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Secretion and Transcriptional Regulation of Transforming Growth Factor-β3 during Myogenesis

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Transforming growth factor-β3 (TGF-β3) mRNA is differentially expressed in developing and mature mouse tissues, including high-level expression in developing and adult cardiac tissue. We show now that TGF-β3 mRNA is also expressed highly in skeletal muscle as well as in the mouse skeletal myoblast cell line C_2C_{12}. We also show that C_2C_{12} cells secrete TGF-β3, and that this TGF-β is able to inhibit C_2C_{12} myoblast fusion after activation. In order to begin to understand how the TGF-β3 promoter is regulated in specific tissues during development, we therefore studied the regulation of TGF-β3 during myoblast fusion. After fusion of C_2C_{12} cells into myotubes, TGF-β3 mRNA levels increased eightfold as a result of increased TGF-β3 transcription. TGF-β3 transcriptional regulation was studied in myoblasts and myotubes by transfection of chimeric TGF-β3/CAT promoter plasmids. Chloramphenicol acetyltransferase (CAT) activity was stimulated in myoblasts by several upstream regions between −301 and −47 of the TGF-β3 promoter and by the TGF-β3 5′ untranslated region. CAT activity directed by the TGF-β3 promoter in myotubes was stimulated by a distinct upstream region located between −499 and −221. Therefore, the high level of TGF-β3 mRNA expression in muscle cells appears to be dependent on multiple regulatory events during different stages of myogenesis.

Three isoforms of transforming growth factor-β (TGF-β), TGF-β1, TGF-β2, and TGF-β3, are found in mammalian species (10–14, 25, 26, 34, 35). Two other cloned TGF-βs, TGF-β4 and TGF-β5, have been found only in chickens and *Xenopus laevis*, respectively (19, 21). TGF-β1, TGF-β2, and TGF-β3 have similar biological activities in most in vitro assays, including stimulation of AKR-2B and inhibition of CCL-64 and MCF-7 cell proliferation, although with somewhat different potencies (18). This is consistent with the finding that activated TGF-β3 competes with TGF-β1 for receptor binding on AKR-2B cells (18). It is noteworthy that TGF-β3 has been shown to be much more potent than TGF-β1 and TGF-β2 in some systems, such as mesoderm induction (33); the reason for these differences is unknown.

Although the activated peptides often share similar biological activities, we and others have noted that TGF-β1, TGF-β2, and TGF-β3 mRNAs are differentially expressed in various mouse tissues during development (11, 15, 25, 30). For example, TGF-β3 is expressed highly in the embryonic heart and lung (11) but minimally in the liver, spleen or kidney. TGF-β1, on the other hand, is expressed most highly in the spleen. Further, TGF-βs are known to have profound effects on cell differentiation (reviewed in references 32 and 34). These studies suggest that differential regulation of the various TGF-βs is involved in the processes of tissue development and cellular differentiation during embryogenesis.

Recently, we have presented evidence that TGF-β3 mRNA expression correlates closely with TGF-β3 promoter activity in a variety of tumor cell lines by using chimeric TGF-β3 promoter/chloramphenicol acetyltransferase (CAT) reporter plasmid constructs (22). These studies revealed the presence of several upstream regulatory regions as well as a cAMP responsive element (CRE) and an AP-2 consensus binding site proximal to a TATA box that is responsible for transcription initiation. Since these initial studies were limited to tumor cell lines, it seemed important to continue the study of TGF-β3 regulation in nontumor cells. In this regard, the high-level expression of TGF-β3 in the heart that was noted above was of particular interest. Since skeletal muscle differentiation has been studied in greater detail in vitro, we investigated whether, similar to cardiac muscle, skeletal muscle expresses high levels of TGF-β3. We found that both mouse skeletal muscle tissue and the mouse skeletal myoblast cell line C_2C_{12} express high levels of TGF-β3.

The differentiation of myoblasts to myotubes has been studied in a variety of myoblast cell lines, and several of the genes regulating differentiation have been characterized. Further, TGF-β1 is known to completely inhibit differentiation of myoblasts (16, 24, 29). Therefore, in order to investigate the role of TGF-β3 during differentiation, we studied TGF-β3 secretion, expression, and promoter regulation during myoblast fusion in vitro. We show that transcription of TGF-β3 in myoblasts is highly dependent on several upstream 5′ flanking regions, including the 5′ untranslated region (UTR), and that a different upstream region is responsible for increased transcriptional activity in fused myotubes, resulting in increased mRNA expression and secretion of TGF-β3 by mature muscle cells.

MATERIALS AND METHODS

Materials. TGF-β1 and TGF-β2 were obtained from R & D Systems Inc. (Minneapolis, Minn.). Recombinant TGF-β3 was purified to homogeneity from stably transfected NIH 3T3 cells by high-pressure liquid chromatography as described previously (33). Dactinomycin was obtained from Sigma Chemical Co. (St. Louis, Mo.).

Cell culture. The mouse skeletal myoblast line C_2C_{12} was obtained from the American Type Culture Collection (Rockville, Md.) and cultured at low cell density (to prevent fusion) in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS; GIBCO Laboratories, Grand Island, N.Y.) in the presence of antibi-

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otics (50 U of penicillin per ml, 50 mg of streptomycin per ml). In order to induce fusion, cells were placed in the same medium, with replacement of the 10% FBS with 1% horse serum (HS; GIBCO Laboratories). Cells were grown in a humidified incubator at 37°C in 5% CO_2.

