Translational repression by the cis-acting element of structure-anchored repression (CAESAR) of human ctgf/ccn2 mRNA

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Abstract The cis-acting element of structure-anchored repression (CAESAR) is a post-transcriptional regulatory element of gene expression, which is located in the 3'-untranslated region (UTR) of the human ccn2 gene (ctgf/ccn2). In this report, the repression mechanism of CAESAR, as well as the structural requirement, was investigated. Removal of minor stem-loops from CAESAR resulted in proportional attenuation of the repression mechanism by RNA secondary structure to determine the basal ctgf/ccn2 expression level was uncovered herein. © 2005 Federation of European Biochemical Societies. Published by Elsevier B.V. All rights reserved.

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1. Introduction

The connective tissue growth factor (CTGF/CCN2) gene is one of the prototypic members of the CCN family [1–6]. Namely, the abbreviation “CCN” stands for the assemblage of the names of the classical members, i.e., ctgf, cyr 61, and nor [1–6]. Currently, the CCN family includes such members as ctgf-3/ctgf-L1wisp-2, wisp-1/elm 1, and wisp-3 [2–6]. The gene products of these members are characterized by a number of conserved cysteine residues and a common modular structure consisting of four independent modules that are encoded by independent exons [1]. In terms of protein function, these modules are believed to play multiple roles under the interactions with other regulatory molecules to enable diverse functions. Such characteristics of CCN family proteins are best represented by CTGF/CCN2. Early studies on this factor disclosed its mitogenic and chemotactic activities for fibroblasts [7], which also provided a rational basis for the role of CTGF/CCN2 in the wound healing process, fibrotic disorders, and malignancies of several different tissues [8–10]. The physiological roles of CTGF/CCN2 are quite well clarified in cartilage tissues. After the re-discovery of CTGF/CCN2 as a specific gene product of the human chondrocytic cell line HCS-2/8 [11], we obtained a series of data defining CTGF/CCN2 as a central growth factor involved in the growth and differentiation of chondrocytes [12–14], osteoblasts [15] and vascular endothelial cells [16,17]. These findings were supported by the defect observed in the growth plate of CTGF/CCN2 knockout mice [18]. Role of CTGF/CCN2 in cell adhesion in collaboration with integrin molecules has been also indicated [2,19].

Immediately downstream of the open reading frame, a 1 kb-long 3'-untranslated region (UTR) follows in the ctgf/ccn2 mRNA. It is widely known that, in a number of eukaryotic genes, 3'-UTRs are engaged in the post-transcriptional regulation of gene expression. Interestingly, we also found that the 3'-UTR of CTGF/CCN2 contained a cis-acting element of structure-anchored repression (CAESAR). CAESAR is a post-transcriptional repressive element located at the very junction of the coding region and 3'-UTR of the CTGF/CCN2 gene [20]. It is an 84-base-long mRNA segment that forms a stable secondary structure. When linked within a transcribed region of a gene in cis, CAESAR strongly inhibits its expression. However, precise structural requirement and mechanism of repressive action have remained to be clarified. Here, we further investigated the structural-functional relationship and the functional properties of CAESAR as a post-transcriptional regulatory element. Based on the obtained findings, we consider CAESAR as an RNA translational repressor that functions through the recognition of secondary structure to determine the basal level of ctgf/ccn2 expression.

2. Materials and methods

2.1. Cells

Cos-7 (a monkey kidney cell line), MDA-231 (a human breast cancer cell line), HSC3 (a human oral squamous cell carcinoma cell line), HeLa (a human cervical cancer cell line), HCS-2/8 (a human chondrosarcoma cell line) and 293T (a human embryonic kidney cell line) cells were maintained at 37 °C in Dulbecco’s modified Eagle’s minimum essential medium (D-MEM) supplemented with 10% fetal bovine serum (FBS) in an atmosphere of humidified air containing 5% CO\textsubscript{2}.

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2.2. Parental luciferase reporter constructs

The SV40 promoter-driven firefly luciferase expression plasmids, pGL3-control, pGL3L (+), and pGL3L (−), were described previously [20]. An HSV-TK promoter-driven Renilla luciferase expression plasmid (pRL-TK; Promega, Madison, WI) was utilized as an internal control. The original chimeric construct containing the full-length 3′-UTR (pGL3UTRS: Fig. 1) or the intact CAESAR (pGL3SA5) at the downstream end of the luciferase gene was constructed as described previously [20].

