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Transcriptional Activation of the Mouse HSP47 Gene in Mouse Osteoblast MC3T3-E1 Cells by TGF-β1

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HSP47 is a 47-kDa collagen-binding heat shock protein, the expression of which is always correlated with that of collagens in various cell lines. We examined the effects of TGF-β1, which is reported to induce the collagen genes, on the expression of HSP47 in mouse osteoblast MC3T3-E1 cells. Treatment of the cells with 5 ng/ml TGF-β1 for 24 h increased the level of HSP47 mRNA three-fold. Dose-dependent induction by TGF-β1 was observed for both HSP47 mRNA and collagen α1(I) mRNA, and actinomycin D inhibited this increase of HSP47 mRNA. To elucidate the TGF-β1 responsive element(s) in the mouse HSP47 gene, we generated a series of 5′-deletion promoters fused to luciferase reporter constructs. Transient transfection assays showed that TGF-β1 induced 4-6 fold the promoter activity of a region approximately −5.5 kbp upstream of the HSP47 gene. Two upstream regions, −3.9 to −2.7 kbp and −280 to −50 bp were shown to be involved in the activation in response to TGF-β1 treatment.

Heat shock proteins (HSPs) are reported to play important roles as molecular chaperones in the folding and assembly of newly synthesized or malfolded proteins (1, 2). HSP47 is a 47-kDa stress protein which specifically binds to collagen (3-7). HSP47 has an RDEL sequence at the COOH terminus, which acts as an endoplasmic reticulum (ER)-retention signal like the KDEL sequence, and binds to newly synthesized procollagen in the ER (5-11). HSP47 transiently binds to procollagen during its transport from the ER to the Golgi, and is retained to bind to conformationally abnormal procollagen for much longer periods. This is the sort of function which has been attributed to molecular chaperones, and acts as a quality control mechanism. In addition to this functional relationship, there are several lines of evidence which suggest the expression of HSP47 is closely related to collagen expression. The expression of both HSP47 and type I collagen is decreased after malignant transformation of fibroblasts caused by Rous sarcoma virus or simian virus 40 (4, 8). Nuclear run on assay shows that this decrease is regulated at the transcriptional level (9). Conversely, the expression of both type IV collagen and HSP47 is induced markedly during the differentiation of F9 mouse teratocarcinoma cells by the treatment with or without retinoic acid plus dibuthylic cyclic AMP (10, 12). Administration of carbon tetrachloride (CCl_4) results in the coexpression of HSP47 and type I collagen in Itoh cells of rat liver, and mRNA levels of both HSP47 and type I and III collagens show a good correlation with the progression of fibrosis (13).

Multifunctional cytokine transforming growth factor (TGF-β) was originally identified in neoplastic cells and subsequently reported to be present in most cells exerting a variety of effects on cell proliferation, cell differentiation and embryogenesis (14-20). It has also been shown to stimulate the production of extracellular matrix components including collagens and fibronectin (21-24), and to play a pivotal role in fibrinogenesis and bone formation (25-29). Indeed, TGF-β is abundant in the connective tissue and bone matrix (30-32). CCl_4-administered rats exhibited increased expression of TGF-β1 and its receptors as well as collagen in the liver (33-36). TGF-β1 is thus considered to be a mediator of collagen production in models of fibrinogenesis. Recently, significant progress has been made in understanding the expression of the collagen genes and their transcriptional regulation by TGF-β. In particular, promoter analyses of the collagen genes revealed that TGF-β1 regulated the transcription of the collagen genes via several cis-elements of their promoters (37-39).

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As the expression of HSP47 mRNA is induced in parallel with that of the collagens, we have examined whether TGF-β1 affects the expression of the mouse HSP47 gene as well as the collagens. In this study, we show that TGF-β1 increases the steady-state level of mouse HSP47 mRNA as well as that of type I collagen in mouse osteoblast MC3T3-E1 cells and is a transcriptional stimulator of the mouse HSP47 promoter.

