomycin experiments. The effect of dactinomycin pretreatment on TGFβ1 autoinduction was examined. Quiescent AKR-2B cells were pretreated for 30 min with dactinomycin (3 μg/ml; Sigma Chemical Co., St. Louis, Mo.). This concentration of dactinomycin inhibited RNA synthesis by greater than 90% in AKR-2B cells (data not shown). At that time, TGFβ1 was added and incubated with AKR-2B cells for 6 h. The stability of TGFβ mRNAs following TGFβ1 treatment of AKR-2B cells was determined in the following manner. Quiescent AKR-2B cells were treated with TGFβ1 (10 ng/ml) for 6 h and washed twice with serum-free medium, and fresh medium containing dactinomycin (3 μg/ml) was added for various times.

RESULTS

Regulation of TGFβ1, TGFβ2, and TGFβ3 mRNA levels by TGFβ1 in AKR-2B cells. Quiescent AKR-2B cells were treated with EGF (10 ng/ml), TGFβ1 (10 ng/ml), or EGF and TGFβ1 for various times. Total cellular poly(A)$^+$ mRNA was extracted from these cells, and Northern blot analysis was performed with a murine TGFβ1 cDNA probe (13). This probe recognizes a 2.3- to 2.5-kilobase mRNA in most of the cell lines and tissues examined (13). Treatment with TGFβ1 alone (or in combination with EGF) resulted in rapid, approximately 25-fold induction of TGFβ1 mRNA levels (Fig. 1A and D). Induction of TGFβ1 mRNA was observed as early as 1 h, with maximal induction between 6 and 12 h. Similar kinetics of TGFβ1 mRNA induction were also observed in proliferating AKR-2B cells (data not shown), suggesting that autoinduction of TGFβ1 mRNA is not dependent on the growth state of the cells. Addition of EGF alone only slightly increased TGFβ1 expression (Fig. 1D). In contrast, TGFβ1 decreased expression of both TGFβ2 and

