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Involvement of *cis*-acting repressive element(s) in the 3′-untranslated region of human connective tissue growth factor gene

Satoshi Kubota, Takako Hattori, Tohru Nakanishi, Masaharu Takigawa*

Department of Biochemistry and Molecular Dentistry, Okayama University Dental School, 2-5-1 Shikata-cho, Okayama 700-8525, Japan

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Abstract To analyze the regulatory mechanism of connective tissue growth factor expression, the 3′-untranslated region (3′-UTR) of CTGF cDNA was amplified from HeLa cell RNA. Direct nucleotide sequencing revealed a single major population in the amplicon, which was nearly identical to other sequences. Subsequently, the effect of the 3′-UTR on gene expression was evaluated. When it was fused downstream of a firefly luciferase gene, the 3′-UTR strongly repressed luciferase gene expression. Interestingly, the repressive effect of the antisense 3′-UTR appeared to be more prominent than that of the sense one. Together with the fact that several consensus sequences for regulatory elements are found in it, these results suggest the involvement of multiple sets of regulatory elements in the CTGF 3′-UTR.

Key words: Connective tissue growth factor; Chondrocyte; Cartilage; 3′-Untranslated region; Gene expression

1. Introduction

The connective tissue growth factor (CTGF) was initially identified as a platelet-derived growth factor (PDGF)-related growth factor from angioendothelial cells [1]. In early studies, the effects of CTGF on mesenchymal cells was analyzed, and observed to be similar to PDGF [1]. Also, it had been suggested that CTGF shares its receptor(s) with PDGF [1,2], although recently, the presence of a CTGF-specific receptor on a human chondrocytic cell line HCS-2/8 has been strongly indicated [3]. Structural similarity of CTGF and PDGF was once implied on the basis of the cross-reactivity of an antibody against PDGF with CTGF, which eventually gave rise to the identification of CTGF [1]. However, the structural relationship between these two growth factors has been found to be relatively distant. CTGF is a cysteine-rich secretory protein with a molecular weight of 38 kDa, which is composed of 349 amino acid residues [4]. In contrast to PDGF, CTGF is a monomeric growth factor, and shares as much as 40% homology with PDGF at the cDNA level [1,4]. As such, it is now widely recognized that CTGF is not an accessory factor of PDGF, but a member of the CCN family, which consists of *ctgf* (human)/*fisp12* (mouse), *cef10* (chick)/*cyr61* (mouse), and *nov* (chicken) [4–6]. Of note, the homology between *ctgf* and *fisp12* is 94% at the amino acid level, suggesting that CTGF is evolutionarily highly conserved, and biologically indispensable in mammalian species [4].

The biological function of CTGF has been shown to be quite diverse. Earlier studies described mitogenic and chemotactic activities of CTGF on fibroblasts, and its role(s) in wound healing were suggested as well [5,7]. However, most integrated functional aspects of CTGF were uncovered by our recent research. After discovering that CTGF is preferentially expressed in a human chondrocytic cell line, HCS-2/8 [8–13], we have obtained sequential evidence that CTGF plays a central role in the growth and differentiation of chondrocytes [12,14]. Namely, differential expression of CTGF mRNA was observed to follow the course of chondrocyte differentiation. In chondrocytes, the highest level of CTGF expression was observed at the hypertrophic stage, whereas relatively lower levels of expression were found in earlier stages [12]. Stimulation of proliferation and proteoglycan synthesis in chondrocytes were also conducted by CTGF [14]. Of importance, CTGF was found to be involved in the proliferation and migration of vascular endothelial cells [6]. Also of note, enhancement of CTGF expression by bone morphogenetic protein 2 (BMP-2) was observed in HCS-2/8 cells in our recent study [12]. Thus, we are re-defining CTGF as a major factor to guide chondrocytes towards endochondral ossification, which may be called ‘ecogenin’.

