Activation of Fibroblast Procollagen α1(I) Transcription by Mechanical Strain Is Transforming Growth Factor-β-dependent and Involves Increased Binding of CCAAT-binding Factor (CBF/NF-Y) at the Proximal Promoter*

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During normal developmental tissue growth and in a number of diseases of the cardiopulmonary system, adventitial and interstitial fibroblasts are subjected to increased mechanical strain. This leads to fibroblast activation and enhanced collagen synthesis, but the underlying mechanisms involved remain poorly understood. In this study, we have begun to identify and characterize mechanical strain-responsive elements in the rat procollagen α1(I) (COL1A1) gene and show that the activity of COL1A1 promoter constructs, transiently transfected into cardiac fibroblasts, was increased between 2- and 4-fold by continuous cyclic mechanical strain. This was accompanied by a ~3-fold increase in the levels of total active transforming growth factor-β (TGF-β) released into the medium. Inclusion of a promoter-specific TGF-β neutralizing antibody inhibited strain-induced COL1A1 promoter activation. Deletion analysis revealed the presence of two potential strain response elements within the proximal promoter, one of which contains an inverted CCAAT-box overlapping a GC-rich element. Both mechanical strain and exogenously added TGF-β1 enhanced the binding activity of CCAAT-binding factor, CBF/NF-Y, at this site. Moreover, this element was sufficient to confer strain-responsiveness to an otherwise unresponsive SV40 promoter. In summary, this study demonstrates that strain-induced COL1A1 promoter activation in cardiac fibroblasts is TGF-β-dependent and involves increased binding of CCAAT-binding factor at the proximal promoter. Furthermore, these findings suggest a novel and potentially important TGF-β response element in the rat COL1A1 gene.

Cells of the cardiopulmonary system are constantly exposed to mechanical stimulation from shear and tensile stresses, and in a number of pathological states, e.g. systemic hypertension, these stresses are significantly exaggerated (1). There is increasing evidence that mechanical strain plays an important role in maintaining normal tissue architecture by influencing cell function and behavior and may also be involved in the pathogenesis of disease (2). This area of research has recently gained much interest with respect to tissue remodeling seen in fibrosis and tissue repair in major organs, including the heart and lung, because increased mechanical force (strain, shear stress, and pressure) has been shown to activate a multitude of intracellular signaling pathways and alter the expression of a large number of genes in cells from these organs (1, 3). In the cardiopulmonary system, interstitial fibroblasts are believed to play a central role in orchestrating tissue remodeling in response to mechanical strain, both by increasing the release of auto/paracrine factors leading to proliferation and by altering their expression of extracellular matrix genes (1).

Type I collagen, the most abundant protein in the interstitium of major organs, plays a critical structural role and influences numerous cellular responses (4). Excessive production of type I collagen is a key feature of fibrotic disorders of the lung, liver, kidney, skin, and heart and multisystem diseases such as systemic sclerosis and hypertension, often leading to severe organ dysfunction (5–7). Novel therapeutic approaches for these disorders will come from a thorough understanding of the molecular mechanisms involved in the regulation of the type I collagen genes in such pathological conditions. The expression of the genes COL1A1 and COL1A2, encoding the α1 and α2 chains of type I collagen, respectively, is controlled primarily at the level of transcription and is tightly regulated to allow physiologic modulation during development and tissue repair (8, 9). Numerous mediators, including transforming growth factor-β (TGF-β),1 connective tissue growth factor, insulin-like growth factor-I, prostaglandine-

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1 The abbreviations used are: TGF-β, transforming growth factor-β; CBF/NF-Y, CCAAT-binding factor; TAK1, transforming-growth factor β-activated kinase; CAT, chloramphenicol acetyl transferase; ELISA, enzyme-linked immunosorbent assay; PAI-1, plasminogen activator inhibitor-1; EMSA, electrophoresis mobility shift assays; TAE, TGF-β activation element; wt, wild type.
brosis is currently being explored. It was recently reported that transforming-growth factor-β-activated kinase, TAK1, a mediator of TGF-β signaling, is activated as a delayed response to mechanical stress in mouse myocardium. Furthermore, a constitutively active form of TAK1 expressed in the mouse heart was sufficient to cause cardiac hypertrophy, interstitial fibrosis, and severe myocardial dysfunction (13). We and others have shown that increased mechanical load enhances the expression of the COL1A1 gene both in vivo (1) and in isolated fibroblasts in vitro (14); however, the underlying molecular mechanisms leading to this change remain poorly understood. There is evidence from studies in mesangial (15) and smooth muscle cells (16) that enhanced COL1A1 expression in response to mechanical strain is TGF-β-dependent. Increased activation of TGF-β has also been demonstrated for fibroblasts subjected to strain (17); however, whether this was sufficient to cause an increase in COL1A1 expression was not examined.