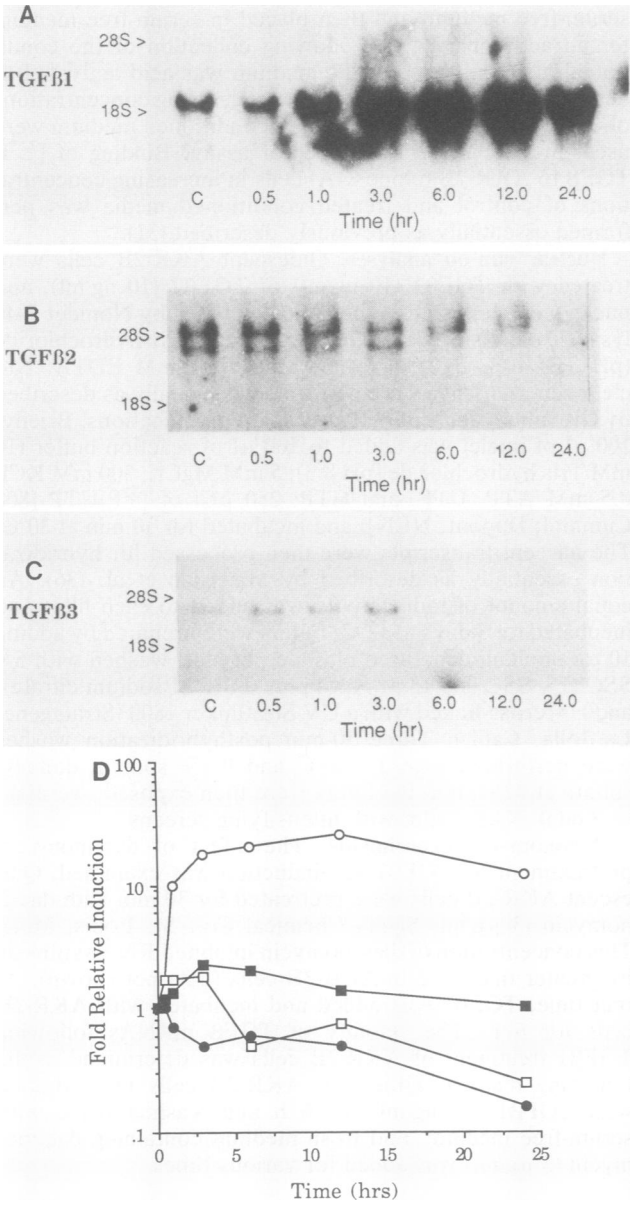

FIG. 1. Regulation of TGFβ1, TGFβ2, and TGFβ3 mRNA expression in quiescent AKR-2B cells by TGFβ1. Quiescent AKR-2B cells were treated with TGFβ1 (10 ng/ml) for various times. At the indicated times, total cellular RNA was collected, and then poly(A)$^+$ RNA was purified by oligo(dT) affinity chromatography and Northern blot analysis was performed. Each lane contained 2 μg of mRNA that was hybridized to a ^{32}P-labeled TGFβ1 cDNA probe (A), a ^{32}P-labeled TGFβ2 cDNA probe (B), or a ^{32}P-labeled TGFβ3 cDNA probe (C). C, Control. Following densitometric scanning and normalization to control 1B15 levels, the effects of TGFβ1 (○) and EGF (■) on TGFβ1 mRNA levels were quantified (D). In addition, the effects of TGFβ1 on TGFβ2 (●) and TGFβ3 (□) mRNA expression were also determined.

TGFβ3 mRNAs in AKR-2B cells. The 4.1- and 5.1-kilobase TGFβ2 transcripts (32) began to decrease after 30 min, and by 12 to 24 h, minimal expression was observed (Fig. 1B and D). TGFβ1 slightly augmented the 3.5- to 3.8-kilobase TGFβ3 transcript (14, 21, 54; Miller et al., in press) from 0.5 to 3 h, but at later times, TGFβ3 expression was also diminished to below control levels (Fig. 1C and D).

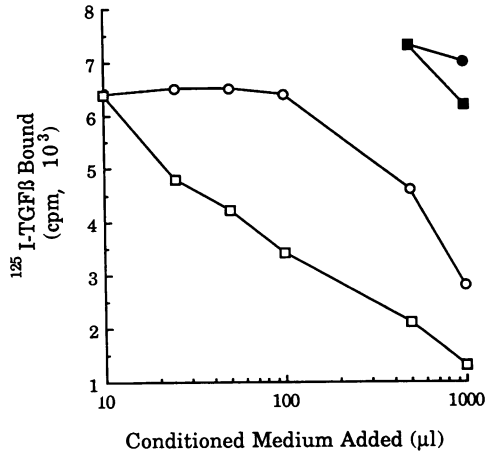

FIG. 2. Increased TGFβ protein release into conditioned medium of AKR-2B cells after TGFβ1 treatment. Quiescent AKR-2B cells were treated with TGFβ1 (10 ng/ml) for 4 h and washed four times with serum-free medium, and serum-free conditioned medium was collected 24 h later. Increasing concentrations of neutral conditioned medium from either control (●) or TGFβ1-treated (■) AKR-2B cells and acid-activated conditioned medium from either control (○) or TGFβ1-treated (□) cells were added and incubated for 2 h at room temperature, and specific binding was determined as previously described (31).

Increased TGFβ protein release into conditioned medium of AKR-2B cells. Treatment of quiescent AKR-2B cells with TGFβ1 also resulted in increased release of TGFβ protein into the conditioned medium. AKR-2B cells were treated for 4 h with TGFβ1, washed four times with serum-free medium, which removes almost all of the added TGFβ (16), and conditioned medium was collected 24 h later. Both neutral and acid-activated samples were assayed for specific competing activity in a TGFβ radioreceptor assay. TGFβ1 increased by about 10-fold the amount of TGFβ protein synthesized and released into the conditioned medium (Fig. 2).

