Regulation of Chicken ccn2 Gene by Interaction between RNA cis-Element and Putative trans-Factor during Differentiation of Chondrocytes*

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CCN2/CTGF is a multifunctional growth factor. Our previous studies have revealed that CCN2 plays important roles in both growth and differentiation of chondrocytes and that the 3′-untranslated region (3′-UTR) of ccn2 mRNA contains a cis-repressive element of gene expression. In the present study, we found that the stability of chicken ccn2 mRNA is regulated in a differentiation stage-dependent manner in chondrocytes. We also found that stimulation by bone morphogenetic protein 2, platelet-derived growth factor, and CCN2 stabilized ccn2 mRNA in proliferating chondrocytes but that it destabilized the mRNA in prehypertrophic-hypertrophic chondrocytes. The results of a reporter gene assay revealed that the minimal repressive cis-element of the 3′-UTR of chicken ccn2 mRNA was located within the area between 100 and 150 bases from the polyadenylation tail. Moreover, the stability of ccn2 mRNA was correlated with the interaction between this cis-element and a putative 40-kDa trans-factor in nuclei and cytoplasm. In fact, the binding between them was prominent in proliferating chondrocytes and attenuated in prehypertrophic chondrocytes. Stimulation of the growth factors repressed the binding in proliferating chondrocytes; however, it enhanced it in prehypertrophic chondrocytes. Therefore, gene expression of ccn2 mRNA during endochondral ossification is properly regulated, at least in part, by changing the stability of the mRNA, which arises from the interaction between the RNA cis-element and putative trans-factor.

Bone is formed by endochondral ossification and intramembranous ossification. During endochondral ossification (reviewed in Ref. 1), chondrocytes first proliferate; then they become mature cells, which produce extracellular matrix compounds such as type II collagen and aggrecan. Thereafter, the cells eventually differentiate into the hypertrophic chondrocytes, which produce type X collagen and alkaline phosphatase.

The terminal stage of endochondral ossification, the cartilage matrix becomes mineralized and is invaded by blood vessels, and the chondrocytes are thought to undergo apoptosis. Through this process, cartilage is replaced by bone. A number of hormones, such as parathyroid hormone (2) and active forms of vitamin D (2–4), and growth factors, such as fibroblast growth factor-2 (5), transforming growth factor-β (TGF-β) (6, 7), bone morphogenetic proteins (BMPs (8–10)), parathyroid hormone-related protein (2,10), and platelet-derived growth factor (PDGF (11)), have been reported to be involved in the proliferation and differentiation of chondrocytes during endochondral ossification.

CCN2 (connective tissue growth factor/hypertrophic chondrocyte-specific gene product number 24; CTGF/Hcs24) is a cysteine-rich secretory protein of 36–38 kDa; it belongs in the CCN family (reviewed in Refs. 12–16), which consists of the cysteine-rich secretory protein of 36–38 kDa; it belongs in the CCN family (reviewed in Refs. 12–16), which consists of the CCN family (reviewed in Refs. 12–16), which consists of the CCN family (reviewed in Refs. 12–16), which consists of the CCN family (reviewed in Refs. 12–16), which consists of the CCN family. In addition to reports concerning the regulatory mechanisms of CCN2, there are several reports indicating that CCN2 may be involved in the differentiation of chondrocytes (19, 27). TGF-β induces the expression of CCN2 (22, 27), and a TGF-β response element was found in the ccn2 promoter region (36, 37). In addition to reports concerning the regulatory mecha-

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This report is dedicated to the memory of Dr. Hitoshi Akedo.

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The abbreviations used are: TGF-β, transforming growth factor-β; BMP, bone morphogenetic protein; PTHrP, parathyroid hormone-related protein; PDGF, platelet-derived growth factor; UTR, untranslated region; CEF cells, chicken embryonic fibroblast cells; FBS, fetal bovine serum; US cells, chicken upper sternum chondrocyte cells; LS cells, chicken lower sternum chondrocyte cells; TBE, Tris-borate-EDTA; REMSA, RNA electromobility shift assay; AU-rich, rich in adenosine-uridine.
nism of ccn2 transcription, we reported that the gene expression is also regulated by its 3'-untranslated region (3'-UTR) at post-transcriptional stages (38-40) and that its repressive effect is functionally conserved between mammalian and avian species (41).

