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Characterization of a Mouse *ctgf* 3′-UTR Segment That Mediates Repressive Regulation of Gene Expression

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We isolated a small segment of the 3′-untranslated region (3′-UTR) in the mouse connective tissue growth factor (*ctgf/fisp12*) gene and evaluated its functionality. Comparison of the nucleotide sequences of human and mouse *ctgf* 3′-UTRs revealed a conserved small segment of 91 bases. The corresponding segments of the 3′-UTRs shared as much as 82.4% homology, whereas the overall homology between the 3′-UTRs was 71.8%. To study the functionality of the conserved segment, the corresponding region of mouse *ctgf* cDNA was amplified from NIH3T3 cells. When it was fused downstream of a marker gene, it showed remarkable repressive effects on gene expression. The repressive effect of the sense form was more prominent than that of the antisense form. Computer analyses of these sequence predicted stable secondary structures, suggesting that they act at the RNA level. The predicted structures of the sense and antisense forms appeared to be slightly different, which is consistent with the difference in repressive function. These findings defined the conserved small element in the mouse *ctgf* gene as a potent negative regulator of gene expression, which may act at a posttranscriptional level.

Key Words: **connective tissue growth factor; CTGF; 3′-untranslated region; 3′-UTR; *cis*-acting elements; secondary structure; gene expression.**

The connective tissue growth factor (CTGF/Hcs24) is a cysteine-rich polypeptide isolated from angioendothelial cells as a growth factor related to platelet-derived growth factor (PDGF) (1–3). It was recognized by anti-PDGF antibodies and has PDGF-like mitogenic and chemotactic activities. Also, CTGF has been shown to be a multifunctional growth factor that potentiates either growth or differentiation of mesenchymal cells, according to biological conditions (1, 4–6). Of note, we have obtained evidence that CTGF plays a central role in the growth and differentiation of chondrocytes, using human chondrocytic HCS2/8 and rabbit primary growth cartilage cells (2, 3).

Gene expression of CTGF *in vivo* is normally at a low level but is induced during particular cytodifferentiation or pathological states (2–8). In chondrocytes, the highest level of CTGF expression was observed at the hypertrophic stage, whereas relatively low levels of expression were found in earlier stages (2, 3). Furthermore, CTGF was found to be involved in angiogenesis (9, 10), which is required at the last stage of endochondral ossification. Namely, differential expression of *ctgf* mRNA seemed to follow the course of chondrocyte differentiation.

Since the level of *ctgf* mRNA is under differential control, multiple regulatory factors should be involved therein. Recently, we reported that the full-length 3′-untranslated region (3′-UTR) of the human *ctgf* gene had repressive effects on gene expression, which is a critical part of the regulation machinery enabling the differential expression (11).

Evidence for critical roles of the 3′-UTR of mRNA in the regulation of gene expression has been accumulating (12). There have been a number of studies on *cis*-acting negative regulatory elements such as silencers and RNA destabilizers in the 3′-UTR of a variety of genes. For example, vascular endothelial growth factor (VEGF) mRNA possesses adenylate-uridylate-rich elements(AREs) in the 3′-UTR (13), which are thought to regulate mRNA stability (14–17). Also, parathyroid hormone (PTH) mRNA is under regulation in that specific sequences of 60 bases in the PTH mRNA 3′-UTR determine the mRNA stability (18).

CTGF is a member of the CCN gene family which includes classical members [*cef10/cyr61, ctgf/fisp12, nov*] and several recently reported genes such as *elm1/*

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wisp1, ctgf-3/ctgf-L/wisp-2/cop1, and *wisp-3* (19–22). Orthologues of this family have been found across vertebrate species from *Xenopus* to human. *Fisp12* is the mouse orthologue of human *ctgf* with 94% homology at the amino acid level. The two are thought to possess fundamental similarities in control mechanisms. Therefore, we speculated that the 3′-UTR of the mouse *ctgf* gene contains repressive regulatory element(s) as reported in the human *ctgf* gene (11).