Mediation of TGFβ1 autoinduction: complex regulatory mechanisms. Protein synthesis inhibitors, such as cycloheximide and anisomycin, block TGFβ1 reduction of c-*myc* and KC in BALB/MK cells (8). The effect of cycloheximide on TGFβ1 autoinduction in AKR-2B cells was also examined. After pretreatment with cycloheximide for 15 min, TGFβ1 (10 ng/ml) was added to AKR-2B cells and incubated for 6 h. Induction of TGFβ1 mRNA was not affected by cycloheximide in AKR-2B cells; cycloheximide alone had no effect on TGFβ1 mRNA expression under these conditions (Fig. 3A). Pretreatment with cycloheximide, however, did block TGFβ1 reduction of TGFβ2 expression (data not shown), suggesting that protein synthesis is not required for TGFβ1 autoinduction but is needed for TGFβ1 to decrease TGFβ2 mRNA levels.

Whether autoinduction of TGFβ1 mRNA in AKR-2B cells occurred at a transcriptional or a posttranscriptional level was next examined. Quiescent AKR-2B cells were pretreated with dactinomycin (3 μg/ml) for 30 min, TGFβ1 (10 ng/ml) was added, and the cells were incubated for an additional 6 h. Pretreatment with dactinomycin blocked TGFβ1 autoinduction (Fig. 3B), indicating that a transcriptional component was involved in regulating TGFβ1 autoinduction. Nuclear run-on assays were also performed to confirm that TGFβ1 autoinduction was transcriptionally regulated. Nuclei were harvested from quiescent AKR-2B

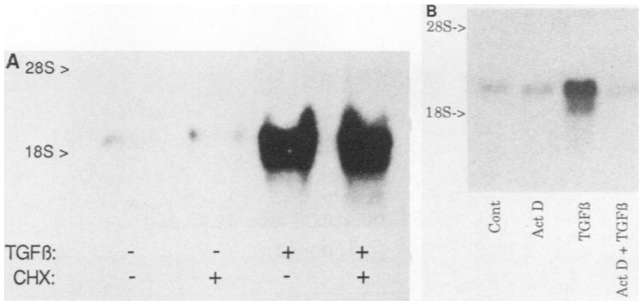

FIG. 3. Effect of cycloheximide (CHX) or dactinomycin (Act D) on TGFβ1 mRNA autoinduction in AKR-2B cells. (A) Quiescent AKR-2B cells were pretreated with 10 μg of cycloheximide per ml for 15 min at 37°C. TGFβ1 (10 ng/ml) was then added to the appropriate condition in cycloheximide and incubated for 6 h. Each lane contained 2 μg of poly(A)$^+$ RNA that was hybridized to a ^{32}P-labeled TGFβ1 cDNA probe. (B) AKR-2B cells were pretreated with dactinomycin (3 μg/ml) for 30 min. TGFβ1 was then added to the appropriate condition and incubated for 6 h. RNA was processed, and each lane contained 1 μg of poly(A)$^+$ RNA hybridized to a ^{32}P-labeled TGFβ1 cDNA probe. Cont, Control.