RNA extraction and analysis. Total RNA was prepared by the disruption of cells in guanidinium isothiocyanate buffer and centrifugation over a CsCl cushion as described previously (8). After a phenol extraction and ethanol precipitation, 10 μg of each RNA sample was separated by electrophoresis through a 1 M formaldehyde–1% agarose gel and blotted onto a Nytran membrane (Schleicher and Schuell, Keene, N.H.). Filters were baked 1 h at 80°C under vacuum. Insert cDNAs were cut with restriction enzymes from plasmids containing mouse TGF-β1, TGF-β2, or TGF-β3 and were purified on gels. Mouse TGF-β1 cDNA was cut from the plasmid pMurb2 (13). Mouse TGF-β2 insert cDNA was cut from pmTGF-β2, clone 17.5. This is a plasmid containing the entire mouse TGF-β2 coding region obtained by polymerase chain reaction (10a). Mouse TGF-β3 insert cDNA was cut from pmTGF-β3, clone 9, containing the entire mouse TGF-β3 coding region (11). Insert cDNAs were labeled with ^{32}P by random primed labeling (kit from Boehringer Mannheim). Prehybridization, hybridization, and washing of the filters were done as described previously (7). Quantitation by transmittance densitometry was performed on an LKB/Pharmacia laser densitometer, model 2202. Graphical analysis was performed with CricketGraph software (Cricket Software, Malvern, Pa.). The mRNA half-life was determined by using the best-line fit for a logarithmic curve in the CricketGraph software program.

TGF-β assay. TGF-β was assayed by a method similar to one previously described (36). CCL64 cells (obtained from American Type Culture Collection) were maintained in tissue culture in DMEM supplemented with 10% FBS. For assaying TGF-β, cells were cultured at 10^4 cells per well in 96-well tissue culture dishes. The next day, the cells were washed with serum-free DMEM and cultured in DMEM supplemented with 0.2% FBS. Either recombinant TGF-β or C_2C_{12} supernatant was then added to the wells, and incubation was continued. C_2C_{12} cell supernatants were either heated to 85°C for 10 min (to measure total TGF-β in the sample) or added without heat activation (to measure only the TGF-β already active in the supernatant). After 24 h, 1 μCi of [^{3}H]thymidine (5 mCi/mmol) was added to each well and incubation was continued an additional 24 h. Following this incubation, cells were washed twice with Ca^{2+}- and Mg^{2+}-free phosphate-buffered saline, 50 μl of trypsin-EDTA was added to each well, incubation was continued for 15 min, and cells were lysed onto glass fiber filters by using a cell harvester. Filters were counted in a scintillation counter after the addition of scintillation fluid. All points (wells) were measured in triplicate. To measure TGF-β isotype, neutralizing antibodies specific for TGF-β1 or TGF-β2 (both provided by D. Danielpour) or for TGF-β1, TGF-β2, and TGF-β3 (R & D Systems Inc.) were added to wells 15 min before the addition of the samples of TGF-β or C_2C_{12} cell supernatant. Controls demonstrating the specificity and neutralizing capacity of the antibodies were carried out in parallel.

Plasmid construction. The chimeric promoter/CAT plasmids pB3−120 and pB3+1083 were constructed by ligation of TGF-β3 5′ flanking region fragments of ph17.4 (22) produced by polymerase chain reaction into the promoterless CAT containing plasmid pGEM4-SV0CAT (20) as previously described (22). The 3′ oligonucleotides used in amplifications to construct pB3−120 and pB3+1083 corresponded to the 20-bp sequences at +110 and +1083 of ph17.4 to which an *Xba*I site and four random nucleotides were added. The 5′ oligonucleotides used in amplifications to construct pB3−120 and pB3+1083 corresponded to 20-bp sequences terminating at positions −301 and −91, respectively, of ph17.4 to which a *Hin*dIII site and four random nucleotides were added. By using these oligonucleotides, fragments were amplified according to the standard protocol of the GeneAmp kit (Perkin Elmer Cetus, Norwalk, Conn.). These fragments were each digested with *Hin*dIII and *Xba*I, gel purified, and ligated into the multiple cloning site of pGEM4-SV0CAT. Approximately 150 bp of sequence was obtained from all promoter clones to verify proper orientation of the inserts.

Cell transfections and CAT assays. Plasmids were prepared for transfection by double CsCl banding (23). Plasmid DNA (10 μg) from each TGF-β3 promoter/CAT chimeric plasmid was cotransfected with 2 μg of pXGH5 (an expression plasmid containing the human growth hormone gene under regulation of the mouse metallothionein I promoter; obtained from Nichols Institute Diagnostics, San Juan Capistrano, Calif.). This plasmid was included as an internal control to allow for normalization of transfection efficiency. DNA was transfected by calcium phosphate coprecipitation (17). The cells were exposed to the precipitate for 10 h and washed until all precipitate was removed. In experiments to examine myotube promoter activity, after transfection the cells were lifted from the transfection plates by trypsin-EDTA, divided between two dishes, allowed to adhere overnight in 10% FBS, and then either grown in media containing 10% FBS or allowed to differentiate in media containing 1% HS. After 3 days (myoblast cultures) or 6 days (myotube cultures), the cells were harvested and CAT activity was determined in the cell lysates (26). Cell transfections were repeated at least three times in all experiments shown, with similar results.

RESULTS

Expression of TGF-βs in skeletal and cardiac muscles. Our previous studies on TGF-β3 expression in mouse tissues had shown high levels of expression of TGF-β3 in the heart. In order to see whether TGF-β3 is also expressed highly in skeletal muscle, tissue was dissected from the quadriceps femoris of an adult mouse and analyzed for TGF-β3 expression. For comparison, RNA from heart tissue was prepared at the same time. TGF-β3 mRNA was expressed at similar levels in both skeletal and cardiac muscle tissues (Fig. 1B, bottom panel). Although both these tissues are heterogeneous, including connective tissue, endothelial tissue, and other cell types, immunohistochemistry showing specific staining for TGF-β3 in cardiac and skeletal myocytes suggests that myocytes are largely responsible for the TGF-β3 mRNA expression shown (15a). These tissue RNAs were also hybridized to TGF-β1 and TGF-β2 cDNAs; TGF-β1 and TGF-β2 were also expressed in both of these muscle tissues (Fig. 1B, top and middle panels). These results are in disagreement with the results of Miller et al. (25), who reported low-level expression of TGF-β1 in the mouse heart. This difference is probably largely the result of the use of different cDNA probe fragments, probe specific activities, hybridization protocols, and radiographic exposure times. Although we cannot compare relative transcript numbers from these experiments, it remains possible that TGF-β3 is the dominant mRNA expressed in muscle despite the higher

