A Novel Transforming Growth Factor β Response Element Controls the Expression of the Connective Tissue Growth Factor Gene

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Abstract
We reported previously that transforming growth factor β (TGF-β) selectively induced high levels of connective tissue growth factor (CTGF) mRNA and protein in human skin fibroblasts. In this study, we investigated the molecular mechanism for TGF-β regulation of CTGF gene expression. Northern blot and run-on transcription assays indicate that TGF-β directly activates transcription of the CTGF gene. Fragments of the 5' flanking region of the human CTGF gene were linked to luciferase reporter constructs. TGF-β induced a 25–30-fold increase in luciferase activity in NIH/3T3 fibroblasts that had been transfected with this construct compared with nontreated cells after 24 h incubation. Other growth factors, such as platelet-derived growth factor or fibroblast growth factor, caused only a 2–3-fold induction. This response to TGF-β occurred only in human skin fibroblasts, fetal bovine aortic smooth muscle cells, and NIH/3T3 fibroblasts but not in the epithelial cell lines tested. Analysis of deletion mutants indicated that an important TGF-β regulatory element is located between positions −162 and −128 of the CTGF promoter sequence. A fragment of the promoter containing this region conferred TGF-β induction to a SV40 enhancerless promoter. Methylation interference and competition gel shift assays mapped a unique 13-nucleotide sequence delineating a novel TGF-β cis-regulatory element. Point mutations in this region result in a complete loss of the TGF-β induction, identifying this sequence as a new TGF-β response element.

Introduction
The formation of new and regenerating tissue requires the coordinate regulation of various genes that produce both regulatory and structural molecules that participate in the process of cell growth and tissue organization. This process proceeds in a cascade, with ordered invasions by different specialized cell types. Each cell type produces a set of factors that act to condition the site of regeneration and repair, to recruit the next wave of cells, and to regulate their behavior during the next phase of the cascade. TGF-β appears to be a central regulatory component of this process. TGF-β is released by platelets (1) macrophages, and neutrophils (2, 3) that are present in the initial phases of the repair process. TGF-β can act as a growth-stimulatory factor for mesenchymal cells (4–6) and as a growth-inhibitory factor for endothelial and epithelial cells (7–10). The growth stimulatory action of TGF-β appears to be mediated via an indirect mechanism of induced autocrine growth factors, such as PDGF-BB (5) or PDGF-AA (11, 12), or CTGF (13, 14).

We initially identified CTGF, isolated from the conditioned media of human umbilical vein endothelial cells due to its PDGF-like biological and immunological activities (15). CTGF is a member of a protein family that includes serum-induced immediate-early gene products such as Cyr61 (16) and Fisp 12 (17)/BIGM2 (18), a v-src-induced peptide (CEF-10; Ref. 19) and a putative avian oncoprotein (nov; Ref. 20). These peptides are characterized by an absolute conservation of 38 cysteine residues that make up over 10% of the total amino acid content. Fisp 12 (BIGM2) is likely to be the mouse homologue of CTGF because it has a 94% amino acid sequence identity to CTGF. Twisted gastrulation (twg), a gene that functions to control cell fate during dorsal ventral pattern formation in Drosophila embryogenesis, is more distantly related to CTGF (21). Thus, CTGF-related gene products appear to function in a wide variety of important biological processes, including normal embryonic development and tissue regeneration, as well as tumor formation and growth.

We wanted to better understand the mechanisms that control the expression of the CTGF gene. To accomplish this, we have examined the molecular mechanisms for the elevation of CTGF transcripts by TGF-β in fibroblastic cells in vitro. The results of our experiments indicate that a major regula-
tory mechanism for the elevation of the CTGF mRNA by TGF-β is increased transcription from the CTGF promoter and have led to the identification of a new TβRE. The TβRE sequence is distinct from previously described TGF-β regulatory elements identified in the α2(I) collagen gene (22), the plasminogen activator inhibitor (PAI-1) gene (23), or the EGF receptor gene (24).

Results

Prolonged Induction of CTGF mRNA by Short-Term TGF-β Exposure. Some immediate-early genes, such as c-fos, that are induced by growth factors exhibit a short burst of expression, although the growth factor remains present in the media (25). Others, such as c-myc, exhibit a more prolonged period of expression (26), and some, such as JE/MCP-1, remain induced as long as the growth factor is present in the media (27). CTGF transcripts remain at high levels for over 24 h after activation of the cells with TGF-β (14). To determine whether the long term elevation of CTGF transcripts was dependent on the continuous presence of TGF-β, we performed the following series of experiments. Confluent human skin fibroblasts were cultured in serum-free DMEM-ITS for 24 h prior to adding TGF-β. After a 1-h exposure to TGF-β, cells were washed in PBS, and the media were replaced with DMEM-ITS, followed by different periods of incubation.