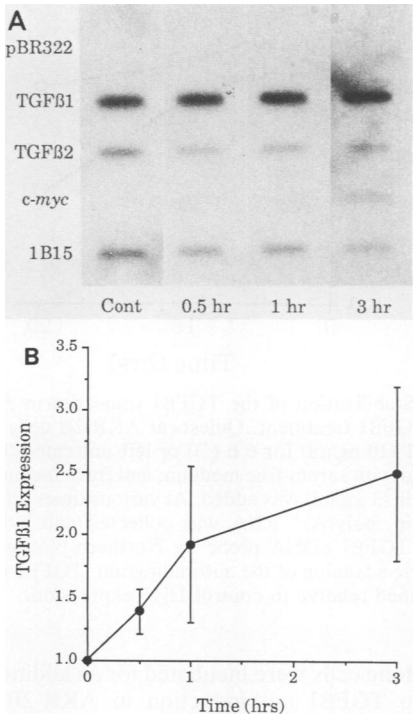

FIG. 4. Nuclear run-on analysis of TGFβ1-treated AKR-2B cells. (A) Nuclei were collected from quiescent AKR-2B cells treated with TGFβ1 (10 ng/ml) for 0.5, 1, or 3 h. The nuclear run-on analysis was performed as described in Materials and Methods. Ten micrograms each of alkali-denatured pBR322, TGFβ1, TGFβ2, pSVc-*myc*, and 1B15 plasmids was spotted per well and hybridized to an equal amount of radioactivity for 4 days at 42°C. Cont, Control. (B) Quantification of the relative increase in TGFβ1 transcription in AKR-2B fibroblasts by laser densitometry. The results represent the means and ranges of three individual experiments at these time points.

cells treated with TGFβ1 for 0.5, 1, or 3 h, and the nuclear run-on transcription assay was performed. Transcription of the TGFβ1 gene was increased in AKR-2B cells only about two- to threefold (Fig. 4A and B). As a positive control, TGFβ1 induced approximately two- to fourfold induction of c-*myc* transcription (Fig. 4A). The observed increase in TGFβ1 transcription probably could not account for the 20-fold increase in steady-state levels. Therefore, potential stabilization of TGFβ1 mRNA by TGFβ1 treatment of AKR-2B cells was examined. Quiescent AKR-2B cells were treated with TGFβ1 for 6 h, the cells were washed, and medium containing dactinomycin (3 μg/ml) was added for 5.0, 10.0, or 20 h. The half-life of TGFβ1 mRNA in untreated cells was approximately 15 h (Fig. 5); however, after treatment with TGFβ1, the half-life of TGFβ1 mRNA increased to greater than 20 h (Fig. 5). In this same experiment, TGFβ1 did not alter the half-life of the messages for either fibronectin or c-*sis* (data not shown). Thus, the data indicate that the large increase in steady-state levels of TGFβ1 mRNA was the result of transcriptional activation of the TGFβ1 gene in addition to increased stability of the TGFβ1 message following TGFβ1 treatment of AKR-2B cells.

Positive regulation of TGFβ1, TGFβ2, and TGFβ3 expression by TGFβ2. Potential regulation of TGFβ1, TGFβ2, and TGFβ3 expression by TGFβ2 was investigated. Quiescent AKR-2B cells were treated with TGFβ2 (10 ng/ml) for different times. Autoinduction of TGFβ2 mRNA was observed, but not to the same magnitude as TGFβ1 autoinduction, and the kinetics were also different. Autoinduction of TGFβ2 expression (two- to fourfold) was seen after 30 min and was maximal from 1 to 3 h (Fig. 6A and D). TGFβ2 also induced TGFβ1 expression. Increased steady-state levels of TGFβ1 mRNA were observed after 3 h, and maximal induction (20-fold) was observed after 12 to 24 h (Fig. 6B and D). Further, eightfold induction of TGFβ3 expression was also observed in AKR-2B cells at 3 h after TGFβ2 treatment (Fig. 6C and D).

The mechanism by which TGFβ2 (auto)regulates expression of various TGFβ genes was examined in a nuclear run-on transcription assay. Nuclei were harvested from quiescent AKR-2B cells treated with TGFβ2 for 0.5, 1, or 3 h. The data demonstrated that TGFβ2 increased transcription of its own gene about two- to threefold at the 0.5- and 1.0-h time points (Fig. 7). In addition, an eight- to ninefold increase in transcription of the TGFβ3 gene was also observed (Fig. 7). As with TGFβ1 autoinduction, TGFβ2 increased TGFβ1 transcription only about 1.5- to 2.0-fold (Fig. 7). As a positive control, transcription of c-*myc* was increased fourfold at 3 h.