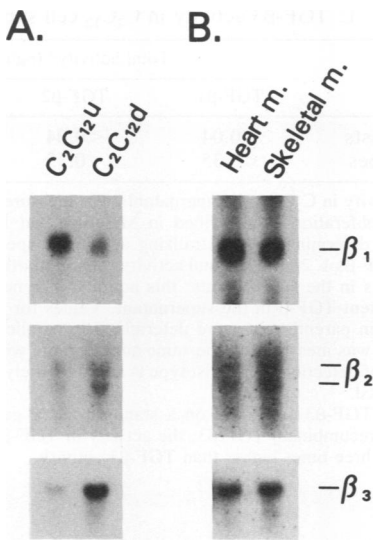

FIG. 1. TGF-β1, TGF-β2, and TGF-β3 expression in mouse muscle in vivo and in vitro. RNA was extracted from C_2C_{12} myoblasts ($C_2C_{12}u$) and C_2C_{12} myotubes ($C_2C_{12}d$) (A) and mouse heart (heart m.) and skeletal muscle (skeletal m.) tissues (B). Ten micrograms of each RNA sample was electrophoresed on a formaldehyde-agarose gel, blotted onto Nytran, and hybridized sequentially with ^{32}P-labeled cDNA of mouse TGF-β1, TGF-β2, and TGF-β3 as described in Materials and Methods.

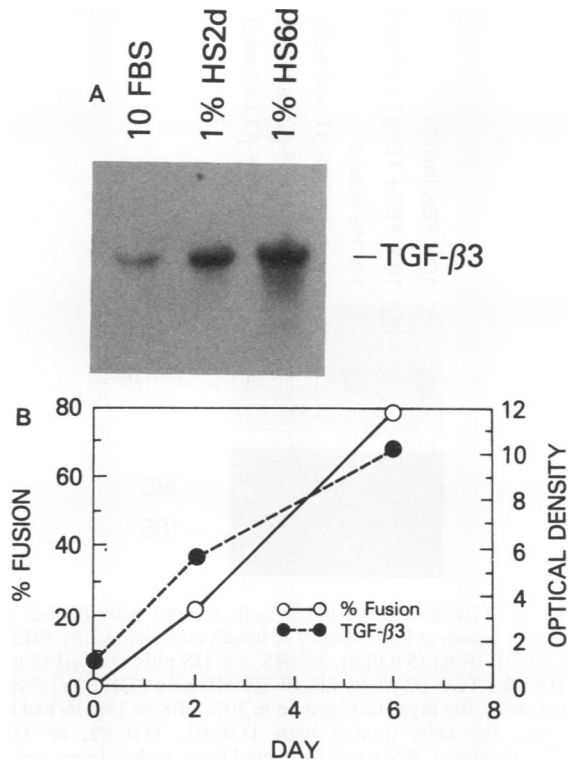

FIG. 2. Kinetics of TGF-β3 induction during fusion of C_2C_{12} myoblasts. C_2C_{12} cells were grown in 10% FBS (10 FBS), in 1% HS for 2 days (1% HS2d), or in 1% HS for 6 days (1% HS6d). The extent of myoblast fusion was estimated by counting nuclei in fused versus unfused cells, and it was found to be 0% in the cells grown in 10% FBS, 22% in the cells grown for 2 days in 1% HS, and 78% in the cells grown for 6 days in 1% HS. RNA was then extracted, and 10 μg of each RNA sample was electrophoresed on a formaldehyde-agarose gel, blotted onto Nytran, and hybridized with ^{32}P-labeled cDNA of mouse TGF-β3. (A) raw data (radiograph); (B) results of densitometric analysis of the radiograph in which the results are plotted together with estimated percentage of cell fusion.

intensity of the TGF-β1 mRNA signal seen in Fig. 1B. The relative importance of TGF-β3 expression by muscle cells is further supported by the observation that TGF-β3 is the predominant form of TGF-β secreted by the mouse myoblast cell line C_2C_{12} (shown below).

Expression of TGF-βs in myoblasts and myotubes in vitro. We have previously observed that TGF-β3 expression increases in whole embryos during early gestation. TGF-β3 mRNA expression has been observed in the heart at 18 days postconception (11) and TGF-β3 protein stained as early as 11 days postconception (15a). These results suggested that TGF-β3 might be regulated during cardiac muscle cell differentiation and, therefore, might also be regulated during skeletal muscle cell differentiation. To test this hypothesis, the mouse (skeletal) myoblast cell line C_2C_{12} was analyzed for TGF-β3 mRNA expression. These cells grow as myoblasts when maintained in media containing 10% FBS at low cell density and fuse to form myotubes in media containing 1% HS or at high cell density. RNA from unfused and fused C_2C_{12} cells was hybridized to TGF-β1, TGF-β2, and TGF-β3 cDNAs (Fig. 1A). Transcripts for TGF-β1, TGF-β2, and TGF-β3 were seen in both unfused and fused C_2C_{12} cells. TGF-β1 transcripts decreased after fusion (0.40-fold by densitometric scanning of the radiograph), while TGF-β2 transcripts increased by 1.7-fold. TGF-β3 transcripts also increased, but to a much greater degree, 7.9-fold.

Kinetics of TGF-β3 expression in fusing C_2C_{12} cells. The relationship between myoblast fusion and TGF-β3 expression was further explored by studying the kinetics of these two events. Myoblasts were placed in 1% HS for 0, 2, or 6 days before RNA was extracted and hybridized to a TGF-β3 cDNA probe. Two days after placing the cells in 1% HS, approximately 22% of the cells were fused (assessed by counting nuclei in fused versus unfused cells). Six days after placing the cells in 1% HS, 78% of the cells were fused. Increased TGF-β3 expression was associated with cell fusion (Fig. 2A and B).