2.3. CAESAR mutant constructs

Among the mutants analyzed, pGL3E23 and GCAESAR were described previously [20]. To remove the central bulge in the secondary structure, two oligonucleotides, E3BP (5′-AATTCGAACTGATTCACTCTCATTTTTCCG-3′) and E3BM (5′-CGGAAAAATGAGTGAATCAGTTCG-3′) were annealed to each other, and ligated between the internal EcoRI and external EcoRV sites of pGL3E23, replacing the latter half of the CAESAR region. The resultant plasmid was designated pGL3E23AB. The other two mutants were designed to remove 1 or 2 minor stem-loops of CAESAR. For the removal of a minor loop (ΔML: Fig. 2), another mutant, E12 [20], was utilized. The chimeric plasmid containing ΔML1 was constructed by the addition of the annealed short oligonucleotides (5′-CTAGCGGAAA-3′ and 5′-CTGATTTCGG-3′) at the XbaI site of pGL3E12 to retrieve the major stem but not the first minor loop. Finally, in order to construct a CAESAR mutant lacking both minor loops, STEMP (5′-CTAGACGGAGAAATGTGAGATTGAATTC-3′) and STEMM (5′-AATTCGAGTCTTAATTCAGTTTTCCG-3′) were synthesized and replaced the former-half of the CAESAR region between the XbaI and internal EcoRI sites in pGL3E23. The resultant plasmid was designated as pGL3E23ATL. The E2 mutant that contained only the upper half of the predicted structure was an E23 derivative, in which the lower half was removed.

2.4. Plasmids for in vitro transcription

A control plasmid, pBSLuc, was obtained by subcloning the 1.85 kbp HindIII–HpaI fragment, which comprises the entire luciferase coding region and a portion of polyadenylation signal region of pGL3-control, between the unique HindIII and SmaI sites in pBluescript SK(−) (Stratagene). Following the same construction procedure, the corresponding HindIII–HpaI fragment from pGL3SA5 was built in pBluescript SK(−) to yield pBSLucSA5.

![Fig. 1](image-url) (A) The repressive effects of the 3′-UTR of human *ctgf*/*ccn2* on gene expression in a variety of primate cells. Structures of the molecular constructs used for the evaluation are shown at the top. Small box below the illustration indicates approximate location of CAESAR in the 3′-UTR. Abbreviations: SVp, SV40 promoter; poly A, SV40 polyadenylation signal; 3′-UTR, human *ctgf*/*ccn2* 3′-UTR cDNA; CAESAR, the cis-acting element of structure-anchored repression. The results of evaluation are shown in percentages as relative activities from the UTR construct versus control with error bars (standard deviations). Names of the cells used are indicated in horizontal axis. Origins of these cells are described in Section 2. (B) Repressive activities of the entire 3′-UTR and CAESAR in Cos-7 cells. These results are the representatives of at least two independent experiments. (C) Nucleotide sequence and predicted secondary structure of CAESAR.
For riboprobe preparation, pBSLuc was subsequently digested by *Hind*II and self-religated to remove the 1.28-kbp major portion of the luciferase-coding region. The resultant plasmid, pBSÆLuc (Fig. 3), contained a 0.35-kbp gene fragment encoding the carboxy terminus of the luciferase followed by a 0.16-kbp sequence from pGL3-control as an external insert.

Fig. 2. Structure-targeted mutational analysis of CAESAR. Nucleotide sequences and possible secondary structures of CAESAR mutants are displayed, together with their repressive activities as measured by the luciferase assay system at the bottom. Mutated nucleotides in E23 or G and C bases in GCAESAR are indicated in boldface, respectively. The results are demonstrated in the same manner as in Fig. 1.
membrane was lysed by incubation on ice for 5 min in an RNex buffer harvested in 5 mM EDTA/phosphate-buffered saline (PBS). Cell lysates were diluted appropriately to give an accurate measurement using transfected cells at an expanded scale with two 10 cm diameter dishes. Isolation of ribosomal complex was carried out, and forwarded to the luciferase assay.

2.7. RNA subcellular fractionation

Within the linear range. The proper structures of all the constructed plasmids were confirmed by automated DNA sequencing (Applied Biosystems/Perkin-Elmer, Foster City, CA).