Northern blot analysis revealed that CTGF mRNA was strongly induced from 4 to 30 h after TGF-β removal (Fig. 1A). Next we investigated the ability of TGF-β to induce long-term expression of the CTGF gene in the presence of the protein synthesis inhibitor cycloheximide. As shown in Fig. 1B, a 1-h stimulation by TGF-β in the presence of cycloheximide was sufficient to induce CTGF mRNA 4 h later as well as 24 h later. Cycloheximide alone induced a transient increase in CTGF mRNA 4 h later, suggesting the possibility of mRNA stabilization, as has been reported for cycloheximide induction of other transcripts such as c-fos or c-myc (28). However, a recent report by Edwards and Mahadevan (29) indicated that the protein synthesis inhibitors cycloheximide and anisomycin, but not puromycin, could act to stimulate transcription of the c-fos and c-jun genes. We investigated the ability of anisomycin and puromycin to elevate CTGF transcripts or to inhibit TGF-β induction of CTGF transcripts (Fig. 1C). Puromycin did not induce the CTGF mRNA at any of the concentrations tested (up to 100 μg/ml), which were 10-fold higher than that needed to completely block protein synthesis in our cells. Even at this high concentration, it had no effect on the ability of TGF-β to induce the CTGF mRNA. Anisomycin did elevate CTGF transcripts (Fig. 1C), as was seen with cycloheximide, although TGF-β treatment raised the level of CTGF mRNA in the presence of anisomycin. These data indicate that TGF-β directly regulates CTGF gene expression via a mechanism that is independent of protein synthesis and that increased transcription plays a role in this process.

To confirm that TGF-β induces increased transcription of the CTGF gene, we performed nuclear run-on assays (Fig. 2). Confluent human skin fibroblasts were cultured in DMEM-ITS for 24 h, and their nuclei were isolated after a 4- or 24-h exposure to TGF-β. We compared the level of CTGF tran-

Fig. 1. A, prolonged induction of CTGF mRNA by short-term TGF-β exposure. Confluent cultures of human skin fibroblasts were incubated with DMEM-ITS for 24 h prior to the addition of TGF-β. After the treatment with 10 ng/ml of TGF-β for 1 h, cells were washed in PBS and incubated with DMEM-ITS for the indicated time periods. B, the effect of cycloheximide on induction of CTGF mRNA. Lanes A and H, nontreated control cells at 4 and 24 h, respectively. Lane B, 4 h cycloheximide (10 μg/ml); Lane C, 4 h TGF-β (10 ng/ml); Lane D, 4 h cycloheximide (10 μg/ml) with TGF-β present for 1 h during hours 1 to 2 of cycloheximide exposure, RNA was prepared at 4 h; Lane E, same as B, with RNA prepared 20 h after removal of cycloheximide; Lane F, 24 h TGF-β (10 μg/ml); Lane G, same as D, with RNA prepared 20 h after removal of cycloheximide (22 h after removal of TGF-β). C, the effect of protein synthesis inhibitors on induction of CTGF mRNA. Cells were treated with puromycin or anisomycin for 4 h. TGF-β was added 1 h after protein synthesis treatment; then cells were incubated for an additional 3 h, and the mRNA was prepared at that time.
sequence comparison showed that the human CTGF promoter has an 80% sequence identity to the murine Fisp 12 promoter in the region 300 nucleotides 5' flanking the transcription start site (Fig. 3B). Further upstream regions exhibit almost no similarity in DNA sequence.

**TGF-β Regulation of the CTGF Promoter.** To determine whether the 5' flanking region of CTGF functions as a TGF-β-inducible promoter, we constructed a fusion gene containing the CTGF promoter (nucleotides −823 to +74) and the coding region of the firefly luciferase gene in the vector pGL2-basic. Luciferase activity was tested in a transient transfection assay using NIH/3T3 cells. This construct conferred a 25–30-fold induction of luciferase activity after a 24-h stimulation by TGF-β (10 ng/ml) compared with control cultures (Table 1). We have reported previously that when either CTGF protein or transcripts (14) were measured, other growth factors, including PDGF, EGF, and FGF, stimulated only a transient and low level induction. Using the luciferase reporter assay, we found that growth factors that act via tyrosine-based receptors, such as PDGF, EGF, and FGF, stimulate only 2–3-fold induction of luciferase activity under identical conditions (Table 1). When this promoter fragment was cloned in the reverse orientation (+74 to −823), only basal levels of luciferase activity were detected, and this level was unaffected by TGF-β or other growth factor treatment of the cells. The same pattern of growth factor induction was observed when human skin fibroblasts, or fetal bovine aortic smooth muscle cells, were used instead of NIH/3T3 cells (Table 1). TGF-β did not induce luciferase activity in several epithelial cell lines (Table 1), demonstrating that TGF-β induction of the CTGF gene appears to occur only in mesenchymal-derived connective tissue cells. The lack of any response by the epithelial cells is not due to a lack of a TGF-β response because the growth of these cells is inhibited by TGF-β (10 ng/ml).4 The induction of luciferase activity under the control of the CTGF promoter only requires a brief exposure of the cells to TGF-β because a 1-h treatment of the cells with TGF-β gives nearly the same fold induction at 4 and 24 h as cells continuously exposed to TGF-β (Table 2). These results confirm the data from the Northern blots described previously and demonstrate that transcriptional regulation plays a primary role in the control of CTGF gene expression by TGF-β.