Effects of TGF-β1, TGF-β2, and TGF-β3 on myoblast fusion and TGF-β3 expression. TGF-β1 is known to inhibit the fusion of cultured myoblasts. Since TGF-β1 mRNA was reduced but TGF-β2 and TGF-β3 were increased after fusion (Fig. 1A), it was important to determine whether the changing mRNA levels of the three TGF-β isotypes might be affecting myoblast fusion through autocrine regulation by secreted TGF-β protein(s). This question was addressed indirectly by testing the effects of activated TGF-β1, TGF-β2, and TGF-β3 on myoblast fusion. TGF-β3 mRNA levels were also determined in these cultures to more completely determine the relationship between myoblast fusion and TGF-β3 expression. Therefore, C_2C_{12} cells were left untreated or were treated with TGF-β1, TGF-β2, or TGF-β3 in media containing 1% HS. In parallel, C_2C_{12} cells were allowed to continue to grow in media containing 10% FBS; these cells were left untreated or were treated with TGF-β1. After 5 days, the cells were examined for fusion and analyzed for TGF-β3 mRNA expression (Fig. 3). After prolonged culture (5 days) at relatively high density (by day 3 of the experiment the cells were confluent), the C_2C_{12} cells fused even in the presence of 10% FBS. This fusion was

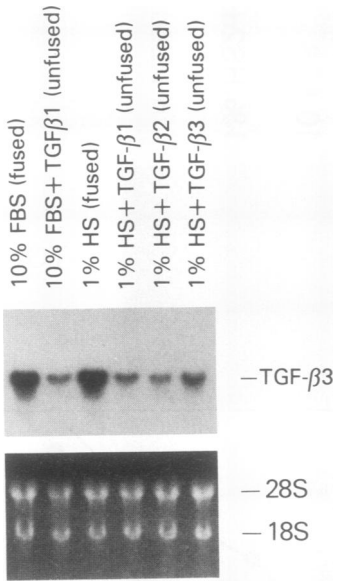

FIG. 3. TGF-β3 expression in cells treated with TGF-β. C_2C_{12} cells were grown at high density in media containing 10% FBS, 10% FBS plus TGF-β1 (5 ng/ml), 1% HS, 1% HS plus TGF-β1 (5 ng/ml), 1% HS plus TGF-β2 (5 ng/ml), or 1% HS plus TGF-β3 (1.7 ng/ml). After 6 days, the myoblasts grown in 10% FBS or 1% HS had fused; however, the cells treated with TGF-β1, TGF-β2, or TGF-β3 remained unfused. RNA was extracted from each culture, and 10 μg of each RNA sample was electrophoresed on a formaldehyde-agarose gel, blotted onto Nytran, and hybridized to ^{32}P-labeled mouse TGF-β3 cDNA. Corresponding ethidium bromide-stained ribosomal RNA of the samples prior to transfer is pictured in the lower panel.

associated with high-level expression of TGF-β3 mRNA; both fusion and TGF-β3 expression were inhibited by exogenous TGF-β1 (Fig. 3, first two lanes). Cells placed in 1% HS also fused, with resulting high levels of TGF-β3 mRNA (Fig. 3, third lane). TGF-β1, TGF-β2, and TGF-β3 each inhibited both fusion and TGF-β3 expression in these cells.

Regulation of TGF-β3 mRNA by TGF-β1 has not been seen in other cell types we have examined, including A375 and HT1080 cells (21a), although both TGF-β1 and TGF-β2 have been reported to regulate TGF-β3 mRNA in AKR-2B cells at early time points after stimulation (1). We suggest that in C_2C_{12} cells, the TGF-βs inhibit TGF-β3 expression indirectly by inhibiting fusion. Further, these results show that the change in serum is likewise not directly responsible for the change in TGF-β3 expression, since cells that fuse in 10% FBS also show increased TGF-β3 expression. Therefore, it appears that some pathway in the fusion process stimulates TGF-β3 expression.

C_2C_{12} cells secrete latent TGF-β3. The inhibition of fusion by exogenous TGF-β1, TGF-β2, and TGF-β3 indicates that active forms of these TGF-βs are not produced by fusing myoblasts in concentrations sufficient to inhibit fusion despite high-level mRNA expression. This apparent paradox could be explained by the secretion of primarily latent TGF-β(s). We therefore measured the level of TGF-βs secreted by C_2C_{12} cells. These assays revealed that both unfused and fused C_2C_{12} cells produce TGF-β and that this TGF-β is, within the sensitivity of the assay, entirely TGF-β3 (Table 1). The level of TGF-β3 secreted by myotubes was 8.7 times higher than that secreted by myoblasts.

TABLE 1. TGF-β3 activity in C_2C_{12} cell supernatants

Supernatant source	Total activity[a] (ng/ml) of:		
	TGF-β1	TGF-β2	TGF-β3[b]
C_2C_{12} myoblasts	<0.04	<0.04	0.8 (4.7%)
C_2C_{12} myotubes	<0.35	<0.35	7.0 (<2%)

[a] TGF-β activity in C_2C_{12} cell supernatants was measured by inhibition of CCL64 cell proliferation as described in Materials and Methods. Isotype specificity was determined by neutralizing antibodies specific for TGF-β1, TGF-β2, or TGF-βs 1, 2, and 3. Total activity refers to both latent and active TGF-β activities in the supernatants; this activity was measured after heat activation of latent TGF-β in the supernatant. Values for the percentage of active TGF-β (in parentheses) were determined in parallel assays in which TGF-β activity was measured in the same supernatants without heat activation. The limit of detection of each isotype is approximately 5% of the TGF-β activity measured.

[b] Values for TGF-β3 are based on a standard curve generated by using highly purified recombinant TGF-β3; the activity of TGF-β3 in this assay is approximately three times higher than TGF-β1 activity.

These results suggest that the increased transcription of TGF-β3 mRNA as described above leads to increased secretion of TGF-β3. Most of the TGF-β3 activity in the myoblast supernatant (95.3%) and all of the TGF-β3 activity in the myotube supernatants was latent. The observation that fusion of myoblasts was not inhibited by the secreted TGF-β suggested that the secreted TGF-β must be activated in order to inhibit myoblast fusion.