A major TGF-β1 response element has been reported at position −1624 in the rat COL1A1 gene (18), but the functional importance of this site has subsequently been questioned (19). Moreover, results from two studies, including the original report, suggest the existence of other, as yet unidentified, response elements (18, 20). Several additional regulatory elements and their cognate transcription factors have been implicated in TGF-β transactivation of type I collagen genes in other species. Because of the high sequence homology in certain regions of the type I collagen promoters across species, these elements may represent potential additional candidates for mediating the response to TGF-β and mechanical strain in the rat COL1A1 gene. These elements have been shown to bind transcription factors Sp1, NF-I, and AP1 (1), and more recently also Smad proteins (21), the major effector molecules responsible for propagating TGF-β signaling following receptor activation (22). Early activation of the human procollagen α1(I) (COL1A2) gene by TGF-β was shown to involve interaction of Smad3/Smad4 with Sp1 in both fibroblasts (23) and mesangial cells (24). Smad proteins can also interact with components of the AP1 complex, as has been demonstrated in the activation of the collagenase I promoter (25). In addition, there is accumulating evidence that Smad3 plays a major role in mediating the fibrotic responses of TGF-β (26), but whether Smad complexes are directly involved in the activation of the type I collagen genes during prolonged cellular stress leading to fibrosis remains to be established.

With this background we hypothesized that transcriptional activation of the rat COL1A1 gene in cardiac fibroblasts subjected to mechanical strain is mediated by autocrine stimulation by TGF-β and that this activation involves TGF-β response elements in the promoter region of the gene. To address this hypothesis, rat cardiac fibroblasts were transiently transfected with rat COL1A1 promoter/reporter gene constructs and subjected to mechanical strain. Here we show that cyclic mechanical strain activates the rat COL1A1 promoter in cardiac fibroblasts via a TGF-β-dependent pathway. Furthermore, at least two regions in the proximal promoter, including an inverted CCAAT-box at position −100/−94, appear to be involved in the response. Finally, increased binding of CBF/NF-Y to this inverted CCAAT-box was shown to contribute to the transcriptional activation by mechanical strain. These findings have important implications for our understanding of the mechanism by which mechanical force increases COL1A1 gene activation. Importantly, these findings identify an as yet unappreciated role for CBF/NF-Y in regulating COL1A1 transcription in response to extracellular signals associated with enhanced extracellular matrix deposition.

**EXPERIMENTAL PROCEDURES**

**Cell Culture and Cyclic Mechanical Strain Applied to Fibroblasts—**

Primary cultures of fetal Sprague-Dawley rat cardiac fibroblasts were prepared by collagenase digestion as previously described (27), and maintained in Dulbecco’s modified Eagle’s medium (DMEM) containing 10% fetal bovine serum, 2 mM glutamine, and antibiotics (Invitrogen). Cells were used at passages 6–9 and plated on collagen I-coated elastic silicon membranes in 6-well (25-mm diameter) FlexI-1 plates (FlexCell International). Confluent cells were pre incubated in serum-free DMEM containing serum replacement 1 and HEPES buffer (Sigma), glutamine, and antibiotics. After 24 h, the medium was replaced with fresh serum-free medium, either alone or supplemented with either 50 ng/ml mithramycin (Sigma), 1 ng/ml porcine TGF-β1 (R&D Systems), 75 μg/ml pan-specific TGF-β neutralizing antibody (AB-100-NA, R&D Systems, Minneapolis, MN), or the same concentration of a control antibody. The effect of 1 ng/ml TGF-β1, or equivalent amounts of IgG control antibody, the TGF-β neutralizing antibody and mithramycin was added 1 h prior to subjecting the cells to mechanical strain. The cells were subjected to mechanical strain for 24–48 h using the Flexercell FX3000 apparatus (Flexcell International), which applies controlled cyclic or static strain to cell monolayers in culture. The strain applied was such as to give an average of 10% elongation of the membrane and a cycle frequency of 1 Hz. This strain regimen was chosen because it is similar to physiological/pathological tension experienced by cells in the heart (28).