**Identification of a Region in the CTGF Promoter Required for TGF-β Induction.** To delineate which region of the promoter sequence is responsible for the induction by TGF-β, we constructed deletion mutants of the CTGF promoter using PCR primers designed to delete the known transcription factor consensus elements. Regions of the promoter beginning at the 5'-most region and moving toward the transcription start site were systematically deleted (Fig. 4A). No significant changes in the basal level of expression of the chimera gene were detected for any of the deletion constructs compared to the largest construct used (−823 to +74). With respect to the TGF-β inducibility of the CTGF promoter, removal of the nucleotide sequence from −823 to −363, which included an AP-1 site and the CArG box, had no significant effect on the induction of luciferase activity by TGF-β. An approximate 25% reduction was seen
when the region from -363 to -276 was deleted, which contained the second AP-1 site. Deletion of the DNA sequence (−276 to −128), which contains the NF-1-like sequence, eliminated the TGF-β inducibility of the promoter, suggesting that this region contains an important TGF-β response element. Taking advantage of two BsmI sites, we deleted the nucleotides from −162 to −95, leaving the remaining portions of the promoter intact. This construct exhibited a complete loss of TGF-β inducibility, demonstrating that the nucleotides sequence between positions −162 and −128 appears to be essential for the TGF-β induction of luciferase activity. In this construct, the TIE-like site is deleted, but the NF-1-like site is retained.

We next constructed a fusion gene by placing the nucleotides from positions −275 to −106 of the CTGF promoter upstream from an SV40 enhancerless promoter controlling a luciferase structural element to determine whether this region of the promoter was sufficient to confer TGF-β inducibility (Fig. 4B). The SV40 enhancerless promoter was not regulated by TGF-β. However, the promoter containing the CTGF sequences −275 to −106 conferred nearly a 9-fold induction after TGF-β treatment. Inversion of the fragment resulted in little stimulation of luciferase activity after TGF-β treatment. These data confirm the results of our deletion studies and indicate that sites located between nucleotides −275 and −106 of the CTGF promoter can act independently as TGF-β regulatory elements.

**Nuclear Protein Binding Sites in the TGF-β Response Region of the CTGF Promoter.** A series of competitive gel shift assays were performed to identify sites in this region of the CTGF promoter that were binding nuclear proteins and to determine if there were any differences between the gel shift patterns of nuclear proteins isolated from control and TGF-β-treated cells. Nuclei were prepared from control and TGF-β-treated cells (10 ng/ml for 6 h) and the proteins extracted as described under methods. A fragment of the promoter containing the nucleotides from −275 to −109 was used as the labeled probe. As seen in Fig. 5, an identical pattern of a single shifted band was detected in the gel shift assay when nuclear protein extracts were used from either control or TGF-β-treated cells. Studies comparing extracts prepared from isolated nuclei versus whole cells or cytoplasm indicated...
that the protein was predominantly present in the nucleoplasm (data not shown). The binding to these nuclear proteins appears to be specific because it can be competed with either a fragment of the promoter, which is identical to the probe (−275 to −106), or by a smaller fragment (−205 to −109) but not by nucleotides from −128 to +74 (Fig. 5). Because no significant difference was seen between the control and TGF-β activated cells, we used nuclear extracts from nonactivated cells for all future studies, unless otherwise indicated. These data suggest that a TβRE is located between nucleotide positions −204 and −128. The region of the promoter from −204 to −128 contains the NF-1-like and TIE-like sequences and a portion of the region deleted by BamHI digestion in the P5 promoter construct that lacks TGF-β inducibility (Fig. 4A). We next compared the ability of oligonucleotides that represented various portions of the sequence between positions −204 and −109 to act as competitors in our gel shift assay. A diagram of the probe and the various competitor fragments used is illustrated in Fig. 6. The results of these studies demonstrate that any fragment that contained nucleotides from −169 to −150 could act as a specific competitor for the labeled promoter fragment containing bases from −204 to −106. Oligonucleotides that contained only the NF-1 like or TIE-like sites were not effective as competitors in this assay.