Supernatants from C_2C_{12} cells inhibit myoblast fusion after heat activation of latent TGF-β. To further explore the relationship between C_2C_{12} cell secretion of latent TGF-β3 and C_2C_{12} cell fusion, the supernatant from fused myotubes was added at various dilutions to media containing 1% HS (i.e., media which permit fusion) and unfused myoblasts. Fusion proceeded normally when C_2C_{12} supernatant was added directly to the myoblast cultures, even at the lowest dilutions (Fig. 4, top panel, "non-activated"). However, if TGF-β in the C_2C_{12} cell supernatant was first activated by heating, it completely inhibited myoblast fusion at dilutions of 1/20 or lower (Fig. 4, top panel, "heat-activated"). Fifty percent maximal inhibition occurred at a dilution of approximately 1/50. Fusion of C_2C_{12} cells was also inhibited by the addition of exogenous TGF-β1 or TGF-β3 (Fig. 4, bottom panel). The 50% maximal inhibition of TGF-β3 was 0.11 ng/ml. On the basis of these data, the calculated concentration of TGF-β3 in the C_2C_{12} cell supernatant is approximately 5.5 ng/ml. This value correlates closely with the value measured in the CCL64 cell assay described above (Table 1).

TGF-β3 mRNA half-life in C_2C_{12} myoblasts and myotubes. The marked increase in TGF-β3 mRNA expression in C_2C_{12} cells during fusion must result from either increased TGF-β3 transcription or increased TGF-β3 transcript stability, or from both. To determine whether TGF-β3 transcript stability changes during myogenesis, fused and unfused C_2C_{12} cells were treated with dactinomycin for various times and RNA was extracted and analyzed for TGF-β3 expression. Consistent with the results described above, myotubes expressed 8.7 times more TGF-β3 mRNA than did myoblasts (Fig. 5A and B). After dactinomycin treatment, TGF-β3 expression in both myoblasts and myotubes decreased at nearly the exact same rate, with a half-life of 3.9 h (Fig. 5A and B). The results are plotted in two ways in Fig. 5B. In the left panel, the data are graphed on the basis of the densitometric analysis of the radiograph pictured in Fig. 5A. The 0-h time point of myotube TGF-β3 expression was not graphed because it was not linear with the remainder of the points.

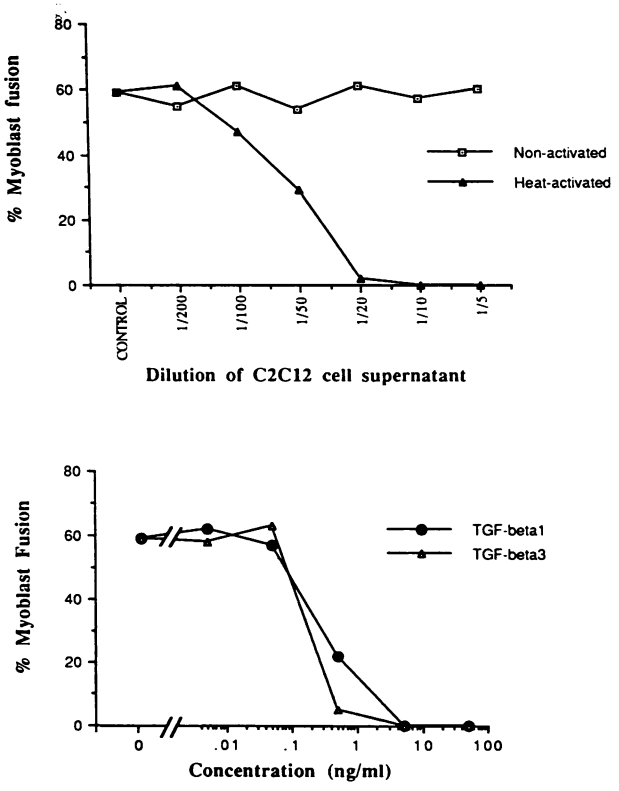

FIG. 4. C_2C_{12} supernatants inhibit C_2C_{12} cell fusion. C_2C_{12} myoblasts were cultured in media containing 1% HS in 24-well tissue culture plates with various dilutions of nonactivated or heat-activated C_2C_{12} myotube supernatant (upper panel) or with different concentrations of recombinant, active TGF-β1 or TGF-β3 (lower panel). The extent of myoblast fusion at each dilution or concentration is the average value determined by counting nuclei in fused versus unfused cells in five different microscope fields.

This is due to saturation of the radiograph and, therefore, inaccuracy in scanning (see below). The 27.5-h time point of myoblast TGF-β3 expression was also omitted since this band was not detected by densitometric scanning. The lines are plotted on a semilog graph, and the best-fit lines were determined as described in Materials and Methods. The right panel shows the results of decreasing the exposure time of the radiograph to approximately 1/8 of that of the left panel (7-h exposure) and plotting the densitometric results of this decreased exposure of the myotube radiograph compared with the longer exposure of the myoblast radiograph shown on the left. In this plot, the 0-h time point from the myotube radiograph lines up with the other points and is shown. In both the myotubes and myoblasts, no signal was detected at 27.5 h, and this time is not shown. The two best-fit lines are virtually coincidental. These data show that the increase in TGF-β3 expression after myoblast fusion does not result from changes in TGF-β3 mRNA stability and, therefore, must be due to increased transcription of TGF-β3 mRNA during myoblast fusion.