**Plasmids and Generation of 5′ Deletion Constructs—**

The intronless pColCAT3.6/1.6 was purchased from MBI Fermentas at position −3518 and +1594 (relative to the transcription start site) of the rat COL1A1 gene, which includes the upstream promoter region, the first exon (the translational start site, ATG, has been converted to a NotI site), and most of the first intron (3.6/1.6 refers to 3.6 kb of upstream promoter sequence and 1.6 kb of downstream sequence). To create the promoter deletion constructs of the reporter gene, the polylinker has been converted to a ClaI site. The construct contains sequences between −3518 and +1594 (relative to the transcription start site) of the rat COL1A1 gene, which includes the upstream promoter region, the first exon (the translational start site, ATG, has been converted to a NotI site), and most of the first intron (3.6/1.6 refers to 3.6 kb of upstream promoter sequence and 1.6 kb of downstream sequence). The ends were blunt ended with Tth111I at −1282 to create pColCAT-1.3/1.6, or MunI at −395 to create pColCAT-0.4/1.6. The ends were blunt ended and the constructs religated. The intronless pColCAT3.6/0 has the first intron and the distal third of the first exon replaced with the 16 S splice donor site from the SV40 virus (compare B47 in ref. 29). The set of proximal promoter deletion constructs were generated using an ExoIII/Mung Bean deletion kit (Stratagene) following the manufacturer’s recommendations. MunI restriction enzyme was used to linearize B47 after which exonuclease treatment generated small deletions of various lengths in both directions. The constructs were subsequently digested with NheI and religated to generate promoter fragments with different 5′ ends but which contained an identical 3′ end cloning site. A set of constructs pColCAT-1.3/1.6 and −0.4/1.6. The exact extents of the deletions and the sequence across cloning sites were determined by automated DNA sequencing (377 DNA Sequencer, Applied Biosystems) using the Dye Terminator Cycle Sequencing Kit (Amersham Biosciences). The sequence numbering in the proximal promoter is according to the rat COL1A1 promoter sequence reported by Lichtler et al. (30) (GenBank™ accession number J04464). The heterologous promoter constructs were generated by ligating double-stranded oligonucleotides containing the wild-type or mutated Region A sequence (Fig. 4A), with additional restriction enzyme sites at either end, to the SV40 promoter-driven (enhancer-less) luciferase pGL2P vector (Promega). The correct sequence was confirmed by DNA sequencing as described above.

**Transcriptional and Promoter Activity Assays—**

Transfections of rat COL1A1 promoter-reporter constructs into rat cardiac fibroblasts (at ~90% confluence) were performed using the calcium phosphate Profection Mammalian Transfection System (Promega) according to the manufacturer’s recommendations 3 h after the addition of fresh serum-containing medium. At least two different plasmid DNA preparations (EndoFree, Qiagen) were tested to eliminate potential preparation artifacts. Cells were transfected with 0.5 μg of DNA per well (25-mm diameter) overnight (16 h), rinsed twice with phosphate-buffered saline and then treated as described above. At the appropriate time points, the cells were lysed and luciferase activity measured as chemiluminescence in a TD-20/20 luminometer from Turner designs (Promega) using the Luciferase Assay System (Promega). The data are presented as relative light units (RLU). Chloramphenicol acetyl transferase (CAT) protein levels in the cell lysates were measured by ELISA.
(Roche Molecular Biochemicals), were within the linear range of the standard curve and expressed as relative absorbance units (RAU). Because many viral promoters driving reporter genes in transfection control plasmids are regulated by mechanical strain (personal observation), a Hirt's assay was performed as described in Clark et al. (31) to determine the relative transfection efficiency between constructs used and between experimental conditions. Briefly, a DNA fragment from the vector backbone part of the COLIA1 promoter/reporter construct was radiolabeled and used as a probe in hybridizations to DNA from 30 μl of cell lysate that had been denatured and immobilized on a nylon membrane. The relative radioactivity signal representing the amount of plasmid DNA in the cell lysate was measured by phosphorimaging densitometry. No significant difference in transfection efficiency was observed between the constructs or between experimental conditions. Transfections were performed three or more times in triplicate wells, unless otherwise indicated in the figure legends. Because the study was performed using primary fibroblasts and significant variation was observed in the fold induction of promoter activity between experiments, the data presented are from one representative experiment showing mean and standard error within that experiment. The statistical significance was evaluated by the Mann-Whitney U test of combined experiments.