To map the binding of nuclear proteins more precisely to this and adjacent regions of the CTGF promoter and to demonstrate that specific sequences were responsible for the binding in the gel shift assays, we performed sequence analysis using methylation interference assays. Initially, a fragment of the promoter from positions −275 to −106 was used to detect binding sites. The methylation pattern was determined for the shifted band (Fig. 7A, Lane S), for the non-shifted free probe (Fig. 7A, Lane F), and for the intact free probe (Fig. 7A, Lane G). As we had seen in the competition gel shift assays, the results of these studies indicated that neither the NF-1 like site or the TIE-like element were interacting with any nuclear proteins. We also did not detect any evidence of protein binding to any sequence between nucleotides −275 and −106, except for the region located at nucleotides from −157 to −147, where four G residues were not methylated in the shifted band, indicating that this was a nuclear protein binding site. Methylation sequence analysis of the complimentary strand indicated that two G residues, which were paired to C residues at positions −153 and −145, were also involved in nuclear protein binding (data not shown). A smaller fragment of the region (nucleotides −169 to −139) was then used to give better resolution of the involved G residues. The results of these studies (Fig. 7B) confirmed our observations with the larger fragment and map G residues on both strands that are located between positions −157 and −145 upstream from the transcription start. This sequence is in the same region of the promoter mapped by the competition gel shift assays and is contained within the region (−204 to −128) delineated by the deletion mutant analysis in the luciferase reporter assays.

We then wanted to compare various oligonucleotides from this region of the promoter to determine which was most effective for binding to the nuclear proteins. Competition binding assays were conducted that compared the effectiveness of oligonucleotides containing the intact sequence determined from the methylation interference assays with oligonucleotides that contained only a portion of the sequence determined by methylation interference and some of the contiguous adjacent sequence. The concentrations of the oligos were adjusted so that equivalent amounts were used as competitors. The results of these experiments are illustrated in Fig. 8. The oligonucleotide that represented the CTGF promoter sequence from positions −159 to −143 and contained the intact sequence determined by methylation interference sequence analysis was a highly effective competitor with 10 ng of oligonucleotide completely blocking the binding to the labeled probe (nucleotides −169 to −139). Oligonucleotides that lacked the 3-prime TC sequence of the mapped element were 10-fold less effective competitors, and those lacking the 3-prime region from −151 to −145

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Table 1 Cell type and growth factor regulation of the CTGF promoter

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<th>Cell</th>
<th>No ADD*</th>
<th>TGF-β</th>
<th>PDGF</th>
<th>FGF</th>
<th>EGF</th>
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<td>NIH/3T3</td>
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<td>2.5</td>
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<td>6.8</td>
<td>0.9</td>
<td>1.2</td>
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<td>0.5</td>
</tr>
<tr>
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<td>1.4</td>
<td>ND</td>
<td>1.3</td>
<td>1.4</td>
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<tr>
<td>HEP G2</td>
<td>1.36</td>
<td>1.8</td>
<td>ND</td>
<td>1.9</td>
<td>2.4</td>
</tr>
</tbody>
</table>

* No ADD, no additions; ND, not determined; HSF, human foreskin fibroblasts (primary); VSMC, fetal bovine aortic smooth muscle cells (primary); HBL100, human breast epithelial cell line (nontumorigenic); HEP G2, human hepatic epithelial cell line (nontumorigenic).

Numbers in parentheses, (23.4) fold induction over No Additions Control luciferase activity, photons/min × 10⁻⁴.

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Table 2 Short-term TGF-β exposure stimulates long-term CTGF promoter activity

<table>
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<tr>
<th>Duration of TGF-β exposure</th>
<th>Time of assay of luciferase activity</th>
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<tr>
<td>4 h</td>
<td>24 h</td>
</tr>
<tr>
<td>Continuous</td>
<td>3.8</td>
</tr>
<tr>
<td>1 h</td>
<td>3.5</td>
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</table>

* Fold Induction of luciferase activity determined as described in the Table 1 legend and in "Materials and Methods." NIH/3T3 cells were used for these experiments. Results are the average of duplicate transfections, where the values varied by less than 10%. These studies were repeated in three separate studies, all of which gave similar results.
were 100-fold less effective competitors than the oligonucleotide containing the intact sequence. An oligonucleotide that contained only the region from −150 to −145 and lacked the 5-prime portion of the element was nearly 1000-fold less effective as a competitor. These data indicate that both of these regions of the sequence are important for recognition by the nuclear proteins that are binding to this element.