Transcriptional regulation of TGF-β3 in C_2C_{12} myoblasts. In order to investigate the transcriptional regulation of TGF-β3 in C_2C_{12} myoblasts, chimeric TGF-β3 promoter/CAT reporter plasmids were transfected into these cells. The chimeric plasmids, pB3−499, pB3−301, pB3−220, pB3−91, and pB3−38. used for these experiments have been described previously (22). They consist of a series of plasmids each starting 110 nucleotides 3′ of the TGF-β3 transcription start site (+110) and extending 5′ to the number indicated in the plasmid name (Fig. 5A). For example, pB3−499 contains a TGF-β3 promoter fragment spanning −499 to +110 of the transcriptional start site ligated to a CAT gene. Another chimeric plasmid, pB3−120, was also constructed as described in Materials and Methods; similarly, this construct contains a TGF-β3 promoter fragment with its 3′ terminus at +110 and 5′ terminus at −120. Finally, a 5′ flanking region/CAT chimeric plasmid (pB3+1083) was constructed which contains −91 to +1083 of the TGF-β3 5′ flanking region ligated to the CAT gene. This reporter plasmid, therefore,

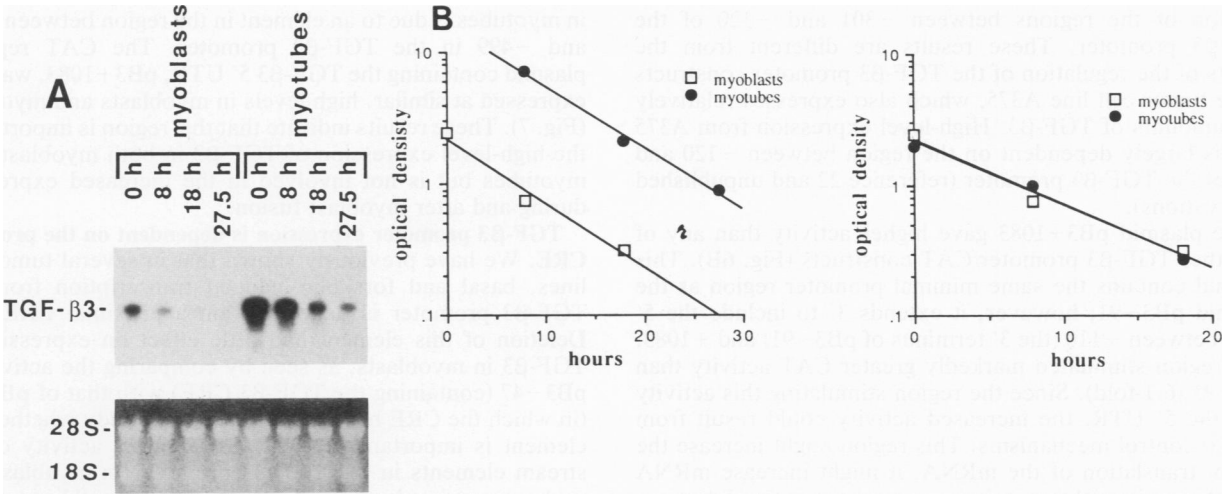

FIG. 5. TGF-β3 mRNA stability in fused and unfused C_2C_{12} cells. (A) C_2C_{12} myoblasts and myotubes (after 6 days in 1% HS) were treated with dactinomycin (10 μg/ml) for 0, 8, 18, or 27.5 h to inhibit new RNA synthesis. RNA was then extracted, and 10 μg of each RNA sample was electrophoresed on a formaldehyde-agarose gel, blotted onto Nytran, and hybridized to ^{32}P-labeled mouse TGF-β3 cDNA. Corresponding ribosomal RNA stained with ethidium bromide of the samples prior to transfer is pictured in the lower panel. (B) At left, data are graphed on the basis of densitometric analysis of the radiograph in panel A. The graph at the right shows the results of decreasing the exposure time. See Results for details.

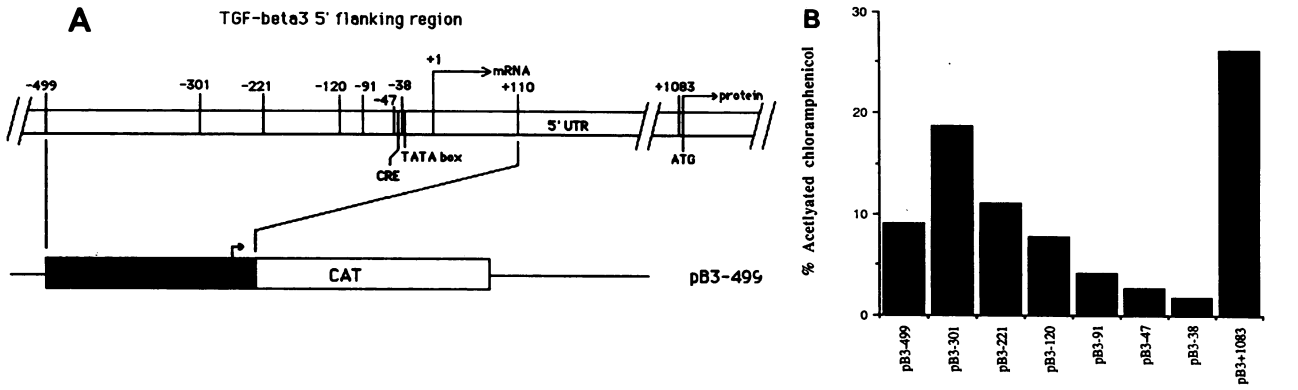

FIG. 6. TGF-β3 promoter regulation in C_2C_{12} myoblasts. (A) A diagram of the TGF-β3 5′ flanking region. The transcriptional and translational start sites and the regions containing the TATA box and CRE are labeled. The 5′ flanking region is also labeled at positions corresponding to the ends of the promoter fragments cloned in front of CAT genes in the TGF-β3 promoter/CAT reporter plasmids. Promoter fragments used to construct the promoter/CAT plasmids pB3−499, pB3−301, pB3−221, pB3−120, pB3−91, pB3−47, and pB3−38 span from the number given in the plasmid name to +110. pB3−499 is diagrammed. pB3+1083 contains the 5′ flanking region spanning −91 to +1083. (B) C_2C_{12} myoblasts were transfected with TGF-β3/CAT chimeric reporter plasmids, and CAT activity was determined 48 h later. The resulting CAT activity was normalized to growth hormone produced by cotransfected pXGH5.

contains all but 21 bp of the 1,104-bp 5′ UTR of the TGF-β3 gene and a minimal portion of the TGF-β3 promoter region (91 bp) linked to a CAT reporter gene.

C_2C_{12} myoblasts were transfected with the reporter constructs pB3−499, pB3−301, pB3−220, pB3−120, pB3−91, pB3−47, pB3−38, pB3+1083, and pSV2CAT. Transfections were completed as described in Materials and Methods, and CAT activity in the cell lysates was determined. The CAT activity of each transfection was normalized to growth hormone produced by cotransfected pXGH5, a plasmid containing the growth hormone gene ligated to the strong constitutive metallothionein II_A promoter.