MATERIALS AND METHODS

Cell culture and recombinant human TGF-β1. Mouse MC3T3-E1 cells were cultured in α-minimum essential medium (MEM), supplemented with 10% fetal calf serum, penicillin (50U/ml) and streptomycin (50mg/ml) under humidified 5% CO_2 and 95% air. Recombinant human TGF-β1 was kindly provided by Dr. Tatsuhiko Ikeda (Morinaga Institute of Biological Science, Yokohama, Japan) and was diluted in PBS(−) containing 4 mM HCl and 2 mg/ml BSA. The cells were plated at a density of 6.0×10^5 cells in each 10-cm dish. After 24 h, the medium was replaced by serum-free α-MEM containing TGF-β1, which was added at the concentration indicated for an additional incubation period of 12 to 36 h. An equal volume of medium without TGF-β1 was added to the control dishes.

Northern blot analysis. Total RNA was isolated from control and TGF-β1-treated cells by the AGPC method with minor modifications (40). RNA was analyzed by Northern blot or Northern slot blot hybridization as described previously (13). The RNA-transfered membranes were hybridized with ^{32}P-labeled mouse HSP47 cDNA (F2 fragment), mouse collagen α1(I) cDNA (0.9 kbp of PstI fragment) or human 28S ribosomal RNA genomic DNA (7kbp of BamHI fragment of pHr14E3, gifted from Dr. Muramatsu).

Plasmid construction. A fragment between −5.5 kbp upstream to +38 bp of the mouse HSP47 gene was fused to luciferase reporter plasmid pGL2-BASIC (Promega) and was designated as pLUC-5.5. A series of 5′-deletion mutants were constructed using restriction enzymes and Nested deletion kit (Pharmacia). The plasmids for the transfection were CsCl-banded supercoiled DNA and each construct was independently prepared at least twice.

Transient transfection and luciferase assays. MC3T3-E1 cells were plated at 0.7×10^5 cells/3.5-cm dish in complete medium. After 24 h, DNA transfections were performed by the calcium phosphate precipitation technique (41). Ten hours later, the medium was removed and the cells were washed twice with PBS(−) and re-incubated for 30-38 h in the fresh medium with or without TGF-β1 at the concentrations indicated in the figure legends. Finally, the cells were collected and lysed in 0.25M Tris (pH7.5) by the freeze-thaw method. Luciferase activity was measured using picaGene assay kit (Toyo ink.) and Lumat 9800 luminometer (Bertold). As an internal control, pSV-β gal was co-transfected and transfection efficiencies were normalized by β-galactosidase activity.

RESULTS

TGF-β1 increases steady state levels of mouse HSP47 and collagen α1(I) genes. We examined the effect of TGF-β1 on the steady state level of HSP47 mRNA of and collagen α1(I) expressed in the mouse osteoblast MC3T3-E1 cells, which is known to be sensitive to TGF-β1. As shown in Fig. 1, treatment of the cells with 5 ng/ml TGF-β1 increased about 3 fold the steady-state level of HSP47 mRNA as well as collagen α1(I) mRNA.