RESULTS

Cyclic Mechanical Strain of Cardiac Fibroblasts Leads to Transactivation of the Rat COLIA1 Promoter—Previous work has shown that COLIA1 mRNA levels are increased in rat cardiac fibroblasts subjected to mechanical strain (14). To begin to elucidate the transcriptional mechanisms involved, we initially used four rat COLIA1 promoter CAT-reporter constructs in transient transfection assays and exposed rat cardiac fibroblasts to continuous cyclic mechanical stretch (strain) for 48 h. All responses were compared with cells transfected with the same construct, but kept static under otherwise identical conditions (rigid controls) (Fig. 1). The full-length construct, pColCAT-3.6/1.6, contains 3.6 kb of the 5′ upstream promoter sequence and 1.6 kb of sequence downstream of the transcription start site, including the first exon and most of the first intron. This construct was chosen because these regions of the COLIA1 gene, including the first intron, are known to contain potentially important positive and negative regulatory sequences (29, 35–37), and it therefore provides a suitable starting point for modeling responses of the endogenous gene. Cells transfected with the full-length pColCAT-3.6/1.6 construct displayed an ~2-fold increase in CAT protein levels in response to continuous cyclic strain for 48 h compared with rigid control cells. Similar responses were obtained with a deletion construct in which sequences upstream of position −1.3 kb had been removed (pColCAT-1.3/1.6), whereas the response with a construct lacking sequences upstream of −0.4 kb (pColCAT-0.4/1.6) was significantly greater (p < 0.01). Taken together, these results indicate that repressor element(s) located in the region between −0.4 and −1.3 kb may dampen the stimulatory effect of mechanical strain. We also tested a construct in which the first intron had been deleted, pColCAT-3.6/0. The response to strain was greater compared with that obtained with construct pColCAT-3.6/1.6 containing the intron, suggesting that the first intron is unlikely to contain important strain response elements and that sequences within the intron may be involved in modulating the magnitude of the transcriptional response.
The data suggest that increased COL1A1 gene expression by mechanical strain is mediated, at least in part, by increased transcription and that regulatory sequences around the transcription start site, within the −0.4/1.6 construct, are involved in mediating this response. In addition, because the pColCAT-3/1.6 and pColCAT-1.3/1.6 constructs responded in a similar manner, the only previously reported TGF-β activation element (TAE) in the rat COL1A1 gene at position −1624 (18) appears not to be involved. This raised two possibilities: either the induction of COL1A1 transcriptional activation by mechanical strain in rat cardiac fibroblasts is independent of TGF-β or other important TGF-β response elements, apart from the site reported (18), are present in the rat COL1A1 gene.

TGF-β Activity Is Increased by Cyclic Mechanical Strain in Cardiac Fibroblasts and Is Necessary for Strain-induced COL1A1 Transcriptional Activation.—To examine whether TGF-β activity is necessary for strain-induced transactivation of the COL1A1 promoter, we determined the effect of mechanical strain on the activity of a promoter construct lacking the previously described TAE at position −1624 (pColCAT-1.3/1.6) in the presence of a pan-specific TGF-β neutralizing antibody. As shown in Fig. 2A, shows that mechanical strain increased activation of the pColCAT-1.3/1.6 construct ∼3-fold and that this increase was almost completely blocked in the presence of the antibody. In addition, the activity of this promoter construct was strongly induced by TGF-β1 added exogenously as a positive control. Involvement of TGF-β was further examined by determining levels of total active TGF-β in the media from mechanically strained and rigid control cells using a TGF-β bioassay based on Mv1Lu stably transfected with a TGF-β-responsive PAI-1 promoter luciferase reporter (32). The amount of TGF-β in conditioned medium from strained cells was 2.6-fold higher compared with the rigid control (p < 0.01 by Mann-Whitney). These luciferase activities were equivalent to 12 versus 39 pg/ml of TGF-β1 in medium from rigid and strained fibroblasts, respectively, as extrapolated from a standard curve from mink lung cells treated with exogenous porcine TGF-β1.

Based on these results, we propose the model illustrated in Fig. 3B as a mechanism for transactivation of the rat COL1A1 promoter by mechanical strain. The region between position −247 and −197 (Region C) contains a strong repressor element, which under basal conditions counteracts a positive element in the region between −197 and −129 (Region B). During mechanical strain the repression in Region C is lifted (indicated by the up-arrow in Fig. 3B) leading to activation of transcription. In addition, a significant portion of the strain response appears to be mediated via sequences downstream of position −129. Taking into account evidence from our initial experiments, which excluded the first intron as containing important region(s) for promoter transactivation in response to...
mechanical strain (Fig. 1), as well as previously reported studies on basal COL1A1 transcription (see below), we propose that the fragment denoted Region A (−129 to −84) may be involved in mediating the strain response observed using construct −129. Transcriptional activation of the COL1A1 gene by mechanical strain is, therefore, a complex process involving at least two regions (Region A and B/C) in the proximal promoter, which, estimating from the activities of the individual constructs, contribute approximately equally to the response.