2.5. DNA transfection

Twenty hours prior to transfection, cells were seeded into 35 mm tissue culture dishes or 12-well tissue culture clusters. Cationic liposome-mediated DNA transfection was performed with each pGL3 derivative in combination with 0.5 μg of pRL-TK (internal control), according to the manufacturer’s optimized methodology (LipofectAMINE; LifeTechnologies, Rockville, MD; FuGene; Roche Diagnostics, Mannheim, Germany). Forty-eight hours after transfection, the cells were lysed and forwarded to the luciferase assay.

For RNA analysis, transfection was carried out at an expanded scale in a 10 cm diameter tissue culture dish with 1.6 × 10⁶ cells and 8 μg of each pGL3 derivative. Isolation of ribosomal complex was carried out, using transfected cells at an expanded scale with two 10 cm diameter dishes per sample.

2.6. 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 [20]. Cell lysates were diluted appropriately to give an accurate measurement within the linear range.

2.7. RNA subcellular fractionation

Forty-eight hours after transfection, cells were washed and harvested in 5 mM EDTA/phosphate-buffered saline (PBS). Cell membrane was lysed by incubation on ice for 5 min in an RNex buffer (0.14 M NaCl, 1.5 mM MgCl₂, 10 mM Tris–HCl, 0.5% Nonidet P40, 1 mM DTT, 20 mM vanadyl ribonucleoside complex). The cell nuclei were pelleted by centrifugation and lysed for RNA extraction in a commercially available reagent (Trizol: LifeTechnologies, Rockville, MD). The cytoplasmic fraction was treated by proteinase K and the RNA was collected by 2-propanol precipitation after phenol–chloroform–isoamylalcohol extraction, as described previously [21].

2.8. Riboprobe preparation and ribonuclease protection analysis

The riboprobe was transcribed in vitro by bacteriophage T3 or T7 RNA polymerase in the presence of [γ-³²P]UTP (Dupont/NEN, Boston, MA). The transcription template, pBLLuc, was linearized by HindIII prior to transcription. Twenty micrograms of total RNA was hybridized with 7 × 10⁹ cpm of radiolabeled probe in a hybridization buffer containing 50% formamide at 42 °C for 20 h. After hybridization, the RNA mixture was digested by a cocktail of RNase T1 and RNase A at 30 °C for 40 min, as described previously [22,23]. The digested mixture was analyzed by 6% PAGE under denaturing conditions in 1× Tris–borate–EDTA (TBE) buffer. Quantitatively of the assay system was ascertained by the fact that signal intensity of the strongest protected band was less than 10% of that of the total input probe.

2.9. Ribosomal sedimentation

Brief summary of the procedure is illustrated in Fig. 4A. The transfected 293T cells (3.2×10⁶) were washed with PBS and suspended in 1 ml of Buffer A (0.25 M sucrose, 25 mM KCl, 5 mM MgCl₂, 2 mM dithiothreitol, 50 mM Tris–HCl, pH 7.4). Cytoplasmic membrane was lysed by the addition of NP-40 at a final concentration of 0.7%, and the nuclei were pelleted down by a centrifugation at 750 × g. After the removal of the nuclei, the supernatant was further centrifuged at 12500 × g to remove mitochondria. The supernatant containing ribosome was supplemented to contain 0.5 M KCl and loaded onto a 1 M sucrose cushion in a ultracentrifuge tube. Final centrifugation was performed at 245000 × g for 10 h. The ribosome-associated RNA was then extracted by the same procedure as described above.

2.10. In vitro translation assay

Preparation of luciferase RNA with or without CAESAR was performed in vitro with pBSLuc, or pBSLucSA5, respectively, as a template. The template plasmid was linearized by BanHI digestion (Fig. 5), and 1 μg of DNA was forwarded to in vitro transcription reaction under the same reaction and purification procedure as used for the riboprobe.

In vitro translation analysis was carried out in a 30 μl volume that contained 21 μl of nuclease-treated rabbit reticulocyte lysate (Promega) supplemented with a total amino acid mixture. To start the reaction, 21 μl of the supplemented reticulocyte lysate was mixed with 20 μl of total RNA in 9 μl of H₂O. After 10 and 20 min of incubation at 37 °C, 5 μl of out of 30-μl translation mixture was forwarded to the firefly luciferase assay, as described elsewhere.