In the present study, we found a small conserved segment at the junction of the coding region and the 3′-UTR in the mouse *ctgf* cDNA, which displays significantly higher homology with the corresponding portion of the human orthologue than other portions of the 3′-UTR. Assuming the conserved segment to be a putative functional element, we isolated and evaluated its functionality, utilizing an established transient assay system with COS-7 cells.

MATERIALS AND METHODS

RNA extraction and reverse transcriptase-mediated polymerase chain reaction (RT-PCR). Total RNA was extracted from log-phase growing NIH3T3 cells by an acid guanidinium phenol–chloroform method as previously described (23). Reverse transcription by avian myelosarcoma virus reverse transcriptase was carried out using a commercially available kit (Takara Shuzo, Tokyo, Japan) with 0.5 μg of total RNA and a mouse *ctgf*-specific elongation primer, fisp12AS (Fig. 2). Subsequent PCR amplification was also performed with the same experimental kit, following the manufacturer's protocol. Primers used for amplification are illustrated in Fig. 2, where flanking *Xba*I and *Eco*RI cutting sites are inscribed for the sense and antisense primers, respectively. Each amplification cycle consisted of 30 s at 94°C, 30 s at 50°C and 1 min at 72°C. After 30 cycles of chain reaction and subsequent incubation at 72°C for 5 min, PCR products were analyzed and purified through 1.5% agarose gel electrophoresis.

Molecular clones. We constructed the firefly luciferase–mouse *ctgf* chimeric genes using the method described by Kubota *et al.* Briefly, the parental pGL3-control (Promega, Madison, WI) was modified to be two pGL3-control derivatives with multiple cloning sites (MCS) in different orientations, pGL3L(+) and pGL3L(−). Both derivatives give the same levels of luciferase expression as pGL-control, as previously observed (11). The 100-bp amplicon from NIH3T3 cells was digested with *Xba*I and *Eco*RI, and was subcloned between the corresponding enzymatic sites in pGL3L(+) or pGL3L(−). The resultant plasmids with the sense and antisense fragment of mouse *ctgf* 3′-UTR were named pGL3f UTRS and pGL3fUTRA, respectively. The structures of these plasmids are displayed in Fig. 3A. An internal control plasmid for monitoring transfection efficiency was purchased and utilized. This plasmid, pRL-TK (Promega), contains a *Renilla* luciferase gene under the control of the herpes simplex virus thymidine kinase gene promoter for constitutive and relatively weak expression of *Renilla* luciferase.

Cell culture and DNA transfection. Fetal bovine serum (FBS) in humidified air with 5% CO_2. Twenty hours prior to transfection, 2×10^5 COS-7 cells were seeded in a 35-mm tissue culture dish. Liposome-mediated DNA transfection was performed with 1 μg of each pGL3 derivative in combination with 0.5 μg of pRL-TK, according to the manufacturer's optimized methodology (Lipofectamine: Lifetechnologies, Rockville, MD). Forty-eight hours after transfection, the cells were lysed in 500 μl of a passive lysis buffer (Promega). Cell lysate was directly forwarded to the luciferase assay system.

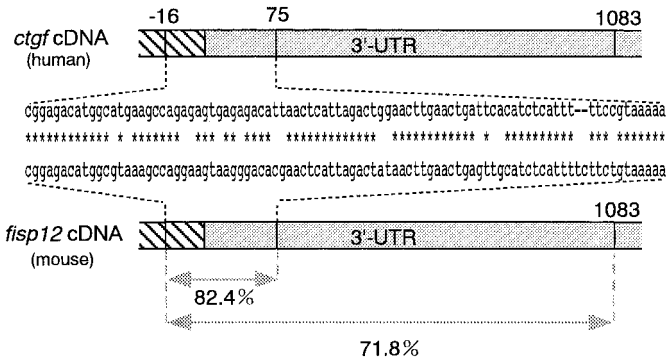

FIG. 1. Alignment of the highly homologous segments in 3′-UTRs between human and mouse (*fisp12*) *ctgf*cDNAs. Nucleotide sequences conserved between human and mouse *ctgf* are displayed as a combined diagram. Identical nucleotides between *ctgf* and *fisp12* in the corresponding region are indicated by asterisks. The 3′-UTRs are indicated by stippled boxes, and the coding regions are indicated by hatched boxes. The numbering of nucleotides starts at the first nucleotide of the 3′-UTRs. Homology scores of the entire 3′-UTR and the highly homologous segment are shown in percentages below the diagram.