In this study we have focused on Region A and explored the possibility that changes in transcription factor binding in this region during mechanical strain is responsible for increased transcriptional activity. Based on previously reported studies on basal COL1A1 promoter regulation and activation by TGF-β, the region between −129 and −84 (Region A) is of particular interest. It contains an inverted CCAAT-box at position −100 to which either CBF (38) or NF-I (39) can bind. It is flanked by GC-rich binding sites, shown to bind Sp1 (39), or an inhibitory factor designated IP2 (40). The binding of CBF to this site is important for basal COL1A1 promoter activity, as demonstrated by previously reported mutation analyses (38) and more recently for the COL1A2 gene using a dominant-negative form of CBF-A (41). CBF has also been suggested to play a critical role in the coordinate regulation of the two type I procollagen genes (42). Moreover, both NF-I and Sp1, which can act as transactivators at this site, have been implicated in TGF-β-induced collagen type I gene transcription in other species (43, 44); however, their binding activities at this particular site have not been investigated with respect to TGF-β stimulation or in response to mechanical strain. Finally, the fact that COL1A1 promoter activation via the mitogen-activated protein kinase (MAPK) ERK1/2 signaling pathway has been mapped to this specific region in activated hepatic stellate cells (45) and that the ERK1/2 pathway can be activated both in response to TGF-β (46, 47) and mechanical strain (48) lend further support for the involvement of this site in mediating the transcriptional activation of COL1A1 by mechanical strain.

Mechanical Strain and TGF-β1 Increases Binding Activity of CBF/NF-Y to the Inverted CCAAT-box in the Proximal Promoter. A, upper-strand of double-stranded oligonucleotides used as probes in EMSAs. The Region A oligonucleotide contains the sequence between −119 and −89 of rat COL1A1 according to Lichtler et al. (30) (GenBank accession number J04464). The core binding sites, including the inverted CCAAT-box (italics) are bold, and mutated bases are underlined. B, EMSA of Region A, left panel; left lane shows radioactively labeled Region A oligonucleotide as a probe, one predominant complex formed, Complex I–III. The intensity of the Complex I band was increased by 75% in response to both stimuli in three separate experiments, one of which is shown here. Middle panel, competition analysis using cold consensus binding site oligonucleotides (as indicated above the lanes) at 125-fold excess and Region A oligonucleotide as a probe or Region A mut and CBF oligonucleotides as probes (as indicated below the panel), suggesting that Complex I contains CBF. Right panel, EMSA using specific antibodies against CBF and NF-I and control IgG antibodies as indicated above the panel, and Region A oligonucleotide as probe confirming that Complex I contains CBF.

**FIG. 3.** Identification of two putative strain response regions in the proximal promoter. A, promoter activity of deletion constructs of the proximal promoter in rigid (white bars) and mechanically strained (black bars) cells. The number under each set of bars indicates the 5′-end position (in base pairs from the transcription start site) of the promoter fragment for that construct. Asterisk denotes a significant increase compared with rigid controls for each construct at p < 0.01 estimated by the Mann-Whitney U test from combined experiments. B, proposed mechanisms of transactivation of the rat COL1A1 promoter under basal condition and during mechanical strain (arrows). Up arrow indicates lifting of repression in Region C and down arrow indicates increased transactivation in Region A by mechanical strain. Angled arrow indicates the transcription start site.

**FIG. 4.** Mechanical strain and TGF-β1 increases binding activity of CBF/NF-Y to the inverted CCAAT-box in the proximal promoter. A, upper-strand of double-stranded oligonucleotides used as probes in EMSAs. The Region A oligonucleotide contains the sequence between −119 and −89 of rat COL1A1 according to Lichtler et al. (30) (GenBank accession number J04464). The core binding sites, including the inverted CCAAT-box (italics) are bold, and mutated bases are underlined. B, EMSA of Region A, left panel; left lane shows radioactively labeled Region A oligonucleotide as a probe, one predominant complex formed, Complex I–III. The intensity of the Complex I band was increased by 75% in response to both stimuli in three separate experiments, one of which is shown here. Middle panel, competition analysis using cold consensus binding site oligonucleotides (as indicated above the lanes) at 125-fold excess and Region A oligonucleotide as a probe or Region A mut and CBF oligonucleotides as probes (as indicated below the panel), suggesting that Complex I contains CBF. Right panel, EMSA using specific antibodies against CBF and NF-I and control IgG antibodies as indicated above the panel, and Region A oligonucleotide as probe confirming that Complex I contains CBF.
(Complex I) and two weaker broad complexes (Complexes II and III) were detected by EMSA (Fig. 4B). The intensity of the major complex was greater (indicating increased binding activity) using nuclear extracts from both strained and cells treated with TGF-β1, compared with nuclear extract from rigid control cells (Fig. 4B, left panel). Densitometric quantitation by phosphorimaging analysis showed that the intensity of the Complex I band was increased by ~75% in response to both stimuli for 24 h. Furthermore, in parallel with enhanced formation of Complex I, the formation of one of the weaker complexes, Complex III, appeared to be diminished in response to TGF-β1 and mechanical strain. This is likely because of either competition for binding at this site between the factors forming Complexes I and III or an actual reduced binding activity of Complex III (e.g. caused by reduced abundance or change in phosphorylation) in response to the two stimuli. Binding specificity and the identity of the factor(s) present in Complex I were further investigated by competition and supershift assays using unlabeled consensus sequence oligonucleotides and specific antibodies to known transcription factors. In competition assays (Fig. 4B, middle panel), a CBF consensus oligonucleotide abolished Complex I formation only, whereas a consensus oligonucleotide for NF-1 diminished Complex II formation. These data are consistent with CBF being present in Complex I. In addition, neither a mutated CBF (CBF mut) oligonucleotide nor a wild-type Sp1 consensus oligonucleotide competed with any of the complexes (Fig. 4B, middle panel). Moreover, when a Region A oligonucleotide with two base pair substitutions in the inverted CCAAT-box (Region A mut) was used as a probe, neither Complex I nor II formed. In contrast, when a CBF consensus oligonucleotide was used as a probe, only Complex I formed (middle panel, far right lane), confirming that Complex I formation involved binding to the CCAAT-box and again, strongly implicating CBF in Complex I formation. To confirm the presence of CBF within Complex I, DNA protein binding assays were performed with specific antibodies. The right panel in Fig. 4B shows that a CBF antibody supershifts Complex I, whereas control IgGs and an antibody to NF-1 have no effect.