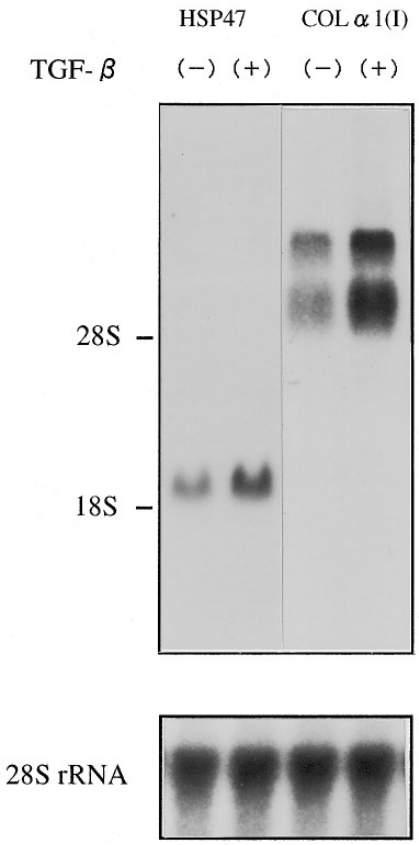

FIG. 1. Analysis of mRNA levels for HSP47 and collagen α1(I) following the treatment with TGF-β1. MC3T3-E1 cells in the growing phase were plated in 10-cm dishes at a density of 6.0×10^5 cells. After 24 h, the medium was replaced by serum-free α-MEM with or without 5 ng/ml TGF-β1. The cells were harvested after 24 h and total RNA was prepared by the AGPC method with minor modifications. Ten μg of total RNAs were electrophoresed on 1% agarose gel containing formaldehyde and transferred onto a nylon membrane. Blotted filters were hybridized to either ^{32}P-labeled mouse HSP47 cDNA probe, mouse collagen α1(I) cDNA probe or human 28S ribosomal genomic DNA. The position of 28S and 18S ribosomal RNA are indicated.

Fig. 2a and 2b show Northern slot blot analyses of RNA from MC3T3-E1 cells treated with TGF-β1 for 12, 24 or 36 h. Amounts of mRNA levels hybridized with specific probes for HSP47 or collagen α1(I) were normalized using 28S ribosomal RNA. An increase of approximately 2 fold in HSP47 mRNA was observed in 12 h after the addition of TGF-β1 and high levels of HSP47 mRNA, of more than 3 fold, were sustained for 24 h and 36 h. The increase in collagen α1(I) mRNA level was similar to that of HSP47 (Fig. 2a and 2b). Next, MC3T3-E1 cells were treated with various concentrations of TGF-β1 for 24 h (Fig. 2c and 2d). Both HSP47 and collagen α1(I) mRNAs were induced in a dose-dependent manner for the concentrations of TGF-β1. Maximum induction was observed with TGF-β1 with a concentration of 5 ng/ml (Fig. 2d).