To link this binding element to the TGF-β regulation of the CTGF gene, we constructed intact promoters with PMs that were contained within the element from positions −157 to −145 (Table 3). The generation and fidelity of these PM constructs were confirmed by DNA sequence analysis, and the mutant promoters were tested in the luciferase reporter assay. We chose to alter G residues that had been identified by the methylation interference assay as sites that would most likely have an impact on TGF-β responsiveness of the promoter. Four different constructs were produced that contained either single-base PM2 and PM4 changes or two-base PM1 and PM3 changes. None of the PMs significantly altered the basal level of expression of the CTGF promoter. However, all mutant constructs exhibited significant reductions in the TGF-β inducibility of the chimeric gene. The PM4 construct, which contained a single change from the normal sequence with a T in place of a C at position −153, exhibited a 45% reduction in TGF-β induction of luciferase activity compared to the normal promoter. All three of the other constructs, PM1 (two G residues replaced with T at positions −155 and −157), PM2 (G replaced with a T at position −148), and PM3 (two G residues replaced with T at positions −148 and −157), exhibited little if any detectable induction of luciferase activity by TGF-β compared to the normal promoter sequence. These results demonstrate that PMs in this region can inactivate the response of the CTGF promoter to TGF-β and identify this sequence as a TgRE.

Discussions

The regulation of CTGF gene expression is distinct from that of other genes that have been demonstrated to be directly regulated by growth factors in two ways: (a) the gene is strongly induced by TGF-β but not by other growth factors,
factor or serum-induced genes, including a CaRG box and AP-1 and SP-1 sites. It has been reported that TGF-β can activate expression of other immediate-early genes, such as c-jun and jun-B (35), and of its own gene (36) through AP-1 (TRE) sites. However, these elements do not appear to be essential for TGF-β up-regulation of the CTGF gene because a fragment of the promoter that does not contain any of these elements (−275 to −109) can confer TGF-β induction on a previously nonresponsive promoter (Fig. 4B). Two regions of the promoter contained within this sequence are related but not identical to the reported TGF-β control elements, NF-1 and TIE. NF-1 sequences have been reported to control, in part, the up-regulation of the α2(I) collagen gene by TGF-β (22) and the PAI-1 gene (23). The TIE [consensus sequence (GNTTGGtGa); Ref. 30] are present in a number of genes, such as the stromelysin gene promoter (37), where this element acts to repress transcription in response to TGF-β. A closely related sequence is present in the c-myc promoter (TIE), which inhibits the expression of this gene in response to TGF-β (38). However, there are no sequences present in the CTGF promoter that are identical to the consensus elements of either the NF-1, TIE, or TCE sequences. Furthermore, the sequences present in the CTGF promoter that are similar to these regulatory elements do not appear to play any active role in TGF-β response. The results of deletion analysis using the luciferase reporter assay demonstrate that fragments of the promoter that contain either the NF-1-like site (P5) or the TIE-like site (P4) are not inducible by TGF-β (Fig. 4A). Competitive gel shift and methylation interference assays confirm that these regions of the promoter are not binding any nuclear proteins in either control or TGF-β-treated cells. A sequence that lies between the NF-1-like and TIE-like sites is the region that is binding to nuclear proteins. This region is deleted in all reporter constructs that lack TGF-β responsiveness.

The sequence we have identified as the TβRE element (GTGTCAAGGGGTC) in the CTGF promoter has not been described previously as a control element. The sequence is absolutely conserved in the murine Fisp 12 gene. A search of the GenBank promoter data base did not indicate any closely related elements that have been identified at the current time. We also did not find the sequence in the Cyr61 promoter or in the promoters of other immediate-early genes that have been identified. Other TGF-β-regulated genes, including collagen, PAI-1, and fibronectin, do not contain this or any closely related sequence in the promoter region. These data indicate that the regulation of CTGF gene expression by TGF-β may function by a distinct mechanism than other TGF-β-regulated genes. The proteins that bind to this sequence are constitutively present in the fibroblast nucleoplasm, as anticipated from the studies demonstrating that the CTGF gene is directly regulated by TGF-β and can be induced in the presence of protein synthesis inhibitors. These data suggest that some type of posttranslational modification of these proteins is needed for the alteration in transcription rates of the CTGF gene induced by TGF-β. The biochemical basis for this is under investigation.

CTGF is a member of a growing family of related peptides. All of the members of this gene family that have been iden-
Fig. 6. Competitive gel shift assays to delineate the binding site for nuclear proteins in the −205 to −109 region of the CTGF promoter. A nucleotide fragment consisting of the region from −205 to −109 of the CTGF promoter was end labeled with 32P and used in competitive gel shifts with the indicated oligonucleotides. Arrow, the specific gel shifted band. A diagram of the sequences used indicates the position of the oligonucleotides used relative to the NF-1- and TIE-like elements. The numbered fragments in the diagram indicate the lane number in the competitive gel shift assay with the specific nucleotide sequence indicated above the lane (i.e., 3, −205−109). Unlabeled competitors were used at a 250-fold molar excess over the labeled fragment. Only oligonucleotides containing the region from −169 to −150 acted as specific competitors. Neither the NF-1- nor TIE-like regions competed in this assay.

tified in vertebrates share high levels of amino acid sequence homology and an absolute conservation of the number and spacing of 38 cysteine residues. All of these peptides are secretory proteins and have multifunctional domains suggesting a mosaic structure and function of these molecules. Sequence analysis of the various members indicates that all have four distinct domains. These domains are (from the amino to carboxyl terminal of the protein): (a) an insulin-like growth factor binding protein-like domain; (b) a von Willebrand type C repeat; (c) a thrombospondin type I repeat, which may be involved in glycosaminoglycan binding; and (d) a COOH-terminal domain, which is homologous to other extracellular mosaic proteins and shows potential structural relationship to TGF-β (see Ref. 39 for a detailed discussion of these relationships). Interestingly, the genomic organization of the five exons of the CTGF gene corresponds with the coding regions of these four domains with the first exon encoding the signal peptide. The functional analysis of these domains in the CTGF protein should provide more clues to its biological activities.