2.11. Reverse-transcription and real-time quantitative polymerase chain reaction (PCR) analysis of mRNA

Polyadenylated RNAs were selectively reverse-transcribed at 50 °C for 30 min from 500 ng of each total RNA by using an avian myeloblastosis virus reverse-transcriptase (AMV-RT: Promega) and an oligo(dT) octadecamer. The copy numbers of firefly luciferase mRNA were evaluated by real-time PCR with a Lightcycler system (Roche Diagnostics). The nucleotide sequences of the primers as follows: Luc-LCS (sense), 5'-CCGCCCTGAAGTCCTGATTA-3'; Luc-LCA (anti-sense), 5'-TCCACAAACACAAACTCTGCC-3'. The amplification procedure under the optimized protocol consisted of a denaturation at 95 °C for 15 min, 45 cycles of amplification reactions at 95 °C for 15 s, 64 °C for 10 s, and 72 °C for 10 s. Identity of all of the PCR products was confirmed by analyzing the melting curves.

2.12. Secondary structure prediction of RNA segments

Computer-associated prediction of putative RNA secondary structure was performed with a software program (GENETYX ver. 8.0: Software Development Co., Ltd., Tokyo, Japan).
3. Results

3.1. Universal repressive potential of CAESAR in a variety of human cell lines

In our previous report, the repressive effects of the 3′-UTR was confirmed in few cell lines. In advance to further analysis, we here evaluated the repressive potential of the human ctgf/ccn2 3′-UTR in a variety of human cell lines. In the present study, a variety of human cell lines were subjected to similar analysis by means of transient reporter gene expression assay. As a result, all of the human cell lines of different origins – breast cancer, oral squamous cell carcinoma, cervical cancer and chondrosarcoma – commonly revealed strong repressive effects of the 3′-UTR (Fig. 1). As such, it was indicated that the 3′-UTR-mediated repression was a fundamental mode of gene regulation to restrain the basal gene expression at low levels.

In the 3′-UTR, CAESAR at the upstream end was previously identified as a major element that conduct the observed repressive effects. Of note, in Cos-7 cells, almost all of the repressive activity could be ascribed to the action by CAESAR alone, as represented by the comparable effect of CAESAR to that of the entire 3′-UTR in this particular cell line. Therefore, we utilized Cos-7 cells for subsequent structural–functional dissection of CAESAR.

3.2. Structural requirement of CAESAR for the repressive function

To examine the possible role of the major-loop nucleotide sequence in the function, we introduced mutations in the CAESAR to obtain E23 mutant. Not only this mutant provided evidence that the primary nucleotide sequence in the major loop was not a determinant of the function (Fig. 2), but also it was utilized to construct the other CAESAR mutants to dissect structural requirement of the repressive function. First, we constructed a CAESAR mutant in which the bulge structure in the center of CAESAR was removed. Functional evaluation of this CAESAR mutant, DB, clearly showed that CAESAR could be fully functional without the central bulge (Fig. 2). Together with the entire retention of the repressive function in a mirror image CAESAR analogue (GCASAR: Fig. 2) that possess totally unrelated primary nucleotide sequence, no primary sequence and the central bulge were found to be required for the CAESAR function. Next, we pursued the role of the multiple stem-loop structure in the repressive function. Initially, we removed a minor loop at the upstream half of the CAESAR and evaluated the functionality of this CAESAR mutant, DML1. As a result, DML1 still retained significant repression potential, but its functionality was modestly attenuated. Then, we further removed the other loop and examined whether the major stem-loop only was sufficient
for the CAESAR function, or not. The result of the luciferase-reporter assay revealed a remarkable reduction in repressive activity on gene expression. Finally, total removal of the stem-loop structure resulted in complete loss of the repressive effect (Fig. 2B). Therefore, the observed gradual loss of the repressive function along with the sequential removal of minor loops indicates the requirement of multiple stem-loop structure as a determinant of CAESAR function.