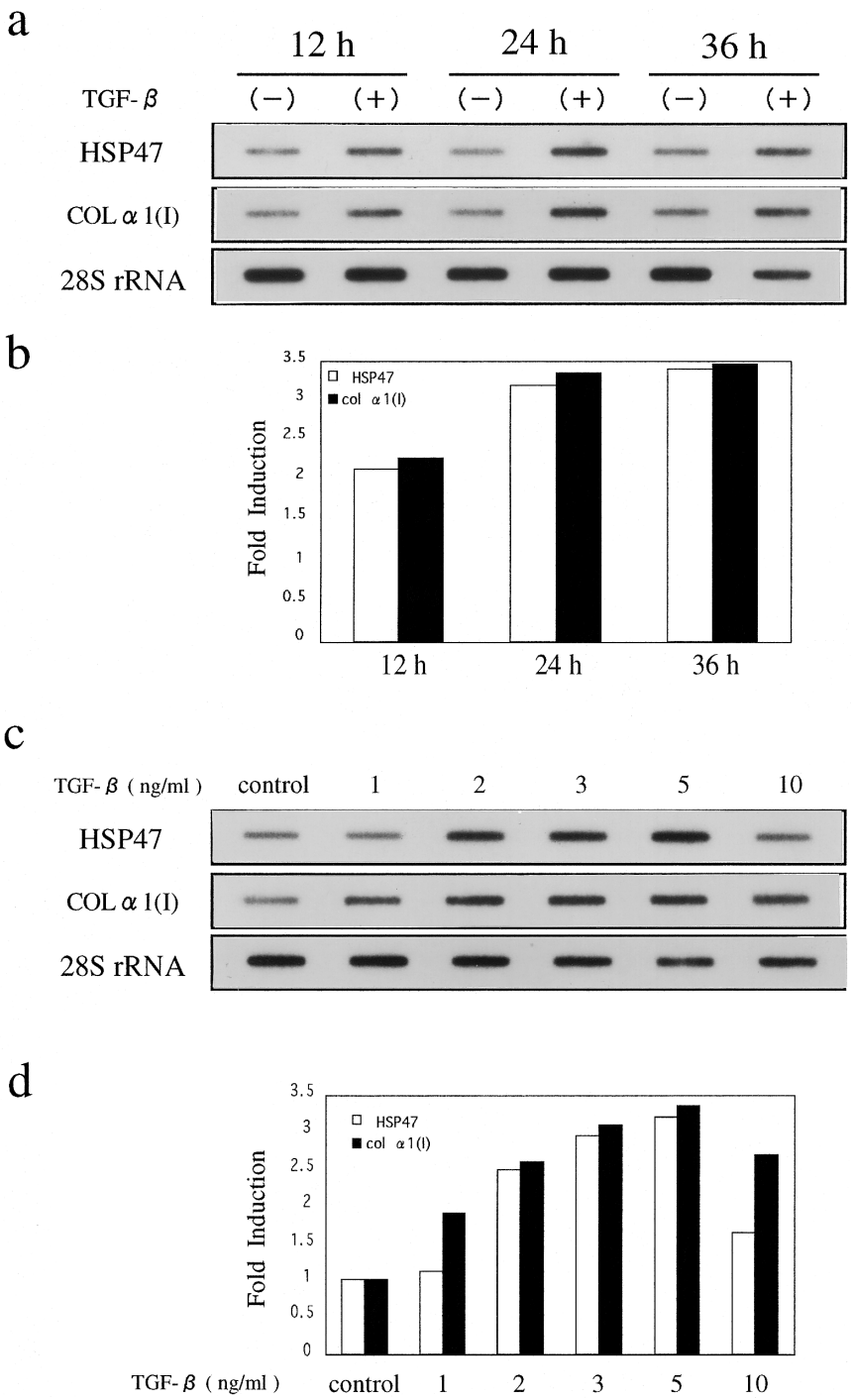

FIG. 2. Quantitative analysis of HSP47 and α1(I) collagen mRNAs by RNA slot blotting. MC3T3-E1 cells were cultured in the serum-free medium supplemented with 5 ng/ml TGF-β1. After the indicated length of time, the cells were harvested and total RNA prepared as described above was analyzed by slot blotting (a and b). The density of the bands hybridized to each ^{32}P-labeled probe were evaluated using BIORAD Molecular imager. MC3T3-E1 cells were cultured in serum-free medium supplemented with various concentrations of TGF-β1. The cells were harvested after 24 h and total RNA was similarly analyzed by slot blotting (c and d).

Effect of actinomycin D on the induction of HSP47 mRNA by TGF-β1. When the cells were treated with TGF-β1 in the presence of 100 ng/ml actinomycin D, an RNA polymerase inhibitor, the increase in HSP47 mRNA was almost completely blocked (Fig. 3). Therefore, the induction of HSP47 mRNA with TGF-β1 treat-

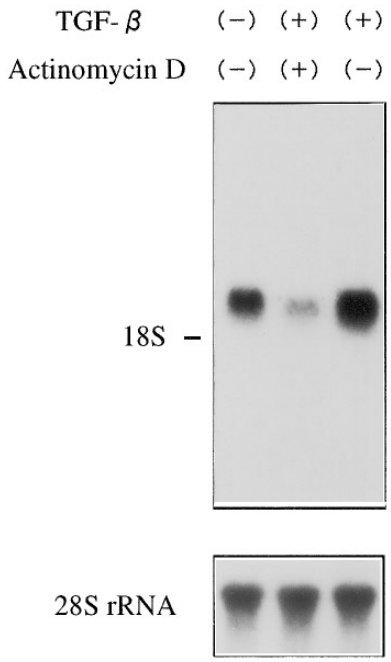

FIG. 3. Effect of actinomycin D on the induction of HSP47 mRNA after the treatment with TGF-β1. Cultured cells were treated with 5 ng/ml TGF-β1 in the presence or absence of 100 ng/ml actinomycin D. After 24 h, the cells were harvested and total RNAs prepared were subjected to Northern blot analysis. The blotted filter was hybridized to ^{32}P-labeled mouse HSP47 cDNA probe.

ment is thought to be regulated at the transcriptional level, similar to the regulation of the collagen genes (24).