In the present study, we reveal that gene expression of chicken ccn2 mRNA is regulated not only transcriptionally, but also post-transcriptionally during the differentiation of chondrocytes and that a cis-element in the 3'-UTR of ccn2 mRNA and its putative trans-factor counterpart collaboratively play an important role in the post-transcriptional regulation by determining the stability of ccn2 mRNA.

**EXPERIMENTAL PROCEDURES**

**Cell Isolation and Culture**—Chicken embryonic fibroblasts (CEF cells) were isolated from a 10-day-old whole chicken embryo and maintained in Dulbecco’s modified Eagle’s minimum essential medium supplemented with 10% fetal bovine serum (FBS) in humidified air containing 5% CO2 at 37 °C. Chicken upper sternum chondrocytes (US cells) and lower sternum chondrocytes (LS cells) were isolated from the cephalic 1/3 portion and caudal 1/3 portion, respectively, of the sternal cartilage of 10-day-old chicken embryos. Dulbecco’s modified Eagle’s minimum essential medium supplemented with 10% FBS in humidified air containing 5% CO2 at 37 °C, as described previously (41).

**Growth Factors**—Recombinant human BMP-2 and recombinant human PDGF-BB were purchased from R&D Systems (Minneapolis, MN) and Sigma-Aldrich, respectively. Human recombinant CCN2 was prepared as described previously (34).

**Preparation for Hybridization**—Clone A1/5 containing a full-length chicken ccn2 was utilized as a probe for Northern blot analysis of the ccn2 gene, as described in a previous study (41), and a 3' 100-base fragment of ccn2 3'-UTR, which is described in another subsection of this report, was utilized as a probe in the RNase protection assay. The probes of chicken α2I(1), α1I(1), and α1X collagen genes, β-actin gene, and mouse c-myc gene were isolated by reverse transcription-mediated PCR, utilizing total cellular RNA of CEF cells (for α2I(1) collagen gene) or US cells (for the other genes) as a template. The nucleotide sequences of the sense primer and antisense primer for α2I(1) collagen (42) were 5'-TTA CTC CTC GGC CAG GTC TGA TG-3' and 5'-GCT CAG CAC GAA CAC CTT GAA-3', respectively. For α1I(1) collagen (43), the sense and antisense primer sequences were 5'-GCA GAG ACC ATC AAC GGC GGT-3' and 5'-GAC CCG GCA CTA CCT CCA G-3', respectively; and those for α1X collagen (44), 5'-AAG GGG CCA CAC TTT CTA-3' and 5'-CTC TGC CAG TTT CCT CCA-3', respectively. These 3 primer pairs were designed and previously utilized by Nakata et al. (45). For chicken β-actin (46), the nucleotide sequences of sense and antisense primers were 5'-TGG ATT TCG AGC AGG AGA TGG CC-3' and 5'-TTA CTC CTC GGC CAG GTC TGA TG-3', respectively. For chicken 18 S rRNA (47) were 5'-GAC TCC GGT TGT ATT TTG TTG G-3' and 5'-ACT AGT TAG CAT GCC AGA GT-3', respectively.

The five amplicons were subcloned into pGEM-T Easy (Promega, Madison, WI) by a TA-cloning method. Proper construction of all plasmids was confirmed by nucleotide sequencing, and one of the plasmids by which the antisense strand mRNA can be transcribed from the bacteriophage T7 polymerase promoter was selected and prepared for each gene.

For hybridization, the plasmids of ccn2, α2I(1), α1I(1), α1X collagen, and 18 S rRNA genes were utilized for the riboprobe preparation. The plasmids were linearized by SalI (for 18 S rRNA) or SpeI (for other genes) and transcribed in *vitro* by bacteriophage T7 RNA polymerase in the presence of 50 μl of [α-32P]UTP (3000 Ci/mmol, Amersham Biosciences) for the preparation of radiolabeled antisense RNA. The transcription reaction was performed at 37 °C for 1 h with reagents supplied by the manufacturer (Promega), followed by RNase A digestion. The riboprobe of firefly luciferase was prepared as described in a previous study (40). After transcription in *vitro*, all of the riboprobes were subjected to spin-column (ProbeQuant G-50, Amersham Biosciences) purification. Total RNA was denatured by glyoxal, separated on 1% agarose gels, and then blotted onto nylon membranes (Hybond N, Amersham Biosciences). After blotting, the membrane was fixed with 5% acetic acid and stained with 0.02% methylene blue to visualize ribosomal RNAs, hybridized, washed, and subsequently autoradiographed. The optical density of each signal band of the autoradiography was quantified by using a commercial computer software (Quantity One, PDI Inc., New York, NY).