These results demonstrate that CBF, an activator of COL1A1 transcription, is the predominant factor binding to the inverted CCAAT-box in Region A in rat cardiac fibroblasts. Furthermore, this is the first report showing that binding of CBF to the proximal inverted CCAAT-box in the COL1A1 promoter is enhanced by mechanical strain, as well as by TGF-β1, and is consistent with the notion that the strain-induced activation of COL1A1 transcription, at least at this site, is a direct effect of the increase in TGF-β activity.

A Region A Oligonucleotide Can Confer Strain-induced Transactivation on a Heterologous Promoter—We further assessed the functional role of the inverted CCAAT-box in Region A in mechanical strain-induced promoter activation by mutation analysis. Because specific mutations in the inverted CCAAT-box within the COL1A1 promoter have been reported to strongly reduce basal transcriptional activity (38), we decided to investigate the functionality of this site by assessing its ability to confer strain response to an otherwise unresponsive heterologous promoter (SV40). The wild-type Region A oligonucleotide (Region A wt) containing the overlapping CCAAT- and GC-boxes used in the EMSAs (Fig. 4) was cloned into the luciferase reporter gene vector pGL2P (Fig. 5B). Fig. 5A shows that promoter activity of this construct was significantly higher in mechanically strained cells, compared with rigid controls, and similar in magnitude to that obtained with construct −129 in which Region A is located within the context of the COL1A1 promoter. In contrast, pGL2P itself did not respond to strain, indicating that the response obtained with the Region A wild-type construct is controlled by sequences within the oligonucleotide itself. Furthermore, a construct containing the Region A oligonucleotide with a mutated CCAAT-box (Figs. 4A and 5B, Region A mut) displayed a significantly reduced strain response (p < 0.01), confirming that factor(s) binding to the CCAAT-box play a significant role in the activation. Although much smaller in magnitude than the response with the wild-
type construct, the strain-induced promoter activation observed with the Region A mut construct was still significant. This suggested that factors binding to another site, such as the GC-box, may also contribute to transcriptional activation induced in response to strain, at least under circumstances when there is no binding at the inverted CCAAT-box. To test this, mithramycin, an inhibitor of protein binding to GC-rich sequences, was added to the culture medium. This resulted in an overall reduction in promoter activity of all three constructs, which is likely because of inhibition of protein binding to Sp1 sites (GC-boxes) in the SV40 sequence, known to drive transcription from this promotor. Nevertheless, there was still a significant induction of the activity of the Region A wt construct with mechanical strain, confirming the important contribution by the CCAAT-box in mediating a strain response. In contrast, mithramycin caused a total loss of induction of the Region A mut construct, suggesting that the remaining small activation obtained in response to mechanical strain in the absence of CBF binding is contributed by factors binding to the GC-box in Region A. Because the only binding activity detected by EMSA using the mutated Region A oligonucleotide as a probe is Complex III, which consistently displayed diminished binding during mechanical strain (Fig. 4B, left panel), it is possible that this protein complex contains a transcriptional repressor and that activation may involve lifting of this repression. A model describing transcription factor binding to the Region A sequence, and the effects of mutations in the CCAAT-box and addition of mithramycin (M) on binding, is illustrated in Fig. 5B where R represents the putative repressor binding to the GC-box.

**DISCUSSION**

Enhanced type I collagen expression plays an important role in driving excessive matrix deposition in a number of fibrotic disorders. Despite a long-established correlation between increased mechanical strain on tissues and extracellular matrix accumulation, and more recent in vitro studies confirming a direct link, very little is known about the underlying mechanisms of this phenomenon. The present study begins to elucidate a complex regulation of the COL1A1 gene by mechanical strain by demonstrating for the first time that strain-induced transcriptional activation in cardiac fibroblasts is: (a) dependent on the release of active TGF-β, (b) mediated by at least two regions in the proximal promoter, and (c) involves increased binding of CBF to an inverted CCAAT-box.