Activation of the mouse HSP47 promoter by TGF-β1. Previously we reported the sequence of the −1.1kbp upstream fragment of the mouse HSP47 gene subcloned from a mouse genomic library (42). To explore the regulatory mechanism of HSP47 expression in more detail, we have recently subcloned another genomic clone containing approximately −5.5 kbp upstream region of the mouse HSP47 gene (manuscript in preparation). This fragment spanning from −5.5 kbp to +38 bp was fused to luciferase reporter plasmid (pLUC-5.5) for promoter analysis (FIG. 4a). Fig. 4c shows that TGF-β1 induced the luciferase activity of pLUC-5.5 approximately 5 fold, while induction of pGL2-control driven by SV40 early promoter was measured 1.5 fold after TGF-β1 treatment. To confirm whether this activation is HSP47 promoter specific, we generated a series of 5′-deletion mutants of pLUC-5.5 (Fig. 4a). As shown in Fig. 4b and 4c, when the region between −3.9 kbp and −2.7 kbp was deleted from the pLUC-3.9 construct, the induction of luciferase activity by TGF-β1 was reduced to about 3 fold. pLUC-50 is a construct containing only the TATA box and pLUC-13 is a TATA box-less construct (Fig. 4a). TGF-β1 failed to induce the luciferase activity of either pLUC-50 or pLUC-13 (Fig.4 b and 4c). Fig. 4c clearly indicates that two distinct regions, the −3.9 to 2.7 kbp fragment and the −280 to −50 bp fragment, are involved in the activation of the mouse HSP47 promoter by TGF-β1 treatment.

DISCUSSION

HSP47 is an ER-resident stress protein which can specifically bind to newly synthesized procollagen. HSP47 is considered to be a unique molecular chaperone because, in addition to the substrate specificity for binding, its synthesis is always closely correlated with that of its substrates, various types of collagens. This parallel expression of HSP47 and collagen and their functional relationship suggest the importance of this molecular chaperone in collagen-related pathophysiological conditions. Indeed, the involvement of HSP47 in the progression of liver fibrosis has been reported (13).

In this study, we reported that TGF-β1 caused an increase in the steady-state level of HSP47 mRNA as well as that of type I collagen α1(I) mRNA. This finding emphasizes that spacio-temporal parallel induction of both HSP47 and collagen might be important for the appropriate production of various types of collagens in the process of cell differentiation and tissue development.

The multifunctional cytokine TGF-β is an important transducer of the extracellular matrix (ECM) production during bone formation and fibrinogenesis (21-29). Actually, TGF-β1 is abundant in bone and connective tissue (30-32). Injection of TGF-β1 onto the periostea of parietal bones of neonatal rats stimulated the formation of periosteal woven bone and increased the thickness of the treated parietal bones (43). This increase in bone matrix fomation was observed in fetal rat calvaria when cultured in the presence of TGF-β1 (44). In addition, subcutaneous injection of TGF-β in the newborn mouse causes the appearance of a proliferative nodule composed principally of ECM and fibroblasts (21). Moreover, TGF-β1 is also considered to be one of the most important mediators in bleomycin-induced lung fibrosis (45-48) and CCl_4-induced liver fibrosis (33-36). Previously we demonstrated that HSP47 mRNA was markedly induced in parallel with collagen α1(I) and α1(III) mRNAs during the progression of rat hepatic fibrosis induced by the administration of CCl_4 (13). Clarke *et al* reported that TGF-β increased the synthesis in collagen α1(I) and α2(I) as well as colligin, a rat homolog of HSP47 in rat skeletal myoblast L6 cells (49). In this study, we showed that the expression of both HSP47 and collagen α1(I) was stimulated in mouse osteoblast MC3T3-E1 cells treated with TGF-β1. MC3T3-E1 is an osteogenic cell line derived from newbone mouse calvaria (50). Therefore, the upregulation of HSP47 expression also appears to be important for the production of collagen in bone formation.