3.3. Nuclear export as well as overall stability of mRNA was not affected by CAESAR linked in cis

CAESAR has been supposed to function during the process of nuclear export of mRNA or during the translation events after the nuclear export. Thus, the former possibility was initially examined. Firefly luciferase mRNA with or without CAESAR was expressed in Cos-7 cells, and total RNA was fractionated into subcellular cytoplasmic and nuclear populations. Successful fractionation was confirmed by the findings that distinct signals for the 45S ribosomal RNA precursor were observed in the nuclear fraction, which originates from nucleolar RNA, while such a signal was totally absent in the cytosolic fraction (Fig. 3, panel B). In those fractionated RNAs, the fate of the luciferase mRNA with/without CAESAR was pursued.

Ribonuclease protection analysis was employed for the study of both the mRNA quantity and structure around the CAESAR-addition point. A riboprobe around the CAESAR-addition point at the 3′-UTR was prepared and utilized. As summarized in Fig. 3A, the RNA from the pGL3-control was expected to protect the entire 510-base segment that originated in pGL3-control. In contrast, the portions of the probe that would be protected by pGLSA5-derived mRNA were 350- and 160-base segments, owing to the inserted CAESAR sequence. As shown in Fig. 3C, the results were totally consistent with this prediction. More importantly, the intensities of specific signals from either the cytosolic or nuclear fraction showed no significant difference, regardless of the involvement of CAESAR (Fig. 3C). Therefore, we concluded that CAESAR did not alter the nuclear export status of cis-linked mRNA as well as its intracellular stability and that the CAESAR-mediated repression may be mainly conducted by cytoplasmic events.

3.4. Interference of ribosomal association by cis-linked CAESAR

After the nuclear export, mature mRNAs have to be assembled onto ribosomal complexes for initiating their translation. Since several cis-elements in 3′-UTRs have been known to control gene expression through this process [24], we investigated the effects of CAESAR on ribosomal association of the cis-linked reporter mRNA. For this objective, we employed 293T cell line, which is a human analogue of Cos-7 as a provider of cellular background. We transiently expressed the same reporter mRNAs as described in the previous subsection and chased their fate in the cytoplasm. Since 293T is a human cell line, we could monitor the experiments by verifying the equivalent level of ribosomal association of an internal control (β-actin mRNA), which is difficult with monkey Cos-7 cells. The results of real-time quantitative RT-PCR analysis of ribosome-associated portion of mRNAs indicated the interference of ribosomal association by the cis-linked CAESAR (Fig. 4).

3.5. Translational repression by CAESAR at the 3′-UTR in vitro

As indicated by the results above, CAESAR functions as a repressor of ribosomal association, which is mostly conducted by the 5′-cap-mediating recruitment of mRNAs by eukaryotic initiation factors. Nevertheless, with the system above, no information concerning other translation parameters, such as translation speed after ribosomal assembly, could be obtained. It is still possible that CAESAR may repress translation at stages after ribosomal association as well. Therefore, in order to examine if CAESAR affects mRNA translation after 5′-cap-mediated primary association, we employed an in vitro rabbit reticulocyte lysate translation system to evaluate the translation efficiency. Since this system works even with 5′-cap-free mRNAs, we could rule out the effects of CAESAR upon the cap-mediated recruitment of mRNA onto the 43s initiation complex by eIF-4F. The 5′-cap-free firefly luciferase mRNA with or without CAESAR at the 3′-UTR was transcribed in vitro by bacteriophage T7 RNA polymerase by using corresponding plasmids as templates (Fig. 4A), and equivalent amounts of RNAs were subjected to in vitro translation analysis. As a result, in vitro evaluation of translation efficiency of the cap-free mRNAs under unsaturated conditions revealed significant difference at 20 min. At 10 min, the difference between the two was not significant, which suggests CAESAR may not exert any effect on the early steps of such an atypical 5′-cap-independent translation procedure. As such, together with the data concerning the effects of CAESAR on the ribosomal association of intact mRNA, these data indicated that CAESAR interfered with mRNA translation process at multiple steps.

4. Discussion

CAESAR was discovered in the 3′-UTR of the human cct8l/ccn2 gene as a major cis-element conferring post-transcriptional repressive effects on gene expression. In this study, comprehensive analyses to dissect out the structural-functional relationship have been carried out. Moreover, we here disclosed that the repressive ability of CAESAR is exerted at the translation procedure.