Expression of TGF-β3 mRNA is relatively high even in undifferentiated C_2C_{12} myoblasts (Fig. 1 to 4). Similarly, C_2C_{12} myoblasts transfected with the TGF-β3 promoter constructs pB3−499 and pB3−301 expressed relatively high CAT activity (Fig. 6B). This activity declined gradually upon deletion of the regions between −301 and −120 of the TGF-β3 promoter. These results are different from the results of the regulation of the TGF-β3 promoter constructs in the tumor cell line A375, which also expresses relatively high amounts of TGF-β3. High-level expression from A375 cells is largely dependent on the region between −120 and −91 of the TGF-β3 promoter (reference 22 and unpublished observations).

The plasmid pB3+1083 gave higher activity than any of the other TGF-β3 promoter/CAT constructs (Fig. 6B). This plasmid contains the same minimal promoter region as the plasmid pB3−91; however, it extends 3′ to include the 5′ UTR between +110 (the 3′ terminus of pB3−91) and +1083. This region stimulated markedly greater CAT activity than pB3−91 (6.1-fold). Since the region stimulating this activity is in the 5′ UTR, the increased activity could result from several control mechanisms. This region might increase the rate of translation of the mRNA, it might increase mRNA stability, or it might contain an enhancer to stimulate transcription. Although we do not have direct evidence of the particular mechanism operative in C_2C_{12} myoblasts, we have studied this in A375 and HT1080 cells. These two cell lines show similar increases in CAT activity after the addition of the 5′ UTR, and this increased activity in HT1080 cells can be accounted for by an increase in CAT mRNA levels (21a). These results indicate that the 5′ UTR has an important role in either the transcription or stability of TGF-β3 mRNA.

Transcriptional regulation of TGF-β3 during myogenesis. To study the transcriptional regulation of TGF-β3 during myogenesis, plates of transfected C_2C_{12} myoblast cells were divided after transfection, and 2/3 of the cells were allowed to fuse in medium supplemented with 1% HS. In order that the activities of the TGF-β3 promoter constructs in the myoblast and myotube cultures could be compared, one plate was transfected with the plasmid pSV2CAT and was also divided into myoblast and myotube cultures. TGF-β3 promoter activity was enhanced only in the promoter construct which included the TGF-β3 promoter region to −499 (Fig. 7). The shorter constructs pB3−221, pB3−91, and pB3−38 were expressed similarly in both myoblasts and myotubes. Therefore, the enhanced transcription of TGF-β3 in myotubes is due to an element in the region between −221 and −499 in the TGF-β3 promoter. The CAT reporter plasmid containing the TGF-β3 5′ UTR, pB3+1083, was also expressed at similar, high levels in myoblasts and myotubes (Fig. 7). These results indicate that this region is important in the high-level expression of TGF-β3 in both myoblasts and myotubes but is not involved in the increased expression during and after myoblast fusion.

TGF-β3 promoter expression is dependent on the proximal CRE. We have previously shown that in several tumor cell lines, basal and forskolin-induced transcription from the TGF-β3 promoter is dependent on a proximal CRE (22). Deletion of this element had little effect on expression of TGF-β3 in myoblasts, as seen by comparing the activity of pB3−47 (containing the TGF-β3 CRE) with that of pB3−38 (in which the CRE has been deleted). To study whether this element is important in the transcriptional activity of upstream elements in the TGF-β3 promoter in myoblasts, as we have previously shown for several tumor cell lines, C_2C_{12} cells were transfected with either pB3−499 or pB3−499Δ, a TGF-β3/CAT plasmid containing the identical promoter region as in pB3−499 except that two of the nucleotides in the CRE have been changed. Mutation of these two nucleotides reduced expression of this plasmid to nearly that of pB3−38

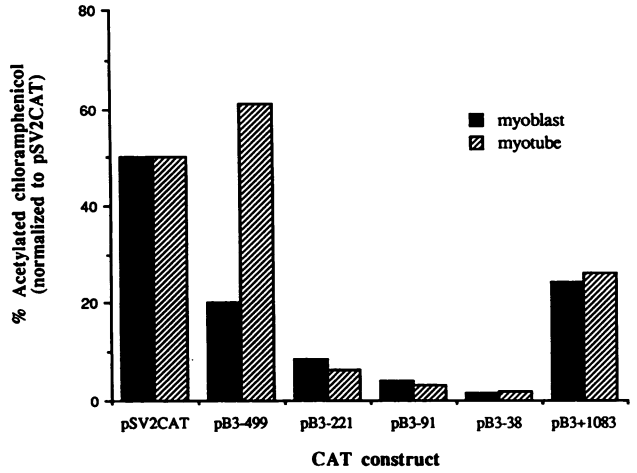

FIG. 7. TGF-β3 promoter regulation in C_2C_{12} myotubes. C_2C_{12} myoblasts were transfected with TGF-β3/CAT chimeric reporter plasmids, divided between two dishes, and allowed to form myotubes (1% HS) or grown as myoblasts (10% FBS). After 3 days (myoblasts) or 6 days (myotubes), CAT activity was determined in cell lysates, and the resulting CAT values were normalized to growth hormone produced by cotransfected pXGH5 in myoblast cultures and then renormalized to an SV2CAT value of 50% conversion of [^{14}C]chloramphenicol to [^{14}C]acetyl chloramphenicol. The results shown are the average values from two experiments.

(Fig. 8). Similar decreased CAT activity was seen in myoblasts allowed to fuse to form myotubes (data not shown). These results indicate that this proximal CRE is critical for expression of promoter activity by distal regions of the promoter in both myoblasts and myotubes.