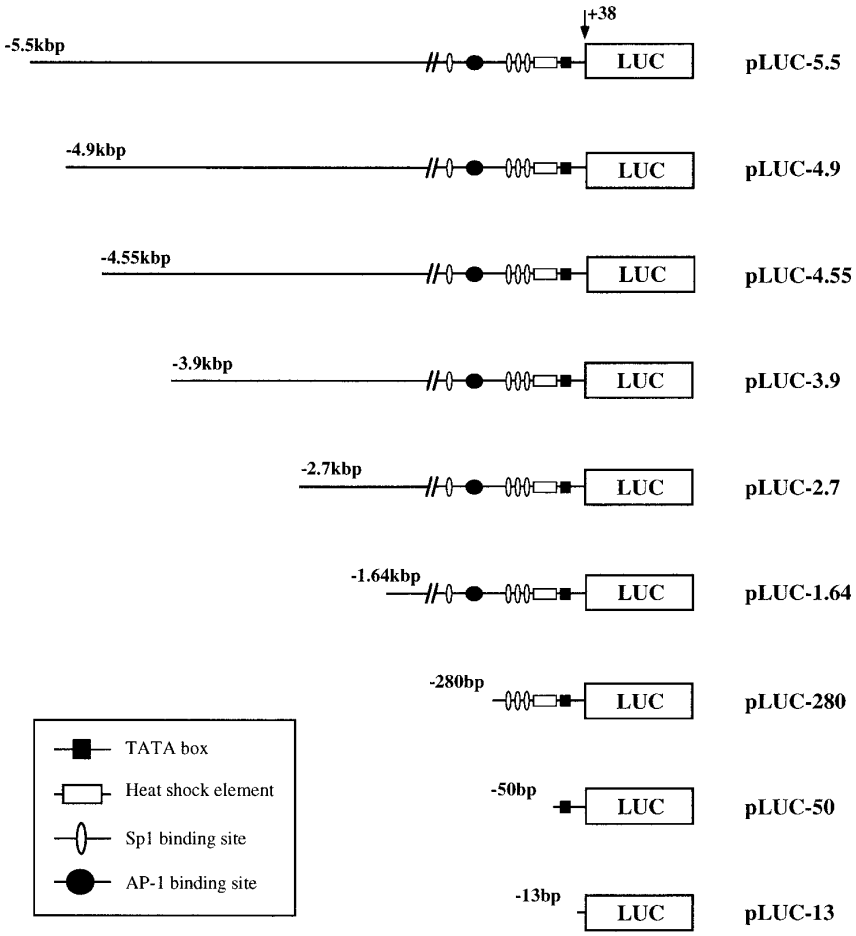

FIG. 4. Analysis of the mouse HSP47 promoter activity. a, Schematic representation of a series of 5′-deletion mutants of pLUC-5.5 used in transfection assay. b, Relative luciferase activities of the deletion constructs transfected in MC3T3-E1 cells. After transfection, the cells were treated with 5 ng/ml TGF-β1 for 30-38 h in the medium containing 1% FCS. The transcriptional activity (luciferase activities) from duplicated transfections was measured and normalized by β-galactosidase activity. The experiments were performed independently at least three times and error bars represent SEM. c, Fold induction represents the ratio of luciferase activities in the extract of TGF-β1-treated cells divided by that in the vehicle-treated cell extract. Error bars represent SEM.

We also demonstrated that the RNA polymerase inhibitor, actinomycin D blocked the protion of HSP47 mRNA induced by TGF-β1 treatment. This observation suggests that TGF-β1 enhances the expression of HSP47 at the transcriptional level. To confirm this hypothesis, we examined whether TGF-β1 can stimulate the promoter activity of the mouse HSP47 gene. Transfection assay showed that TGF-β1 activated the HSP47 promoter approximately 5 fold (Fig. 4c). Many investigators have reported that TGF-β1 regulates α1(I) and α2(I) collagen genes at the transcriptional level. These promoter analyses have identified several TGF-β responsive *cis*-elemets in the collagen promoters and revealed the involvement of various transcription factors, namely NF-1, AP-1 and Sp1 (37-39). As shown in Fig. 4, two distinct regions of the HSP47 promoter were engaged in the TGF-β response. The proximal region between -280 bp and -50 bp contained three putative Sp1 binding sites (42). However, there were no putative NF-1, AP-1 or Sp1 binding sequence found in the distal region between -3.9 kbp and -2.7 kbp, suggesting that HSP47 expression was activated through novel TGF-β signal pathway(s). Now we are trying to identify TGF-β responsive element(s) in the distal region of the mouse HSP47 promoter and transcription factor(s) that activate(s) the mouse HSP47 gene through TGF-β1.