Post-transcriptional control of gene expression is mostly conducted by specific elements on mRNAs, in collaboration with their specific binding counterparts. In a number of well-characterized cases, these RNA elements are known to form stable secondary structures. Several signal sequences in eukaryotic mRNAs that enable efficient nuclear export have been identified as well. Interestingly, they are all located in the 3′-UTR [24,25]. Post-transcriptional cis-elements in 3′-UTRs can also determine the fate of certain mRNAs by controlling their degradation [26]. Finally, it has been also known that certain RNA segments play critical roles in the regulation of the protein translation process [24]. We initially examined the effects of CAESAR on mRNA export and stability. However, neither the nuclear accumulation of the reporter mRNA, nor a reduction in the steady-state mRNA levels was induced by cis-addition of CAESAR. Then, we moved forward to evaluate whether cis-addition of CAESAR affected ribosomal association, or not. Usually, ribosomal assembly of particular mRNAs is evaluated by fractionating cytosolic RNA from free to polysome-associated RNA through the gradient of sucrose density by ultracentrifugation. This methodology is excellent for the analysis of endogenous and viral mRNAs. However,
Unfortunately, such conventional methods were not applicable to our case with DNA transfection, because of the contamination of plasmid DNA. We could finally remove the background signals by pelleting down only the polysomes through ultracentrifugation on a sucrose cushion. This approach was cleaner, but might be less sensitive. Obviously, the observed interference in this evaluation (Fig. 4B) does not fully account for the repressive potential by CAESAR in vivo (Fig. 1B). This discrepancy may be ascribed to the insensitivity of the evaluation system only with polysomal pellets. Establishment of an alternative expression/analysis method is currently in progress. Additionally, in order to further dissect the effect of CAESAR on translation thereafter, we employed a cap-independent in vitro translation assay system. After all, we eventually found that cis-linked CAESAR repressed gene expression by interfering with translation at multiple steps.

Translation control by a cis-element together with its binding counterparts is best represented in the regulatory system using Nanos and Pumilio gene products in Drosophila [27]. According to a number of previous reports, these two proteins bind to a 32 base element in the $3^\text{UTR}$ of the mRNA for the transcription factor Hunchback, to repress its translation [27]. This system is structurally and functionally similar to the CARSA-R-mediated repression system. Under such a system, Nanos and Pumilio actually play critical roles in conducting germline development. According to the findings obtained in this study, the CAESAR function is assumed to be based on the recognition of the multiple stem-loop structure without sequence specificity. Therefore, a number of RNA segments with CAESAR-like elements may well be under the same regulation conducted by the same cellular counterpart(s). It is noteworthy that Nanos shows relatively flexible target selectivity.

It is now clear that cis-addition of CAESAR represses the basal translation level without affecting pre-translational status of mRNA. However, it is still possible for the built-in CAESAR to alter the half-life of mRNA in response to certain stimuli. Such an example is found in the $3^\text{UTR}$ of the parathyroid hormone (PTH) gene [28]. This system is driven by cytosolic factors that bind specifically to the structured RNA target in the $3^\text{UTR}$ to control its degradation efficacy [28]. Furthermore, we recently reported this type of dynamic regulation in chicken $ctgf/ctn$ gene, which was actually mediated by the $3^\text{UTR}$. In this case, $ctgf/ctn$ gene expression was duly regulated along with chondrocytic differentiation, utilizing a $3^\text{UTR}$-mediated control of mRNA degradation [29]. Extensive investigation to clarify the dynamic roles of CAESAR in gene control under particular biological process is currently underway.

$CTGF/CCN2$ is a key player in endochondral ossification, a process that determines the growth of longitudinal bone [4]. Differential expression of the $ctgf/ctn$ gene is observed in growth cartilage and other connective tissues during the development of vertebrates [4]. Under physiological conditions in adult animals, a very limited population of the cells has been shown to express $CTGF/CCN2$. Therefore, the novel CAESAR-mediated translation repression system clarified here may be a fundamental component of gene regulatory machinery to determine the basal level of $ctgf/ctn$ gene expression. Since such long $3^\text{UTRs}$ are also found in other $ccn$ family members, this particular mode of $3^\text{UTR}$-mediated regulatory system may have evolved along with this particular family of genes encoding proteins with similar structure and function.

Comprehensive analysis of all of the CCN family members will carve out the common origin and nature of these genes and their translational products.

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