Luciferase assay. The Dual Luciferase system (Promega) was used for the sequential measurement of firefly and *Renilla* luciferase activities with specific substrates of beetle luciferin and coelenterazine, respectively. Quantification of both luciferase activities and calculation of relative ratios were carried out manually with a luminometer (TD-20/20: Turner Designs, Sunnyvale, CA).

DNA sequencing. The purified 100-bp PCR product of mouse *ctgf* cDNA 3′-UTR was directly subjected to automated DNA sequencing by a dye terminator method (Bigdye: Applied Biosystems/Perkin–Elmer, Foster City, CA). Nucleotide sequencing was performed, utilizing the mouse *ctgf*-specific primers shown in Fig. 2 (fisp12S and fisp12AS). The nucleotide sequence of the corresponding region in pGL3f UTRS and pGL3fUTRA was verified by the same procedure with the same primers.

RESULTS

Comparison of the Nucleotide Sequence of Human ctgf 3′-UTR to That of Mouse and Alignment of the Highly Homologous Segments

Comparison of the nucleotide sequences of the *ctgf* 3′-UTRs revealed a highly conserved small segment of 91 bases at the very junction with the coding region. The corresponding segments of the 3′-UTRs showed as much as 82.4% homology between human and mouse, whereas the overall homology between the 3′-UTRs was no more than 71.8% (Fig. 1). Thus, this sequence was observed to be significantly conserved among mammalian species, suggesting its indispensable role in proper *ctgf* gene expression.

Detection of Mouse ctgf mRNA in NIH3T3 Cells and Isolation of the Conserved 3′-UTR Segment

Based on a published nucleotide sequence of mouse *ctgf* cDNA, we synthesized two mouse *ctgf*-specific

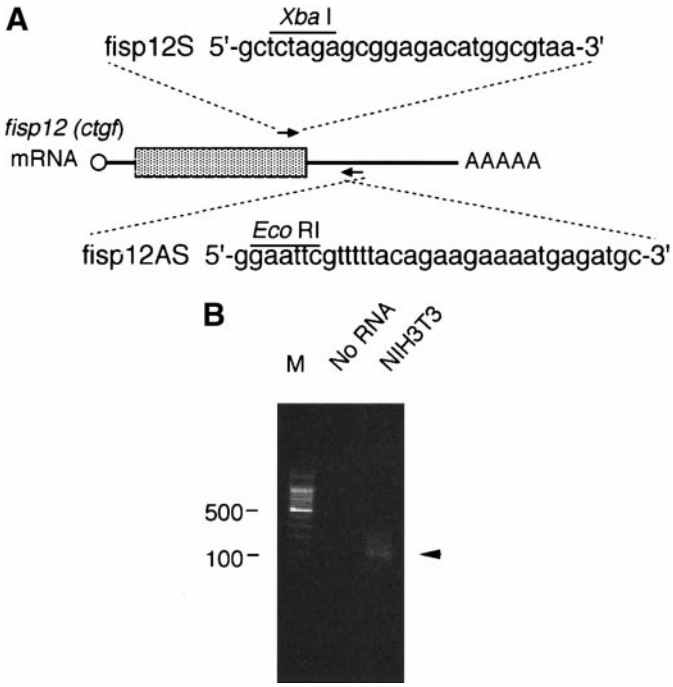

FIG. 2. Detection of *ctgf* expression in NIH3T3 cells by RT-PCR by amplifying the highly homologous portion of the 3′-UTR. (A) Organization of mouse *ctgf* (*fisp-12*) mRNA and primers used for RT-PCR. The rod at the center represents a mouse *ctgf* mRNA. The small open circle and "AAAAA" at the left and right ends denote the 5′-cap structure and poly(A) tail, respectively. The stippled box represents the coding region, while solid lines indicate untranslated regions. Names, locations for recognition, and nucleotide sequences of the primers used in the PCR are given. The flanking restriction endonuclease recognition sites for cloning are also shown. (B) Agarose gel electrophoresis analysis of the amplicon. Five microliters out of a total reaction mixture of 100 μl was analyzed. M, 100-bp DNA ladder marker. Sizes of a few bands in the marker are shown in base pairs at the left of the panel. The major amplicon is indicated by an arrowhead at the right.