In conclusion, the present study demonstrated that TGF-β1 could activate the mouse HSP47 at the transcriptional level as well as increase the steady state level of mRNA. As mentioned before, HSP47 and collagens are coexpressed in collagen producing cells, and the expression of HSP47 is induced in parallel with the increase in that of collagens. Although we have not elucidated the mechanisms of this coexpression, we

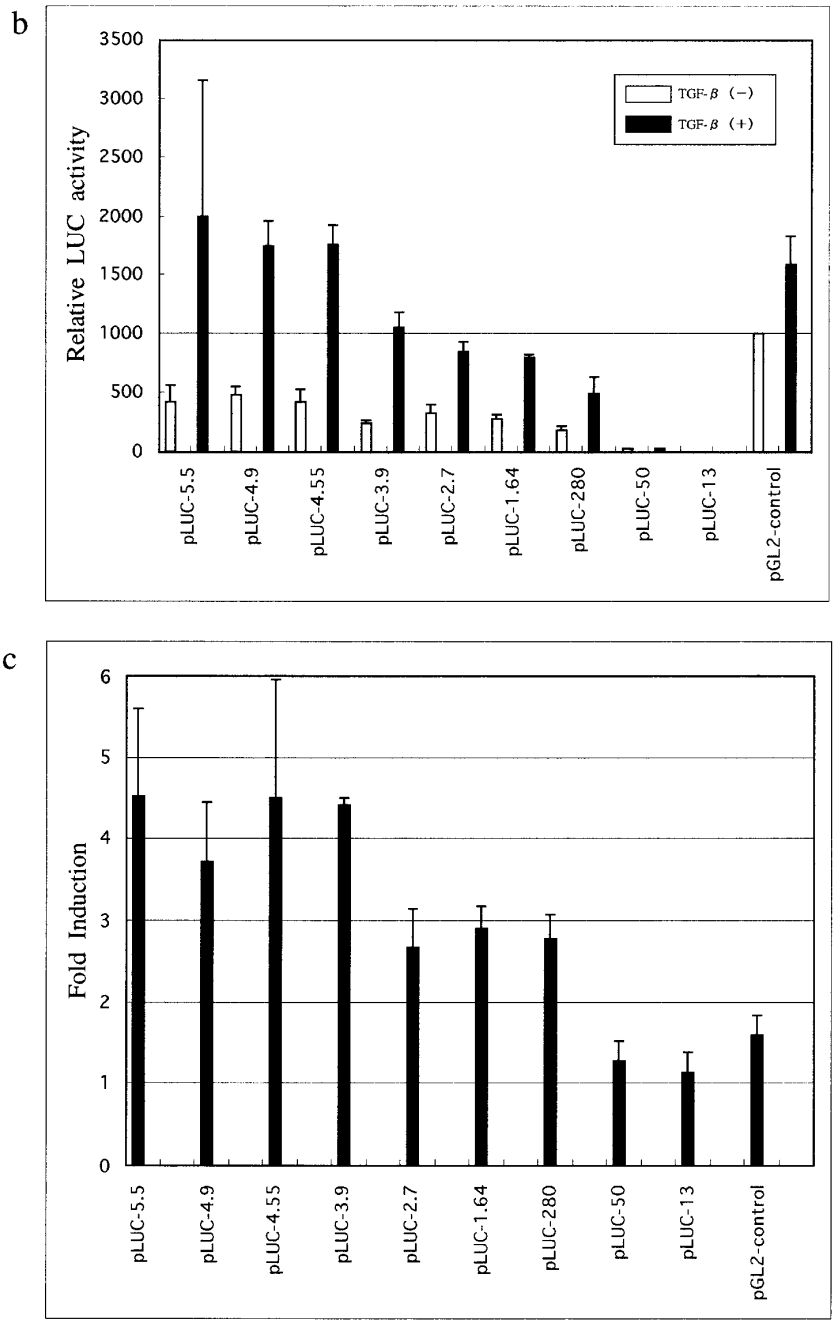

FIG. 4—*Continued*

have found that TGF-β1 operates as a common transcriptional stimulator of the gene expression of both HSP47 and collagen.

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