With the exception of CTGF, which was originally identified by its mitogenic and chemotactic activity for connective tissue cells (15), the biological activities of the other CTGF-related gene products, such as Cyr61/CEFH0 or nov, is not well defined. In the adult, the Fisp 12 and Cyr61 genes show similar patterns of expression, with the highest levels detected in the lung, although these are 10–20-fold below the level of expression in induced fibroblasts in culture (16, 17). Similarly, nov is expressed at detectable levels in the lung and brain of adult chickens but is expressed in the embryo at high levels only in kidneys (20). We did not find that either Cyr61 or nov is significantly induced by TGF-β in human skin fibroblasts or NIH/3T3 cells. In adult mammals, CTGF is expressed at high levels during wound repair (14) and at sites of connective tissue formation in a variety of fibrotic disorders (40). The expression of CTGF transcripts in all of the in

Fig. 7. Methylation interference assay of the −205 to −109 region of the CTGF promoter. A, sequence analysis of the region from −205 to −109. The sequence from −200 to −113 is shown. Assays were performed as described in "Materials and Methods." Lane G is the G sequence of the intact labeled probe; Lane S is the sequence of the shifted band; Lane F is the sequence of the nonshifted free probe from the same sample. The only region containing missing G residues is from positions −157 to −147. B, sequence analysis of the region −159 to −142 using a smaller fragment of the promoter (−169 to −139). Lanes are the same as in A. Arrows, competed G residues in this sequence. ●, G residues detected in analysis of complementary strand (data not shown). Symbols * and # are for orientation with the sequence in A.

5 G. R. Grotendorst, K. Frazier, and M. Glassberg, unpublished observations.
vivo studies we have made is highly correlated with the expression or presence of TGF-β. Injection of TGF-β into the subdermal layer of newborn mouse skin induces a high level of CTGF transcripts in fibroblasts present at the site within 24 h, based on in situ hybridization studies. In vitro studies in our laboratory with recombinant CTGF indicate that it is a mitogen for connective tissue cells and that it may mediate many of the effects of TGF-β on synthesis of extracellular matrix proteins, including collagen and fibronectin. These findings lead us to speculate that CTGF may be a mediator of some of the action of TGF-β on connective tissue cells.

The coordinate expression of TGF-β and CTGF during connective tissue regeneration in mammals supports a cascade model where initiators of the process stimulate production of secondary factors that sustain and regulate specific cellular processes in the regenerating tissue. Our observations demonstrating the prolonged induction of CTGF expression after a transient exposure to TGF-β are consistent with such a cascade model. Other experimental studies in our laboratory indicate that a brief exposure of cells to TGF-β are sufficient to induce biological effects that persist long after the removal of the TGF-β. TGF-β was initially described based on its unique ability to stimulate the growth of normal rat kidney fibroblasts to grow in an anchorage-independent culture system (agarose suspension; Ref. 41). We have investigated whether a transient exposure (1 h) of normal rat kidney fibroblasts to TGF-β is sufficient to stimulate DNA synthesis in an anchorage-independent growth assays that allows for recovery and washing of the cells to remove the TGF-β (42). The results of these experiments indicate that a 1-h exposure to TGF-β induces a 3.5-fold increase in DNA synthesis 24 h later compared with control cells that were not treated with TGF-β. Continuous exposure to TGF-β under these conditions resulted in a 6-fold increase in DNA synthesis. These data indicate that TGF-β need not be continuously present to induce biological effects on target cells, and a 1-h exposure of cells to TGF-β can result in growth stimulation. The role of CTGF in this process is currently under investigation.

Regulatory cascades involving TGF-β and CTGF-related genes may play an important role in early development as well as in wound repair. In the early Drosophila embryo, a cascade of gene products controls pattern formation during

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Fig. 8. Competitive gel shift titration assay of oligonucleotides in the TβRE. Overlapping and nonoverlapping oligonucleotides containing portions of the −157 to −145 region of the CTGF promoter were tested in the competitive gel shift assay using a 32P-labeled human CTGF promoter fragment (−205 to −109). An oligonucleotide from −159 to −143, which contains the intact sequence, exhibits the highest affinity with complete competition at 10 ng. All other fragments that contain only a portion of this sequence are less effective, with the −150 to −134 region being the least effective. Lanes 14 and 15 are the NF-1- and TIE-like elements, respectively, and show no competition at 5000-fold molar excess of labeled probe.