TGFβ1 autoinduction in mouse keratinocytes. TGFβ1 effects on TGFβ expression in BALB/MK cells, which are reversibly growth arrested by TGFβ1, were also analyzed to determine whether TGFβ1 autoinduction was restricted to fibroblastic cells. Proliferating BALB/MK cells were treated with TGFβ1 (10 ng/ml) for various times, and Northern analysis was performed with the TGFβ1 cDNA probe. TGFβ1 autoinduction was observed, but with a magnitude and kinetics different from those in fibroblastic cells (Fig. 8). Only a two- to fourfold increase was demonstrated, with maximal induction after 12 to 24 h of TGFβ1 treatment. In contrast to AKR-2B cells, TGFβ1 did not decrease but slightly increased the low levels of TGFβ2 and TGFβ3 mRNA expression in BALB/MK cells (data not shown), suggesting that regulation of TGFβ1, TGFβ2, and TGFβ3 expression by TGFβ1 varies depending on the cell type examined.

The effect of cycloheximide (2.5 μg/ml) on TGFβ1 autoinduction was examined. BALB/MK cells were pretreated with cycloheximide for 15 min, TGFβ1 (10 ng/ml) was

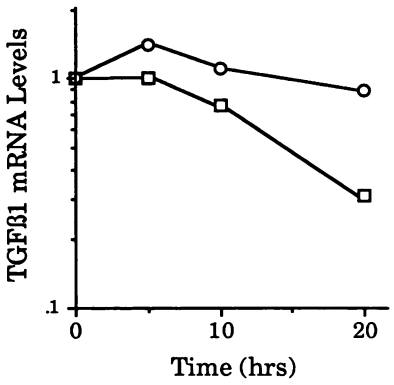

FIG. 5. Stabilization of the TGFβ1 transcript in AKR-2B cells following TGFβ1 treatment. Quiescent AKR-2B cells were treated with TGFβ1 (10 ng/ml) for 6 h (○) or left untreated (□). The cells were washed with serum-free medium, and fresh medium containing dactinomycin (3 μg/ml) was added. At various times after addition of dactinomycin, poly(A)$^+$ RNA was collected and hybridized to a ^{32}P-labeled TGFβ1 cDNA probe by Northern blot analysis. After densitometric scanning of the autoradiogram, TGFβ1 mRNA levels were quantified relative to control 1B15 expression.

added, and the cells were incubated for an additional 12 h. In contrast to TGFβ1 autoinduction in AKR-2B fibroblasts (Fig. 4A), cycloheximide blocked TGFβ1 autoinduction in murine keratinocytes (Fig. 9).

DISCUSSION

Several reports have established that growth factors can regulate cell proliferation by controlling the expression of other positive and negative growth factors and growth factor receptors (1, 7, 15, 27, 33). Furthermore, it has been shown that TGFα (9), platelet-derived growth factor (43), melanoma growth stimulatory activity (4), and TGFβ1 (59) can regulate expression of their own genes as a possible mechanism for further modulation of cell growth. TGFβ1 message (auto)induction in both murine fibroblasts and keratinocytes was examined, and the investigation was extended to include regulation of TGFβ2 and TGFβ3 expression in these cells. Treatment of mouse embryo-derived AKR-2B fibroblasts with porcine TGFβ1 resulted in rapid and substantial autoinduction of the TGFβ1 message. However, the approximately 25-fold induction of steady-state levels of TGFβ1 mRNA was much greater than what has been observed in normal rat kidney, 3T3, or A549 cells (59). Stimulation of AKR-2B cells with EGF had a minimal effect on TGFβ1 expression and had no effect on TGFβ1 mRNA autoinduction; this is consistent with what has been observed in normal rat kidney cells (59). An increase in specific TGFβ receptor competing activity in the conditioned medium of AKR-2B cells was also demonstrated, suggesting that there is not only an increase in TGFβ1 message levels but also an increase in TGFβ protein production.