**Nuclear Run-on Analysis**—Nuclear run-on assays were carried out according to an established described protocol (48), with a slight modification. The nuclei of the cells grown in 10-cm tissue culture dishes were isolated and suspended in 50 mm Tris-HCl (pH 8.0), 0.4% glycerol, 5 mM MgCl2, and 0.1 mm EDTA; aliquots of the suspension were then incubated at 30 °C for 2 h with ATP, CTP, and GTP each at 3 mM, and 100 μCi of [α-32P]UTP (3000 Ci/mmol, Amersham Biosciences) in a reaction buffer consisting of 10 mM Tris-HCl (pH 8.0), 5 mM MgCl2, and 300 mM KCl. After digestion with RNAse and proteinase K (Invitrogen), the radiolabeled transcripts were extracted by using Isogen LS (Nippon Gene) according to the manufacturer’s protocol, and further purified by use of a spin column.

For preparation of membranes, the plasmids containing chicken β-actin and ccn2 were linearized by SpeI; whereas pGEMM2zf (+) (Promega) as a background control was linearized by EcoRI. The plasmids were subjected to alkaline denaturation, and then 10 μg of each was dot-blotted onto a nylon membrane by use of a Bio-Dot apparatus (Bio-Rad, Hercules, CA). Ten million cpm of radiolabeled nuclear transcription reaction mixture containing 1 μg of radiolabeled DNA was then hybridized to the nylon membrane. The hybridization buffer consisting of 5× SSC (pH 7.0: 1× SSC = 0.15 M sodium chloride and 0.015 M sodium citrate), 5× Denhardt’s solution (1× Denhardt’s solution = 0.002% polyvinylpyrrolidone, 0.02% Ficoll 400, and 0.002% bovine serum albumin (Sigma Aldrich)), 2% SDS, 50% formamide, and 100 μg/ml sonicated salmon testis DNA (Sigma Aldrich). After hybridization, the membranes were washed twice at 65 °C, for 20 min each time, in a wash buffer composed of 0.1× SSC (pH 7.0) and 0.1% SDS and then autoradiographed. The optical density of each dot was quantified by using Quantity One.

**RNA Degradation Analysis and RNase Protection Assay**—The cells in 10-cm tissue culture dishes were grown until subconfluent, and then 10 μg/ml actinomycin D (Sigma Aldrich) was added to the cultures to arrest de novo RNA synthesis. After properly timed intervals, total cellular RNA for the RNA protection assay was extracted by the manufacturer’s protocol.

RNase protection assay was carried out with a commercial kit (RPA III Kit: Ambion, Austin, TX), according to the manufacturer’s protocol. Total RNA (3 μg) was hybridized with 1× 106 cpm of ccn2 or firefly luciferase probe in the presence of 1× 106 cpm of 18 S RNA probe as an internal control for 16 h at 42 °C. After hybridization, the RNA was digested by an RNase A/T1 mixture, precipitated with ethanol, and then re-purified by 1% PAGE in the presence of 7 M urea. The RNase EDTA (TBE, 90 mm Tris, 90 mm borate, and 2× EDTA) buffer. The gels were subsequently dried and autoradiographed. The optical density of each signal band of the autoradiography was quantified by using Quantity One.