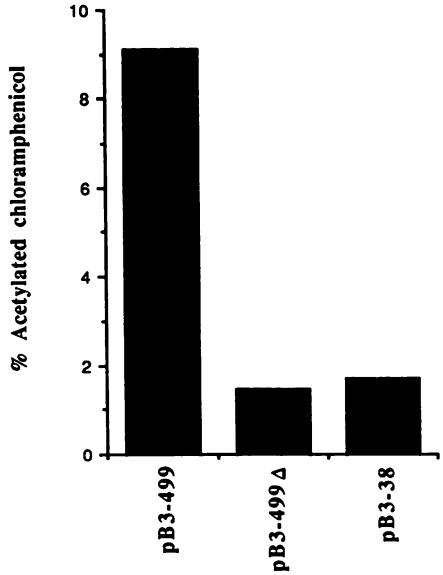

FIG. 8. TGF-β3 promoter expression requires a proximal CRE sequence. C_2C_{12} myoblasts were transfected with pB3−499, pB3−499Δ, or pB3−38, and CAT activity was determined 48 h later. The resulting CAT activity was normalized to growth hormone produced by cotransfected pXGH5.

DISCUSSION

The lack of detectable expression of TGF-β3 transcripts in early-stage whole embryo RNA, followed by the appearance of TGF-β3 expression in embryonic heart tissue (11), indicates that expression of this growth factor increases dramatically during the formation of certain tissues during development. The cultured myoblast cell line C_2C_{12} expresses relatively high levels of TGF-β3 even before fusion (compared with the level of TGF-β3 expression in early-stage whole embryos and a wide variety of normal and tumor cell lines; 11, 21a, 22), suggesting that prior to commitment to a myoblast lineage, mesoderm progenitor cells likely express even less TGF-β3 than committed myoblast cells. The several stages of muscle cell differentiation, therefore, appear to be associated with at least one and possibly two quantum increases in the level of TGF-β3 mRNA. Although we have studied in detail only the increase associated with myoblast fusion, the TGF-β3 promoter studies support and give some insight into TGF-β3 expression possibly associated with earlier mesodermal differentiation. Specifically, TGF-β3 expression in myoblasts is stimulated through several different regions of the TGF-β3 promoter from −301 to −47 and in the long 5′ UTR. These regions are mostly distinct from the region between −499 and −221 responsible for the increased expression of TGF-β3 stimulated during and after myoblast fusion. A reasonable model for the increased expression of TGF-β3 during muscle differentiation might, therefore, postulate that the promoter region between −301 and −47 and/or the 5′ UTR binds a regulatory factor(s) during some stage in the commitment of mesodermal progenitor cells to a myoblast lineage and that the region between −499 and −221 binds an additional factor(s) needed to stimulate high-level expression in the mature myotube.

Understanding regulation of the TGF-β3 promoter in the context of regulation of the promoters of muscle-specific genes will require further study. It is noteworthy that TGF-β3 regulation is more complex since this protein is expressed in both myoblasts and myotubes whereas the promoters of the muscle-specific genes of myosin and creatinine kinase are stimulated only after myoblast fusion. These muscle-specific genes appear to be regulated by at least four regulatory proteins: MyoD (or myf 3), myogenin (or myf 4), myf 5, and MRF-4 (or myf 6 or herculin) (2, 3, 6, 27, 31, 37, 38). Each of these genes, after transfection and expression in fibroblast cells, confers a myoblast phenotype. After placement in a low (1%) serum concentration, cells transfected with any one of these proteins express muscle-specific genes and fuse to form myotubes. These four proteins all belong to the helix-turn-helix family of DNA binding proteins (28), and MyoD and myogenin bind to similar regions of DNA in the creatinine kinase promoters (4, 9). Why four different proteins apparently redundant in function are required for muscle cell differentiation is unclear. Regulation of the TGF-β3 gene by these proteins is a distinct possibility given the pattern of expression we have demonstrated. A computer search of the TGF-β3 promoter has not shown any regions similar to the regions in the muscle creatinine kinase and myosin promoters responsible for MyoD or myogenin responsiveness (21a). It should be of interest to see whether transfection of muscle determination genes stimulates the TGF-β3 promoter.

The functional significance of the high-level expression of TGF-β3 in skeletal and cardiac muscles is unclear. During development, a potential role for TGF-β3 could be in directing some aspect(s) of embryogenesis through intercellular

signaling to induce or inhibit the differentiation of cells in adjacent tissues. The secretion of latent TGF-β3 by myoblasts also gives the potential for adjacent cells to inhibit the final stage in myogenesis by simply secreting a molecule that activates TGF-β3. The role of TGF-β3 expression by adult muscle is obscure but is possibly related to the regulation of its function as an electrochemically activated, contractile tissue.

A complete understanding of the function of the various TGF-βs in vivo will require a more complete understanding of the two possibly related questions of how signal specificity among TGF-β1, TGF-β2, and TGF-β3 is determined and how physiologic activation of the latent forms of each of the TGF-βs takes place. Data on physiochemical activation of TGF-βs have shown that they have similar profiles of activation by either heat or pH changes (5). Data in regard to the only studied potential physiologic activator, plasmin, indicate that plasmin activates only a small percentage of latent TGF-β1 while simultaneously degrading the active molecule; these data suggest that plasmin is not the major activator of TGF-β activation in vivo (5). No data are available on activation of latent TGF-β3 by plasmin. As our data show, although latent TGF-β3 is secreted by both myoblast and myotube cells, this secretion does not result in inhibition of myogenesis as induced by activated TGF-β3 added exogenously. Activation of latent TGF-β3 by adjacent cells could, therefore, inhibit myoblast differentiation. Selective activation of this TGF-β isotype could be responsible for conferring signal specificity. This possibility is suggested by the greater divergence of homology in the regions of the TGF-β proproteins responsible for conferring latency than in the regions corresponding to the active, mature peptides. Alternatively, specificity of receptors for various forms of the TGF-βs could lead to signal specificity.

By studying the regulation of TGF-β expression in a defined, differentiating system, we have demonstrated selective control of the three TGF-β isoforms. Moreover, it has become apparent that regulation of TGF-β3 mRNA levels during myogenesis occurs through multiple regions in both the promoter region and the 5′ UTR. Further study of TGF-β3 regulation during myogenesis should answer several important questions regarding both regulation of myogenesis and TGF-β3 function during developmental processes.

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