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6 G. R. Grotendorst, unpublished observations.
Table 3

<table>
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<tr>
<th>Sequence used</th>
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<td>GTGCAGGGTGC</td>
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<tr>
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<td>PM2</td>
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<td>PM4</td>
<td>0.16 ± 0.03</td>
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* Numbers in parentheses (16) are the percentage of fold induction relative to the native sequence.

** Results in bold, bases mapped by methylation interference.

PMs were generated by designing oligonucleotides with the desired PM linked to a BSM-1 site identical in sequence to the site at positions -167 to -161. This was used with a second oligo to PCR amplify a mutant BSM-1 fragment region, which was subsequently isolated and cloned into a BSM-1-digested P0 (intact, native sequence) CTGF promoter fragment contained in the Bluescript plasmid. Recombinant clones were checked by restriction digestion and the DNA sequence analysis of the entire sequence to ensure that only the PM had occurred. These inserts were then cloned into the pGL2 vector. Transient transfections were performed with NIH/3T3 cells, and the amount of luciferase activity was determined in control (basal) and TGF-β-activated cells.

Dorsal ventral axis organization (43, 44). Several of the genes that have been identified in the dorsal/ventral cascade of the Drosophila embryo, decapentaplegic (dpp; Ref. 45) and screw (scw; Ref. 46), encode proteins that are members of the TGF-β superfamily and are closely related to mammalian genes that have been identified as bone morphogenetic proteins (47). Other studies have indicated that the human bone morphogenetic protein gene sequences can confer normal dorsal ventral patterning in the Drosophila embryo (48). Another gene in the dorsal/ventral cascade [twisted gastrulation (tsg)] is related to CTGF (21). Twisted gastrulation controls the cell fate of medial mesodermal elements in the Drosophila embryo, where it acts in coordinate fashion with the gene products of dpp and scw (21). Collectively, these observations suggest that the genetic pathways controlling tissue formation are highly conserved and that the wound repair cascade may be derived from or possibly identical to the pathways that originated to specify mesodermal tissue formation and organization during embryogenesis in the invertebrate.

Materials and Methods

Cell Cultures. Human skin fibroblasts were grown from explants of skin biopsy specimens. NIH/3T3 cells and Cos 7 cells were obtained from the American Type Culture Collection (Rockville, MD). All cells were cultured in DMEM containing 10% FBS at 37°C in an atmosphere of 10% CO₂ and 90% air. Human skin fibroblasts were used prior to the sixth passage.

Growth Factors. TGF-β1 was a gift from Richard Asoian (University of Miami). Recombinant PDGF-BB was obtained from Chiron (Emeryville, CA). Purified murine EGF was purchased from Biomedical Technologies, Inc. (Stoughton, MA). Purified basic FGF was purchased from Sigma Chemical Co. (St. Louis, MO).

RNA isolation and Northern Blotting. Total RNA was isolated from cultured cells by acid guanidinium thiocyanate-phenol-chloroform extraction, as reported previously (49). Total RNA was electrophoresed on a 1.5% agarose/formaldehyde gel and transferred to nitrocellulose. The CTGF probe was a 1.1-kb fragment representing the CTGF open reading frame obtained by PCR reaction using specific primers H01 (5’-cggactttgtcagccacagtgc-3’) and H02 (5’-cgaatctggcccagttctcaatctc-3’). Hybridizations were performed using 1 x 10⁶cpm/ml of these probes labeled with [α-32P]UTP by using a Random Primer DNA labeling kit (Boehringer Mannheim Biochemicals, Indianapolis, IN). Autoradiography was performed at -70°C for 6 to 72 h by using X-ray films and intensifying screens.

Isolation of Genomic Clones and Sequence Analysis. Genomic DNA was isolated from human skin fibroblasts as described previously (50). Using 4 µg of genomic DNA as a template, a fragment of the CTGF gene was amplified by PCR using primers H02 (5’-cgaatctggcccagttctcaatctc-3’) and H03 (5’-cgaatctggcccagttctcaatctc-3’). PCR products were digested with EcoRI and subcloned into M13. Sequence analysis by the dideoxy chain termination method (51) using the Sequenase kit (U.S. Biochemical Corp., Cleveland, OH) demonstrated a 900-bp fragment that had a 387-bp intron in the middle portion. Using a Human Genomic Library in the Lambda Fix II vector (Stratagene, La Jolla, CA), we screened approximately 1 x 10¹¹ recombinant phages with 3²P-labeled 900-bp genomic DNA fragment as probe and isolated three phage clones that contained the CTGF gene.