primers for the detection of mouse *ctgf* mRNA via RT-PCR. The sense (fisp12S) and antisense (fisp12AS) primers were designed to recognize the corresponding segment as shown in Figs. 1 and 2A. Using these primers, we could isolate a conserved small segment of 91 bases from mouse *ctgf* cDNA, as well as detect its expression. As shown in Fig. 2B, a distinct single band with the expected size was observed on agarose gel electrophoresis of the crude RT-PCR product from NIH3T3 RNA. The band was excised, purified and subjected to DNA sequencing and subsequent plasmid construction, as described in the next subsection. The nucleotide sequence of the major amplicon, which was determined by direct sequencing, revealed its identity as the corresponding portion of the mouse *ctgf* cDNA. The results of direct sequencing showed no significant variation, indicating the uniformity of the PCR product.

cis-Acting Repressive Effect of the Conserved Small Segment of Mouse ctgf 3′-UTR on Gene Expression

To elucidate possible effects of the conserved small segment of mouse *ctgf* 3′-UTR on gene expression, we constructed chimeric genes, in which firefly luciferase genes were fused with the RT-PCR-isolated mouse *ctgf* 3′-UTR fragments at the 3′ ends. Two chimeric expression plasmids, pGL3fUTRS and pGL3fUTRA, were constructed. Construction of these plasmids was performed, following a procedure described previously (11). The resultant pGL3fUTRS contains the small segment of 3′-UTR in the sense direction of the luciferase gene, whereas pGL3fUTRA possesses the same segment in the antisense direction (Fig. 3A). These plasmids were subjected to a calibrated transient expression assay with *Renilla* luciferase co-expression (internal control). As is clear in Fig. 3B, both plasmids yielded much lower levels of expression than a parental vector, pGL3-control (i.e., 65.4% inhibition by the sense form; 46.1% inhibition by the antisense: against pGL3-control). Interestingly, the sense form of the small *ctgf* segment showed as remarkable repressive effects on

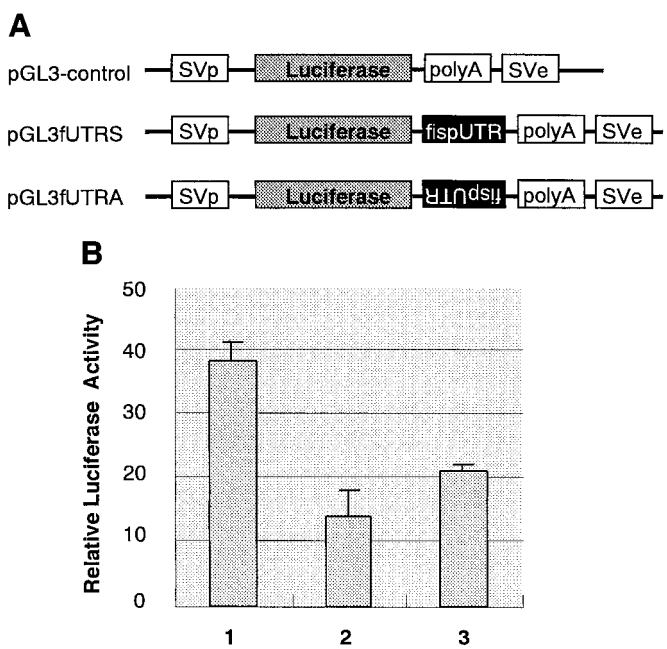

FIG. 3. (A) Structures of the plasmids used in this study. All were derived from pGL3-control (Promega), thus the basic structure of every plasmid is the same. Abbreviations: SVp, SV40 promoter; SVe, SV40 enhancer; poly(A), SV40 polyadenylation signal; fispUTR, mouse *ctgf* 3′-UTR fragment (the major PCR product: Fig. 1B); Luciferase, firefly luciferase gene. (B) Firefly luciferase activity from the plasmids displayed in panel A in Cos-7 cells. Activity is represented as a relative value of the measured luminescence of firefly luciferase versus *Renilla* luciferase from cotransfected pRL-TK (internal control). Mean values of the results of four experiments are displayed with error bars (standard deviations). Lane 1: pGL3-control. Lane 2: pGL3fUTRS. Lane 3: pGL3fUTRA.