It is widely known that, in addition to BMP-2, CTGF expression is induced by transforming growth factor β (TGF-β) [12,15]. Save for a report which clarified a novel TGF-β responsive element in the CTGF promoter [16], little has been published about the molecular mechanism of the regulation of CTGF gene expression. In the present study, we focused on the 3′-untranslated region (UTR) of the human CTGF cDNA, and evaluated its effect on gene expression, utilizing a transient assay system with COS-7 cells.

2. Materials and methods

2.1. RNA extraction and reverse transcriptase-mediated polymerase chain reaction (RT-PCR)

Total RNA was extracted from log-phase growing HeLa cells by an acid guanidinium phenol-chloroform method as previously described [17]. Reverse transcription by avian myelosarcoma virus reverse transcriptase was carried out using a commercially available kit (Takara Shuzo, Tokyo, Japan) with 1.5 μg of total RNA and a CTGF cDNA-specific oligodeoxynucleotide, CT3UTA (Fig. 1A), as an elongation primer. Subsequent PCR amplification was also performed with the same experimental kit, following the manufacturer’s protocol. Primers used for amplification are illustrated in Fig. 1A: CT3UTS and CT3UTA, where flanking *Xba*I and *Eco*RI cutting sites are inscribed, respectively. Each amplification cycle consisted of 1 min at 94°C, 30 s at 50°C, and 1 min at 72°C. After 30 cycles of chain reaction and sub-

*Corresponding author. Fax: (81) (86) 235-6645.
E-mail: takigawa@dent.okayama-u.ac.jp

Abbreviations: CTGF, connective tissue growth factor; 3′-UTR, 3′-untranslated region; PDGF, platelet-derived growth factor; BMP-2, bone morphogenic protein 2; TGF-β, transforming growth factor β; MCS, multiple cloning site; RT-PCR, reverse transcriptase-mediated polymerase chain reaction

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sequent incubation at 72°C for 5 min, PCR products were analyzed and purified through 1.5% agarose gel electrophoresis.

2.2. Molecular clones

Prior to the construction of firefly luciferase-CTGF chimeric genes, the parental vector, pGL3-control (Promega, Madison, WI, USA) was initially modified. A double-stranded synthetic oligonucleotide, which confers multiple cloning sites (MCS), was synthesized, and built in pGL3-control at the unique *Xba*I site immediately downstream of the luciferase gene. Consequently, two pGL3-control derivatives with the same MCSs in different orientations were obtained, and designated pGL3L(+) and pGL3L(−), respectively. The major 1.0 kbp amplicon from HeLa RNA was digested with *Xba*I and *Eco*RI, and was subcloned between the corresponding sites of pGL3L(+) or pGL3L(−). The resultant plasmids with CTGF 3′-UTR at the end of the firefly luciferase gene were entitled pGL3UTRS or pGL3UTRA, respectively. A negative control plasmid was also constructed from pGL3-control. The SV40 promoter was removed from pGL3-control by *Sma*I-*Hin*dIII digestion, then the *Hin*dIII overhang blunted by T4 DNA polymerase and ligase, utilizing a commercially available kit (Takara Shuzo). The promoterless pGL3-control derivative, was then designated as pGL3ΔP. The structures of these plasmids are displayed in Fig. 2. An internal control plasmid for monitoring transfection efficiency was purchased and utilized. This plasmid, pRL-TK, contains a *Renilla* luciferase gene under the control of the herpes simplex virus thymidine kinase gene promoter for constitutive and weak expression of *Renilla* luciferase.

2.3. Cell culture and DNA transfection

COS-7 and HeLa cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% 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: Gibco BRL, Rockville, MD, USA). Forty-eight hours after transfection, the cells were lysed in 500 μl of a passive lysis buffer (Promega, Madison, WI, USA). Cell lysate was directly forwarded to the luciferase assay.

2.4. Luciferase assay

The Dual Luciferase system (Promega) was applied 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, USA).