The activation of TGF-β by mechanical forces has been demonstrated previously in a number of cell types, including mesangial cells (49) and cardiac fibroblasts (17). Because TGF-β is the most potent activator of collagen gene expression identified to date (4), there is a strong possibility that the induction of collagen transcription by mechanical stress is mediated through the autocrine stimulation by TGF-β. Hori et al. (15) proposed experimental evidence that this is the case in mesangial cells, and were supported by a subsequent study of intestinal smooth muscle cells (16). With the results presented here, obtained with cardiac fibroblasts, it is now evident that a mechanism of mechanical strain-induced COL1A1 expression dependent on autocrine TGF-β stimulation exists for a wide range of mesenchymal cells. There are several mechanisms by which active TGF-β may contribute to the strain-induced activation of COL1A1 transcription observed. First, the increase in COL1A1 promoter activity may be directly caused by the increase in TGF-β activity and, therefore, involve a novel TGF-β response element in the COL1A1 gene. Second, TGF-β may have a permissive function and contribute indirectly by providing a cell-signaling pathway (e.g. by stimulating the synthesis of a signaling molecule) without which strain-induced activation of the COL1A1 gene cannot occur. Such synergistic effects have been observed in several recent studies, for example, those involving intracellular signals from growth factor receptors and integrins (50). Given the recognized effect of TGF-β on COL1A1 transcription, combined with our findings that the proximal inverted CCAAT-box mediates strain-induced transcriptional activation, and that TGF-β alone can cause increased binding activity of CBF at this site, it is highly likely that, at least at this site, the COL1A1 transcriptional activation by mechanical strain is a direct effect of TGF-β stimulation.

A major TGF-β activation element has previously been identified by Ritzenthaler et al. (18) at position −1624 in the rat COL1A1 gene. It is, therefore, of interest that in our study mechanical strain did not require this TGF-β response element. The reason for this apparent discrepancy is not clear; however, some differences in the experimental model between our study and that of Ritzenthaler et al. may be significant, for example, the choice of cell-type. We used the rat promoter constructs transfected into rat cardiac fibroblasts, whereas Ritzenthaler et al. used human embryonic lung fibroblasts (IMR-90). Another difference between the two studies, which may be a more likely cause of the discrepancy, is the fact that we employed promoter constructs containing the first intron of the gene. The first intron has been shown to contain important repressor elements (36), and it is therefore possible that in our experiments the TAE at position −1624 is counteracted by these repressor elements. The original study by Ritzenthaler et al. (18), although demonstrating a dramatic decrease in promoter activation in response to TGF-β by deleting the TAE, provided some evidence for the existence of additional response elements because the response was still −2-fold in the absence of the TAE. These observations were subsequently confirmed by a second independent study by King et al. (20) employing rat lung fibroblasts. Interestingly, a recent in vivo study by the group originally reporting the TAE, using transgenic mice carrying 3.6 kb of the rat COL1A1 promoter gene with or without mutations in the TAE, has provided evidence that questions the critical role of this site (19). In this study, both the wild-type and mutated constructs showed a similar up-regulation of reporter gene expression during bleomycin-induced fibrosis, which is mediated in part by TGF-β, supporting the notion that other important TGF-β response elements exist in the rat COL1A1 promoter.

Previously reported studies mapping TGF-β response elements in the two type I collagen genes from different species collectively suggest that the regulation is complex, involving diverse transcription factors binding to several distinct sites (21, 43, 44, 51, 52). Results from our study strongly suggest that the site comprising the proximal inverted CCAAT-box and adjacent GC-box around position −100 is one of the so far unidentified TGF-β response elements in the rat COL1A1 gene. The predominant factor shown to bind to this site, CBF (also known as NF-Y or CP1), is a ubiquitous transcription factor important for efficient basal transcription of several mammalian genes containing CCAAT-box elements, including the serum albumin and major histocompatibility complex class II genes (53). It acts by disrupting the nucleosome structure (54) and cooperatively interacts with promoter-specific transcription factors to activate transcription (53, 55). More recent studies have shown that CBF also plays a role in regulating a number of genes during conditions of cellular stress, for example, in the activation of the multiple drug resistance gene MDR1 by UV radiation (56), and activation of the tissue inhibitor of the metalloproteinases-2 (TIMP-2) gene in response to cAMP (57). A specific role for CBF in the induction of COL1A1
transcription had not previously been investigated. Our findings that CBF binding activity is increased during mechanical strain, and in response to TGF-β1, in cardiac fibroblasts are novel and suggest a wider role for CBF in regulating type I collagen expression. A recent report published during the course of this study supports this notion by demonstrating increased binding activity of CBF in dermal fibroblasts from patients with systemic sclerosis (58), a multisystem fibrotic disorder in which TGF-β has been strongly implicated. Moreover, in a recent study performed in our laboratory using microarray technology, mRNA levels of CBF-C, one of the three CBF subunits, were increased 4.6-fold in fibroblasts exposed to TGF-β1 compared with untreated controls, demonstrating a direct link between TGF-β signaling and CBF regulation. A detailed study of the induction of the CBF subunits in response to TGF-β is in progress in our laboratory.