In contrast to TGFβ1, expression of TGFβ2 and TGFβ3 was decreased by TGFβ1 in AKR-2B cells. It is unclear why TGFβ1 would down regulate expression of TGFβ2 and TGFβ3. It might suggest that TGFβ1 in fibroblastic cells has a more dominant or selective role and that when production of TGFβ1 is increased by some other factor it serves as a negative feedback control to diminish expression of other TGFβ genes. The ability of exogenous factors to regulate TGFβ1, TGFβ2, or TGFβ3 expression selectively might be

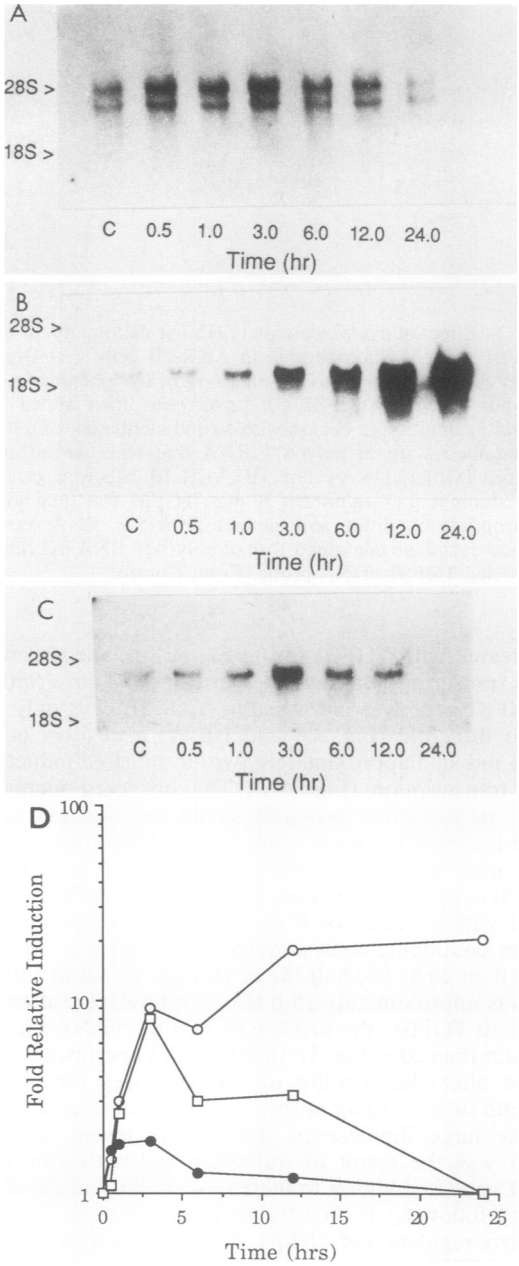

FIG. 6. Regulation of TGFβ2, TGFβ1, and TGFβ3 mRNA levels by TGFβ2 in quiescent AKR-2B cells. TGFβ2 (10 ng/ml) was added to quiescent AKR-2B cells for the indicated times. Two micrograms of poly(A)$^+$ RNA was then processed for Northern blot analysis and hybridized to a ^{32}P-labeled TGFβ2 cDNA probe (A), a ^{32}P-labeled TGFβ1 cDNA probe (B), or a ^{32}P-labeled TGFβ3 cDNA probe (C). C, Control. After scanning densitometry and normalization to control 1B15 levels, the effects of TGFβ2 on TGFβ1 (○), TGFβ2 (●), and TGFβ3 (□) mRNA expression were quantified (D).

significant. This was made evident by recent demonstrations that TGFβ1 and TGFβ2 differentially regulate hematopoietic differentiation, inhibition of endothelial proliferation, or mesodermal induction (23, 41, 48).