2.5. DNA sequencing

The purified major 1 kbp PCR product of CTGF cDNA 3′-UTR was directly subjected to automated DNA sequencing by a dye terminator method (Bigdye: Applied Biosystems/Perkin Elmer, Foster City, CA, USA). Three specific primers for each DNA strand were used therein. The nucleotide sequences of the primers other than CT3UTS and CT3UTA (shown in Fig. 1) are:

1. CTUTS-2: 5′-CGGGATCCTCTAGACCTGTAACAAGCCAG-AT-3′
2. CTUTA-2: 5′-GGAATTCTCGAGCACAAACAACTTTAAAT-TAACTTAG-3′
3. CTUTS-3: 5′-CGGGATCCTCTAGAGTGCCTTTTTATTTTT-GTTTTTAATGC-3′
4. CTUTA-3: 5′-GGAATTCTCGAGTCTGGGGAAGATAGAC-T-3′

The nucleotide sequence of the corresponding region in pGL-3UTRS was also obtained by the same procedure.

3. Results

3.1. Detection of CTGF mRNA in HeLa cells and isolation of the 3′-UTR fragment

Based on a published nucleotide sequence of human CTGF cDNA [1,12], we synthesized two CTGF-specific primers for the detection of CTGF mRNA via RT-PCR. The sense primer (CT3UTS) was designed to recognize the very end of the CTGF-coding frame, the last three nucleotides of which correspond to the termination codon TGA. The antisense primer (CT3UTA) anneals immediately upstream of the putative poly(A)$^+$ addition signal (Fig. 1A). Using these primers, we could not only detect the expression of CTGF mRNA, but also isolate almost the entire portion of the 3′-UTR (approximately 1.0 kbp) from CTGF cDNA. As shown in Fig. 1B, a distinct single major band was observed on agarose gel electrophoresis of the crude PCR product from HeLa RNA. The band was excised, purified and identified as a CTGF cDNA 3′-UTR, as described in Section 3.2.

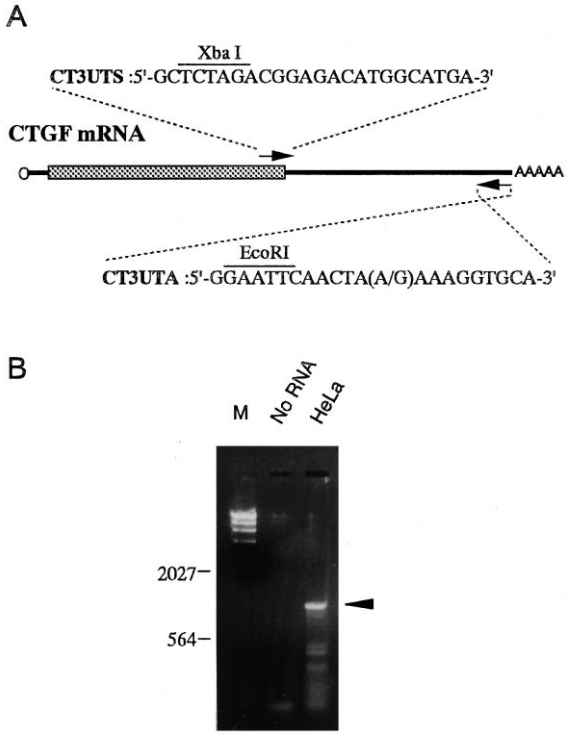

Fig. 1. Detection of CTGF expression in HeLa cells by RT-PCR to amplify the 3′-UTR portion of CTGF cDNA. A: Primers used for RT-PCR and the organization of human CTGF mRNA. The rod in the center represents a human CTGF mRNA. The small open circle and AAAAA at the left and right end denote the 5′-cap 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 in each sequence. B: Agarose gel electrophoresis analysis of the amplicon. Two microliters out of a total reaction mixture of 100 μl was analyzed. M: λ phage DNA *Hin*dIII digest (λ-*Hin*dIII) as a chain length standard. Sizes of a few bands in λ-*Hin*dIII are shown in bp at the left of the panel. The position of the major amplicon is indicated by the arrowhead at the right.