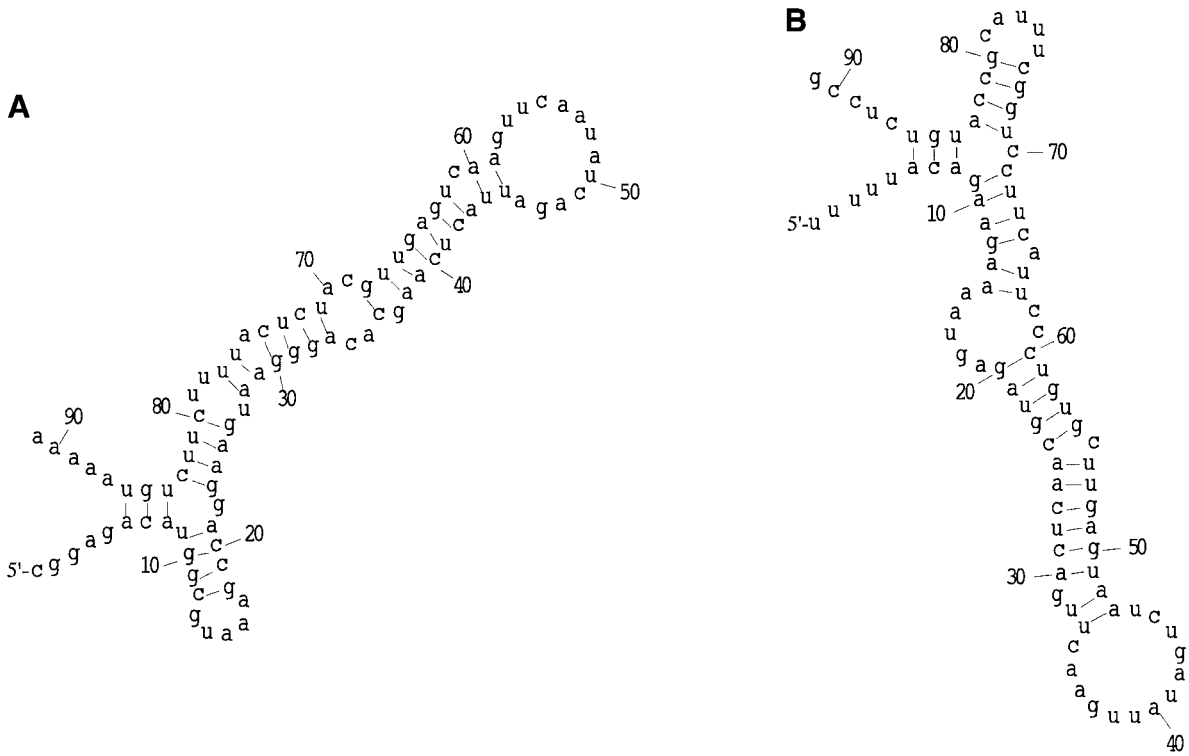

FIG. 4. Predicted secondary structures of the mouse *ctgf* 3′-UTR element mRNA. (A) Sense form. (B) Antisense form. Free energy calculation predicted almost the same stability for the sense form as the antisense form (data not shown). Nucleotides are numbered from the first nucleotide of each element.

gene expression as the sense form of the full-length human *ctgf* 3′-UTR. In addition, the repressive effect of the sense form appeared to be more prominent than that of the antisense, representing its partial orientation dependence.