TGFβ1 autoinduction was also observed in murine skin keratinocytes (BALB/MK cells); however, the magnitude was much less and the kinetics were different in comparison with those observed in AKR-2B fibroblasts. In BALB/MK

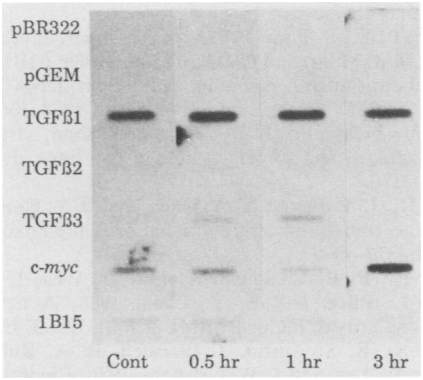

FIG. 7. Nuclear run-on analysis of TGFβ2-treated AKR-2B cells. Nuclei were harvested from quiescent AKR-2B cells treated with TGFβ2 for 0.5, 1.0, or 3 h. Nuclear run-on analysis was performed as described in Materials and Methods. Ten micrograms each of alkali-denatured pBR322, pGEM, TGFβ1, TGFβ2, TGFβ3, pSVc-*myc*, and 1B15 plasmids was spotted per well and hybridized to an equal amount of radioactivity for 4 days at 42°C. Cont, Control.

cells, maximal induction was only two- to fourfold and occurred between 12 and 24 h following addition of TGFβ1. Further differences in gene regulation by TGFβ1 in AKR-2B and BALB/MK cells were illustrated by the fact that expression of TGFβ2 and TGFβ3 was slightly augmented by TGFβ1 in mouse keratinocytes. The signal transduction pathway responsible for TGFβ1 mRNA induction appears to be related to cell type because cycloheximide blocked autoinduction in BALB/MK cells while not affecting TGFβ1 autoinduction in AKR-2B cells.

TGFβ1 transcriptionally regulates expression of several genes, including fibronectin and collagen (20, 49), EGF receptor (55), and osteopontin (40). However, it is important to note that, in some instances, regulation of certain genes, such as fibronectin (12, 44, 46), by TGFβ1 is different and complex, depending on the cell type examined. Therefore, one cannot assume a priori that TGFβ1 autoinduction would occur in a similar fashion in all cells. Therefore, the mechanism by which TGFβ1 regulates its own expression in both AKR-2B and BALB/MK cells was examined. The data indicated that regulation of TGFβ1 mRNA expression in AKR-2B cells is complex, involving both increased transcription of the TGFβ1 gene and stabilization of the TGFβ1 transcript. Several nuclear run-on analyses consistently demonstrated only a two- to threefold increase in transcription of the TGFβ1 gene after TGFβ1 treatment of AKR-2B cells. In addition, the TGFβ1 transcript was found to be highly stable, with a half-life of about 15 h; however, the

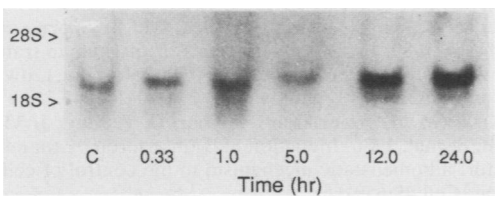

FIG. 8. Autoinduction of TGFβ1 mRNA in proliferating BALB/MK cells. Rapidly growing BALB/MK cells were treated with TGFβ1 (10 ng/ml), and at the indicated times, poly(A)$^+$ RNA was processed for Northern blot analysis. Each lane contained 2 μg of RNA hybridized to a ^{32}P-labeled TGFβ1 cDNA probe. C, Control.

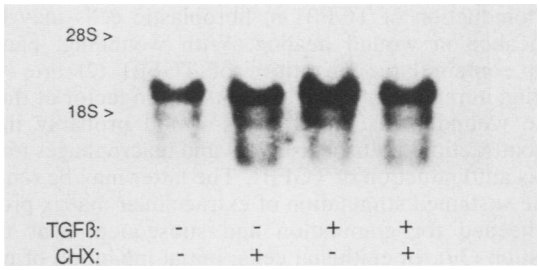

FIG. 9. Effect of cycloheximide (CHX) on TGFβ1 autoinduction in BALB/MK cells. Proliferating BALB/MK cells were pretreated with 2.5 μg of cycloheximide per ml for 15 min at 37°C. TGFβ1 (10 ng/ml) was then added to the appropriate condition, still in cycloheximide, and incubated for 12 h. Poly(A)$^+$ RNA was purified, and Northern blot analysis was performed. Each lane contained 2 μg of mRNA hybridized to a ^{32}P-labeled TGFβ1 cDNA probe.