3.2. Nucleotide sequence of the CTGF 3′-UTR

The nucleotide sequence of the major amplicon, which was determined by direct sequencing of the PCR product, revealed its identity as the 3′-UTR portion of the CTGF cDNA. The results of the automated sequencing by dye terminators showed no significant variation in the major amplicon, suggesting uniformity of the PCR product. The determined sequence was deposited into GenBank under accession number

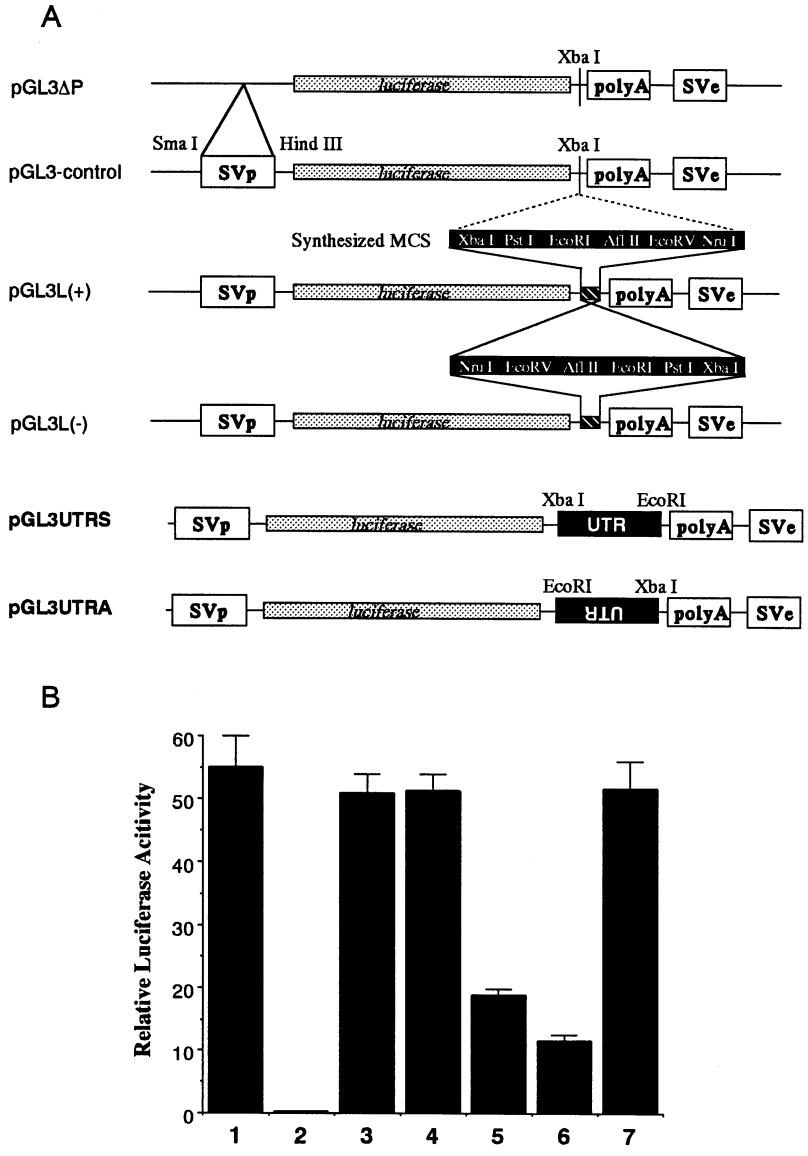

Fig. 2. A: Structures of the plasmids used in this study. All were derived from pGL3-control (second from the top), thus the basic structure of every plasmid is the same. Plasmids pGL3UTRS and pGL3UTRA were constructed from pGL3L(+) and pGL3L(−), respectively. Abbreviations: SVp: SV40 promoter; SVe: SV40 enhancer; poly A: SV40 polyadenylation signal; MCS: multiple cloning site; UTR: human CTGF gene 3′-UTR fragment (the major PCR product appeared in Fig. 1B); luciferase: firefly luciferase gene. B: Firefly luciferase activity from the plasmids displayed in A. Activity is represented as a relative value of measured luminescence from firefly luciferase versus *Renilla* luciferase from co-transfected pRL-TK (internal control). Mean values of the results of four experiments are displayed with error bars of standard deviations except for lane 7, where the data represent the results of two experiments. Lane 1: pGL3-control. Lane 2: pGL3ΔP. Lane 3: pGL3L(+). Lane 4: pGL3L(−). Lane 5: pGL3UTRS. Lane 6: pGL3UTRA. Lane 7: pGL3-control (×0.75 plasmid dose).

AF127918. Multiple alignment with previously reported sequences of corresponding regions of different human origins revealed that they are almost identical (data not shown), suggesting an indispensable role for the 3′-UTR in proper CTGF gene expression.