Predicted Secondary Structures of the Corresponding mRNA Segment

The observed orientation dependence of the repressive effect of the 3′-UTR segment implies a regulatory mechanism other than a conventional DNA silencer function (24). Thus, we suspected the involvement of posttranscriptional regulation in the repressive effects. Interestingly, computer analyses of the corresponding sequences predicted stable secondary structures for both sense and antisense forms of the repressive elements, suggesting that they act at the RNA level (Fig. 4). Of note, the predicted structures of the sense and antisense forms appeared to be slightly different, which is consistent with the observed difference in their repressive function.

DISCUSSION

We previously reported that the 3′-UTR of the full-length human CTGF gene contains repressive regulatory element(s) (11). Assuming that there may be fundamental similarities in the control mechanisms of the expression of these two genes, we compared the nucleotide sequence of human *ctgf* 3′-UTR with that of mouse. Consequently, a small conserved segment with significantly higher homology than other segments was found. These findings implied a critical role for the corresponding segments in the repressive function of the 3′-UTR.

Based on this hypothesis, our subsequent functional analyses of the mouse 3′-UTR segment, for the first time, uncovered repressive effects on gene expression per se. Interestingly, the small *ctgf* segment showed as remarkable repressive effects on gene expression as the full-length human CTGF 3′-UTR. Thus, it is plausible that the isolated small portion of mouse *ctgf* gene is the major functional element for the 3′-UTR-mediated *ctgf* gene regulation.

The repressive effects of the sense form of the *ctgf* segment appeared to be more prominent than those of the antisense form. However, in contrast, our previous study demonstrated that the antisense form of the full-length human *ctgf* 3′-UTR conferred stronger repressive effects than the sense form (11). This led us to assume that the 3′-UTR of the full-length human CTGF gene contains multiple regulatory elements—some of them may be orientation-independent, whereas the others may be orientation-

dependent. Further investigations are needed to clarify how the antisense 3′-UTR displays stronger effects, by characterizing all the regulatory elements involved therein.

The involvement of structured RNA elements within the 3′-UTR in the regulation of gene expression has been described for a variety of genes. VEGF is a potent angiogenic factor and endothelial cell-specific mitogen (25) which is regulated by hypoxia (26). VEGF induction by hypoxia is due to an increase in the steady state level of VEGF mRNA (25, 27). The half-life of rat VEGF mRNA is controlled by AREs in the 3′-UTR, which are thought to regulate mRNA stability. Claffey *et al.* (13) identified an AU-rich sequence in the 3′-UTR of the human VEGF mRNA as the major determinant for RNA-protein interaction. This element has a unique stem-loop structure required for protein recognition. Specific recognition of RNA stem loops by RNA-binding proteins has already been found and characterized in the regulatory systems of other genes, such as the transferrin receptor gene (28, 29).

Analogous to VEGF/VPF, CTGF is also a potent angiogenic factor (6, 9, 10). The steady-state level of *ctgf* mRNA has been shown to be differentially controlled, depending upon the type and state of the cell (2–6). Therefore, the mouse *ctgf* gene may utilize the repressive regulatory system of 3′-UTR as a critical part of the regulation machinery which enables the differential expression. Although VEGF -like AU-rich elements were absent, the identified *ctgf* segment was predicted to yield mRNAs with stable stem loop structures. Of note, the structural and functional relevance of the sense and antisense forms suggests that proper formation of RNA secondary structures is critical for mediating the posttranscriptional repression of mouse *ctgf* gene expression. It is also of our interest to seek RNA-binding proteins that recognize these stable secondary structures.

The gene expression level of *ctgf* is generally low in normal tissues *in vivo*, for which the repressive function of 3′-UTR may be responsible, but strong expression is induced during particular stages of cell growth and differentiation along with development (2, 3, 5, 6), or in pathological states, such as through the processes of wound healing (4) and tumor development (8). Therefore, although we cannot rule out that other regulatory machineries are involved in the repression of the *ctgf* gene, we assume that liberation from repressive regulation by the 3′-UTR leads to a high level of CTGF in times of necessity or in pathological states. It should be noted that in growth cartilage, strong expression of the ctgf gene is observed only in hypertrophic chondrocytes. Further investigation of this point is in progress.

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