**Luciferase Constructs**—The SV40 promoter-driven firefly luciferase reporter plasmid (pGGL3-control: Promega) was used to elucidate the effects of chicken ccn2 3'-UTR fragments, and a herpes simplex virus thymidine kinase (HSV-TK) gene promoter-driven Renilla luciferase expression plasmid (pRL-TK: Promega) was used as an internal control for transcription experiments to monitor the transcription efficiency. Plasmids pGL3L(+) and pGL3L(-) were described in a previous study (38); the luciferase-chicken ccn2 3'-UTR chimeric construct pGGL3-FULL(+) and another chimeric construct with a ccn2 3'-UTR deletion mutant, 3'-400(-) were also described previously (41). Furthermore, in the present study, four other deletion mutants of chicken ccn2 3'-UTR were constructed by using the same backbone. All of the mutants were obtained by utilizing PCR technology from clone A1/5 (41), and the primer sequences used to obtain the respective mutants were as follows: 5'-CGG TAA GGA TCG TGC CTA GTG-3' for the sense primer of 3'-200, 5'-TCT AGA CTA TGG TCT TTT TTT TTT-3' for the sense primer of 3'-100 and 3'-100/50, 5'-TCT AGA CCA AAA GTT ACA TGT TTG-3' for the sense primer of 3'-50, 5'-CCG AAT TAC GTT ATA AAT AAT AAT AAT AC-3' for the antisense primer of 3'-200, 3'-100, and 3'-50, and 5'-GAA TTC AAA CAT GTA ACT TGT GTT C-3' for the antisense primer of 3'-100/50. The sense and antisense primers containing full and an EcoRI site, respectively, were double-digested with XbaI and EcoRI, purified, and subcloned between the corresponding sites in pGL3L(+) or pGL3L(-).

Proper construction of all plasmids was confirmed by nucleotide sequencing and restriction enzymatic digestion analyses. Schematic representations of the molecular constructs are shown in Fig. 3A.

**DNA Sequencing and Computer Analysis**—The cDNA subcloned into each plasmid was sequenced by the dye-deoxy chain termination method.
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(49) with a Big Dye Terminator Cycle Sequencing Ready Reaction Kit version 2.0 (Applied Biosystems, Foster City, CA) and an ABI PRISM 310 Genetic Analyser (Applied Biosystems). DNA sequence alignment and RNA secondary structure predictions were computed by using commercial computer software, GENETYX-MAC version 10 (Software Development, Tokyo, Japan).

**DNA Transfection and Luciferase Assay**—Twenty-four hours prior to transfection, 2 × 10^5 cells were seeded into each of several 35-mm tissue culture dishes. Cationic liposome-mediated DNA transfection was carried out with 1 μg of each pGL derivative in combination with 0.5 μg of pRL-TK, according to the manufacturer’s protocol (Promega, Roche Applied Science). Forty-eight hours after the transfection, the cells were lysed in 500 μl of a passive lysis buffer (Promega), and the cell lysate was directly used for the luciferase assay.

The dual luciferase assay system (Promega) was applied for the sequential measurement of firefly (reporter) and Renilla luciferase (transfection efficiency standard) activities with specific substrates of beetle luciferin and coelenterazine, respectively. Quantification of both luciferase activities was done with a luminometer (TD20/20, Turner Designs, Sunnyvale, CA) and calculation of relative ratios was carried out manually.

**Analysis of RNA Folding in Vitro**—For preparation of template plasmids for *in vitro* transcription of cDNA corresponding to ccn2 3'-UTR, pGL3 derivatives containing the cDNAs were double-digested by XbaI and EcoRI, purified, and subcloned between the corresponding sites in pGL derivatives. The transcripts were made by T7 bacteriophage polymerase in the presence of 50 μCi of [α-32P]CTP (3000 Ci/mmol). For synthesis of antisense RNA, the plasmid was linearized also by XbaI and transcribed by T7 bacteriophage polymerase in the presence of 50 μCi of [α-32P]CTP. After spin-column purification, the radiolabeled RNAs were analyzed by 6% PAGE in the presence of 7 M urea in 1× TBE buffer.

The RNA *in vitro* folding assay was carried out as described previously (40), with a slight modification. 50,000 cpm of radiolabeled RNA was heated at 95 °C for 10 min, then gradually cooled to room temperature in RNA folding buffer consisting of 10 mM HEPES (pH 7.9), 40 mM KCl, 3 mM MgCl$_2$, 1 mM dithiothreitol, 0.5 mg/ml yeast tRNA (Roche Applied Science), and 0.5 mg/ml bovine serum albumin. After having been cooled further to 4 °C, the RNAs were subjected to 6% native PAGE analysis in 0.5× TBE. Subsequently, the analytical gels were dried and autoradiographed.