3.3. *cis-Acting repressive effect of the 3′-UTR on gene expression*

To elucidate possible effects of the CTGF 3′-UTR on gene expression, we constructed chimeric genes, in which firefly luciferase genes were fused with the RT-PCR-isolated CTGF 3′-UTR at the 3′ ends. Prior to subcloning the PCR product, we improved the parental plasmid, pGL3-control, by inserting a multiple cloning site at the end of the luciferase gene, which gave rise to no significant alteration of the luciferase gene expression itself (Fig. 2A: pGL3L(+) and pGL3L(−), Fig. 2B: lanes 3 and 4). Utilizing the improved vectors, two chimeric expression plasmids were constructed and subjected to a calibrated transient assay with *Renilla* luciferase co-expression. One of the resultant plasmids, called pGL3UTRS, contains the 3′-UTR in a sense direction of the luciferase gene, whereas the other plasmid, pGL3UTRA, possesses the same fragment in an anti sense direction. As is clear from Fig. 2B, both plasmids yielded strikingly low levels of expression compared to the other three parental luciferase expressors (i.e. 66.1% inhibition by the sense 3′-UTR;

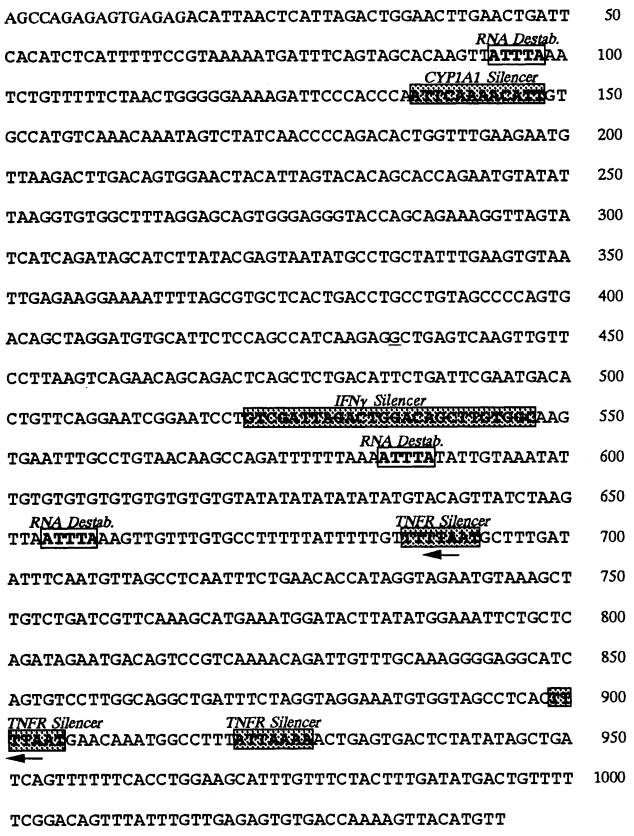

Fig. 3. Putative *cis*-acting negative regulatory elements in the CTGF 3′-UTR. The entire nucleotide sequence (except primers) of the subcloned 3′-UTR fragment in pGL3UTRS is displayed. Only one base was found to be different from the direct sequencing results of PCR product, which is underlined here. Putative RNA destabilizing elements are marked by open boxes, whereas putative silencer elements are indicated by stippled boxes. Arrows pointing upstream denote the elements with consensus sequences in the antisense strand.