Nuclear Run-On Assays. Nuclear run-on transcription assays were performed as described previously (26) with a slight modification. We precipitated RNA by the addition of sodium acetate (pH 5.2) to a final concentration of 0.3 M and ethanol to 70% instead of using trichloroacetic acid. The [3²P]UTP-labeled run-on transcripts were hybridized at 8 x 10⁶ cpmm per filter to filters on which 5 µg of corresponding cDNA was immobilized.

Luciferase Reporter Gene Assays. A fragment of the CTGF promoter containing nucleotides -823 to +74 from one of the human genomic clones was first cloned into the ScaI-XhoI cloning site of pG2L2-Basic vector (Promega). We used this construct (P0) as a template for PCR and made deletion mutants with specific primers as follows: P1 contained nucleotides from -638 to +74, P2 from -363 to +74, P3 from -270 to +74, and P4 from -128 to +74. NIH/3T3 cells were transfected in a 6-well plate with Lipofectin reagent (Life Technologies, Inc.) for 6 h. Each transfection included 2 µg of reporter plasmid and 0.5 µg of pSV-β-galactosidase vector (Promega). Cells were incubated in serum-free DMEM-ITS (Collaborative Biomedical Products) for 24 h after transfection, followed by incubation with growth factors for 4 or 24 h. Luciferase activity was measured by using the Luciferase assay system (Promega) and a scintillation counter (Beckman LS6500SC) which had a single photon monitor. To normalize for differences in transfection efficiency, β-galactosidase activity was measured using a chemiluminescent assay using Galacto-Light (TROPIX, Inc.).

Preparation of Nuclear Extracts. Nuclear extracts were prepared as described by Abmayr and Workman (62). Confluent monolayers of cells were scraped from tissue culture dishes using a cell scraper in Tris-buffered saline (0.125 M NaCl, 25 mM Tris, pH 7.4) and collected by centrifugation at 1500 x g for 5 min. The cell pellets were resuspended in hypotonic buffer (10 mM HEPES (pH 7.9), 1.5 mM MgCl₂, 10 mM KCl, 0.2 mM PMSF, and 0.5 mM DT) and homogenized with 10 strokes of a glass Dounce homogenizer; nuclei were isolated by centrifugation at 3300 x g for 15 min. Nuclear proteins were extracted by suspending the nuclei in an equal volume of extraction buffer (200 mM HEPES (pH 7.5), 25% glycerol, 1.5 mM MgCl₂, 0.8 M KCl, 0.2 mM EDTA, 0.2 mM PMSF, and 0.5 mM DTT) for 10 min. The extract was dialyzed against 200 mM HEPES (pH 7.9), 20% glycerol, 100 mM KCl, 0.2 mM EDTA, 0.2 mM PMSF, and 0.5 mM DTT before use. The protein concentration was determined using the BCA protein assay reagent (Pierce).

Gel Mobility Shift Assays. Fragments of the CTGF promoter were prepared by PCR or restriction endonuclease digestion of the promoter fragment. Double-stranded oligonucleotides were prepared by annealing complementary single-stranded oligonucleotides. All oligonucleotides and fragments were checked by electrophoresis in agarose or polyacryl- amide gels prior to use. Radiolabeled fragments of the CTGF promoter were prepared by end-labeling with Klenow enzyme (Boehringer Mannheim) and poly nucleotide kinase (Boehringer Mannheim). Labeled frag-
ments were purified by electrophoresis in 2% agarose gels or 20% polyacrylamide gel before use in gel mobility shift assay.

The binding reaction mixture contained 1 µg of nuclear extract protein in 20 µl of 10 mM HEPES (pH 7.9), 5 mM Tris, 0.1 mM EDTA, 1 µg poly(dI-dC)poly(dI-dC) (PharMacia), 10% glycerol, 300 µg/ml BSA, and 10,000 cpm 32P-labeled DNA probe. In competition assays, the indicated amount of unlabeled competitor DNA was added and incubated at 4°C for 2 h prior to adding the labeled probe. The labeled probe was incubated for one h at 4°C in the reaction mixture. Electrophoresis was performed using 5% polyacrylamide gel with 50 mM Tris, 0.38 M glycine, and 2 mM EDTA.

Methylation Interference Assay. End-labeled fragments of double-stranded oligonucleotides were prepared as described for the gel mobility shift assay. The oligonucleotides were methylated by dimethyl sulfate (Fisher Scientific) for 5 min at room temperature. DNA-protein binding and gel mobility shift assays were performed as described above using large amounts of labeled probe (100K cpm) and nuclear protein (20 µg). DNA from shifted and nonshifted bands was purified and cleaved with piperidine (Fisher Scientific), and the samples were electrophoresed on a polyacrylamide DNA sequencing gel. The sequences of the shifted and nonshifted fragments were compared with the intact probe sequence using the same methods.

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References