half-life of the message was increased to greater than 20 h following TGFβ1 treatment. Thus, the slight stimulatory effect that was observed in transcription of the TGFβ1 gene, along with the stabilizing effect on the message, could lead to the observed large increase in steady-state levels of TGFβ1 mRNA. In a previous study (57), van Obberghen-Schilling et al. observed only a two- to fourfold increase in TGFβ1 mRNA levels in both normal and transformed cells, and this effect was suggested to occur at the transcriptional level, although the stability of the message was not described in that report. Perhaps the lack of mRNA stabilization in their cells accounts for the low level of induction observed relative to that of AKR-2B cells. Preliminary findings suggest that autoinduction of TGFβ1 in BALB/MK cells might occur through a similar mechanism.

It is now important to define regulatory regions within the TGFβ1, TGFβ2, and TGFβ3 genes that are involved in both induction and repression of these genes by TGFβ1 and TGFβ2. In fact, the promoter region for human TGFβ1 was recently obtained (25), and a region within the promoter that mediates TGFβ1 autoinduction in A549 cells has been identified (26). Because of the specificity and complexity of TGFβ1 regulation of TGFβ gene expression within AKR-2B cells, it would be interesting to examine whether similar regions are involved in TGFβ1 autoinduction in these cells. Further, it is also of interest to determine regulatory regions that mediate TGFβ1 reduction of TGFβ2 and TGFβ3 gene expression. This, however, is only partially involved in regulating TGFβ1 mRNA levels, since TGFβ1 treatment of AKR-2B cells stabilizes the TGFβ1 transcript. In addition, preliminary data indicate that TGFβ2 and TGFβ3 mRNAs are also stabilized within AKR-2B cells after TGFβ1 treatment. This suggests that there is a consensus sequence involved in controlling the turnover of various TGFβ mRNAs.

The results of these studies reveal another circumstance in which TGFβ1 and TGFβ2 elicit different responses. In fibroblastic cells, TGFβ1 induces TGFβ1 mRNA but suppresses TGFβ2 and TGFβ3 expression, whereas TGFβ2 induces all three genes. These results also demonstrate that expressions of the three TGFβ genes are regulated differently. This is consistent with previous data from our laboratory demonstrating different expressions of TGFβ1, TGFβ2, and TGFβ3 in cell lines (14) and in tissues in vivo (36a; Miller et al., in press). These data suggest that one reason mammals have three such closely related TGFβ genes is the need for differential regulation of expression in different physiological and pathological circumstances.

Autoinduction of TGFβ1 in fibroblastic cells may be of significance in wound healing. With wounding, platelets which contain large quantities of TGFβ1 (2) are lysed, resulting in release of large amounts of the factor at the site of the wound. This initial bolus would probably induce chemoattraction of fibroblasts (45) and macrophages (58), as well as autoinduction of TGFβ1. The latter may be required for the sustained stimulation of extracellular matrix production needed for granulation and subsequent scar tissue formation (39). In epithelial cells, initial inhibition of proliferation by TGFβ1 may be beneficial, since it would be desirable to retard overgrowth of the epithelium until there is a substratum for epithelial support. However, sustained inhibition of epithelial growth would be deleterious because eventual coverage of the wound is necessary. Hence, marked autoinduction of TGFβ1 in epithelial cells is probably not needed and was not observed. In vivo studies of TGFβ1 autoinduction are needed to determine the possible role of this interesting phenomenon in wound healing.

ACKNOWLEDGMENTS

We gratefully acknowledge Dot Blue for preparation of the manuscript.

This investigation was supported by Public Health Service grant CA 42572 from the National Cancer Institute. C.C.B. is the recipient of Public Health Service postdoctoral fellowship CA 09592 from the National Cancer Institute.

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