In our experiments, investigating a functional role for Region A in a heterologous promoter construct, a second factor(s) (Complex III), binding to the GC-box immediately downstream of the inverted CCAAT-box, also appeared to be involved in the mechanical strain response. The fact that its binding activity demonstrated in EMSA is consistently reduced in response to mechanical strain and TGF-β (Fig. 4B), suggests that this factor may be a repressor. The identity of this factor is currently unknown, although Sp1 or Sp3 could be excluded because an Sp1 consensus oligonucleotide was unable to compete with its binding in EMSA. Another likely possibility is the repressor IP2, previously reported by Karsenty and de Crombrugghe (40). However, antibodies to this factor are not commercially available. The small but significant contribution from the GC-box to strain-induced transcriptional activation suggests a functional involvement of this site. However, this observation was made in the absence of binding of CBF to the CCAAT-box (i.e., using the Region A mut construct). Competitive and mutually exclusive binding to these two overlapping sites has been demonstrated together with a higher binding affinity of CBF for the CCAAT-box (59). Because CBF is the predominant factor binding to this site in the experimental conditions used here, we propose that the major contribution from this element to the mechanical strain response is attributed to the increased binding of CBF to the inverted CCAAT-box. We also suggest that the reduction in binding activity of Complex III observed in the EMSA in response to TGF-β and mechanical strain is because of competition by the increase in CBF binding. Further experiments, however, will be required to firmly exclude an involvement of the GC-box and Complex III in mechanical strain-induced COL1A1 promoter activation. This study also identified a more distal region in the proximal promoter (Region B/C), which appears to be involved in the mechanical strain response and which, therefore, may also contain a TGF-β activation element. Previous results from studies of basal regulation are in accordance with our proposed model of mechanical strain activation in this region (Fig. 3B). A repressor element, known to bind cKrox (60) or BFCOL-1 (61) under basal conditions, has been mapped to the sequence spanning the junction between regions in the mouse gene equivalent to Regions B and C. However, the role of this element in regulating COL1A1 transcription during cellular stress and in response to TGF-β has not been evaluated. Furthermore, in rat cardiac fibroblasts a strong positive element has been mapped to Region B under basal conditions, and an upstream repressor has been suggested to counteract the effect of this positive element (62). Our data agree with this because the reported repressor element would be disrupted in the ~197 construct and give rise to high basal levels. Furthermore, if the repression is alleviated by mechanical strain, this may explain the increase in activity in response to strain observed with the ~247 construct, as well as the lack of increase of the already high activity seen with the ~197 construct. Moreover, in a study by Jimenez et al. (51), a region in the human gene equivalent to the region containing the positive element identified in the rat gene (Region B) was shown to contain a TGF-β response element. The factors binding at this site have not been characterized, and although there are areas of significant sequence homology between species in this region, TGF-βresponsiveness at this site has not been evaluated in the murine or rat COL1A1 genes. A detailed study is currently underway to characterize transcription factor binding in this region of the rat COL1A1 gene during stimulation by mechanical strain and in response to TGF-β.

In summary, this study demonstrates that activation of the rat COL1A1 gene by mechanical strain involves at least two regions within the proximal promoter. Our data further suggest that this response is TGF-β-dependent and involves increased binding of the ubiquitous transcription factor CBF at the proximal inverted CCAAT-box element. Based on these findings, and the fact that functional CBF-binding sites in both COL1A1 and COL1A2 genes are highly conserved between species, we propose that CBF may play an important role in the up-regulation of type I collagen expression in response to cellular stresses mediated by TGF-β stimulation. Overexpression of the type I collagen genes is believed to be a key event leading to excess deposition of extracellular matrix in tissue fibrosis. A closer investigation of the role of CBF in fibrotic conditions is therefore warranted.

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Activation of Fibroblast Procollagen \( \alpha 1(1) \) Transcription by Mechanical Strain Is Transforming Growth Factor-\( \beta \)-dependent and Involves Increased Binding of CCAAT-binding Factor (CBF/NF-Y) at the Proximal Promoter

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