80.0% inhibition by the antisense: against pGL3-control). Interestingly, the antisense 3′-UTR had the greatest inhibitory effect on the luciferase gene.

Since the addition of the 3′-UTR fragment also results in an increase of the size of plasmids, it might be that chimeric plasmids of the same weight (1 μg) as the parental vectors confer relatively fewer plasmid molecules for transfection, which may affect the interpretation of the results. With this in mind, we examined luciferase gene expression from a smaller amount of a parental plasmid, pGL3-control. Although 750 ng of pGL3-control contained fewer (90%) molecules than 1 μg of pGL3UTRS/pGL3UTRA, it yielded luciferase activities as high as other parental plasmids, strongly indicating that the repressive effect of the CTGF 3′-UTR fragment is striking and significant.

4. Discussion

We have detected the expression of CTGF mRNA, and isolated the 3′-UTR cDNA fragment via RT-PCR. The observed conservation of the non-coding region suggests a critical role for the corresponding region in the regulation of the CTGF gene expression. Since the 3′-UTR of the CTGF mRNA is unusually long (>1000 bases), possible functional elements are suspected particularly in this case. Indeed, our subsequent functional analyses of the 3′-UTR have clearly uncovered its remarkable repressive effects on gene expression. Since the effects have been evaluated under saturated levels of basal transcription (i.e. 750 ng and 1 μg of pGL3-control gave rise to a minimal difference in results), the repressive effects of the 3′-UTR seem to be quite distinct and efficient against active gene expression.

From our results, it is clear that negative regulatory elements exist in the CTGF 3′-UTR. Since there have been a number of studies on *cis*-acting negative regulatory elements such as silencers and RNA destabilizers, we also sought possible candidates for such elements. As a consequence, several sequences that are identical or highly homologous to known negative regulatory *cis* elements were identified, which are summarized in Fig. 3. Three AT-rich elements to destabilize mRNA [18,19] and three silencer elements found in tumor necrosis factor receptor [20], all of which perfectly match the originals, were present in the corresponding area. Two other silencer-like elements can be specified as well. One near the 5′-end of the sense strand retains 90.9% homology to the silencer element of rat CYP1A1 gene [21,22], and the other, a 26 bp element in the middle, shares 65.4% homology with the YY1-binding silencer of the human interferon-γ gene [21,23]. Needless to say, they are potential candidates for the functional repressive elements built in the 3′-UTR. However, in addition, an orientation-dependent positive regulatory element may be present, since the antisense 3′-UTR construct conferred a more pronounced repressive effect than the sense construct. Of note, it is widely recognized that the 3′-UTR of a gene is an appropriate location for mRNA export signals [24,25].

CTGF is a multi-functional growth factor that potentiates either growth or differentiation of various types of mesenchymal cells, according to biological conditions [1,5–7,14]. Moreover, gene expression of CTGF in vivo is normally low but is induced during cytodifferentiation or in pathological states [5–7,12]. For example, CTGF expression in growth cartilage cells increased as the cells differentiated to the hypertrophic stage [12]. The mechanism of this controlled expression of CTGF in chondrocytes is as yet unclear. Nevertheless, it is highly likely that the regulatory function of the 3′-UTR, which has been uncovered by our present work, is involved in this differential regulation of CTGF expression, as a strictly tuned system may require multiple regulatory factors. Investigation of the 3′-UTR function in chondrocytic cells may shed light on critical aspects of the regulatory mechanism of gene expression.

In summary, the CTGF 3′-UTR is quite long and conserved, hence involvement of multiple regulatory elements, including positive effectors, is expected, which may be one of the major parts of the regulation machinery enabling the differential expression of the CTGF gene. Further functional dissection of the 3′-LTR is currently under way.

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