**Results**

**Synthesis and Degradation Patterns of ccn2 mRNA Are Dependent Not Only on the Cell Type, but Also on the Differentiation Stage of Chondrocytes**—Previous studies of ours (22, 41) revealed that ccn2 mRNA is strongly expressed in chondrocytes, particularly in hypertrophic chondrocytes. Therefore, we were interested in whether de novo synthesis and/or degradation patterns of ccn2 mRNA were different among different types of cells or not. Northern blot analysis (Fig. 1A) revealed that ccn2 mRNA was expressed faintly in CEF cells, in which only α2(1) collagen mRNA (a marker of fibroblasts and fibroblast-like cells) was expressed. In LS cells, in which α1(II) collagen (a marker of mature chondrocytes) was moderately expressed, ccn2 mRNA was more strongly expressed than in CEF cells (1.5-fold). Moreover, in US cells, in which not only the α1(II) collagen but also expression of α1(X) collagen (a marker of hypertrophic chondrocytes) was observed, the expression level of ccn2 mRNA was remarkably higher than that in CEF cells (2.5-fold) or in LS cells (1.6-fold). These results indicate that *de novo* synthesis and/or degradation of ccn2 mRNA was different among these cells; hence, we carried out a nuclear run-on assay and RNA degradation analysis to investigate the mechanism. The results of the nuclear run-on assay (Fig. 1B) revealed that the transcriptional activity producing ccn2 mRNA was the strongest in US cells and was moderate in LS cells, the findings of which are consistent with the results of Northern blot analysis. The difference in the relative transcriptional activity between CEF and LS cells was likely to be the same as that in the relative amounts of their steady-state mRNA (right panels of Fig. 1, A and B). However, the transcriptional activity in US cells (right panel of Fig. 1B) was at most 2-fold (versus CEF cells) and 1.4-fold (versus LS cells) higher, indicating that the increased transcriptional activity producing ccn2 mRNA did fully support the increased amounts of steady-state mRNA.

**Differential Response of ccn2 mRNA Stability to Growth Factors during Differentiation of Chondrocytes**—It is reported that Western Blot Analysis—Extracted proteins were heated at 95 °C for 5 min in SDS sample buffer in the presence of 5% 2-mercaptoethanol, separated by 12.5% SDS-PAGE, and transferred to a polyvinylidene difluoride membrane (Hybond P, Amersham Biosciences). The membrane was then blocked with 5% skim milk in Tris-buffered saline for 16 h at 4 °C. After having been blocked, the membrane was next incubated for 1 h at 37 °C with a 1/2,000 dilution of monoclonal anti-ß-tubulin antibody (Sigma Aldrich) or a 1/1,000 dilution of monoclonal anti-lamin B1 antibody (Zymed Laboratories Inc., South San Francisco, CA) in Tris-buffered saline containing 0.05% Tween 20; thereafter, it was incubated with a 1/20,000 dilution of a peroxidase-conjugated goat anti-mouse IgG antibody (American Qualex, La Mirada, CA) in Tris-buffered saline containing 0.05% Tween 20 for 1 h at 37 °C. Subsequently, the blot was visualized by using an ECL Western blotting Analysis System (Amersham Biosciences) with chemiluminescence detection.

**Differential Response of ccn2 mRNA Stability to Growth Factors during Differentiation of Chondrocytes**—It is reported that...
several growth factors stimulate the expression of *ccn2* mRNA in rabbit growth plate chondrocytes (22). Therefore, next we analyzed whether the effects of the growth factors depended on the regulation of mRNA stability and *de novo* synthesis of mRNA or not. LS and US cells were stimulated with BMP2 (200 ng/ml), PDGF (10 ng/ml), or CCN2 (30 ng/ml) for 24 h in the presence of 10% FBS, and then the cells were subjected to Northern blot, nuclear run-on, and RNA degradation assays. In the presence of 10% FBS, 24-h stimulation by the growth factors significantly decreased mRNA stability. Most surprisingly, stimulation by the growth factors significantly decreased *ccn2* mRNA stability in US cells, an effect contrary to that on the LS cells. Of note, both the effect of increasing the stability in LS cells and that of decreasing it in US cells were observed when the stimulator was CCN2 itself. Taken together, these results suggest that the post-transcriptional regulation may play important roles in the precise control of the expression of *ccn2* mRNA by altering the stability of the mRNA.

**Locating the Minimal *cis*-acting Repressive Element in 3′-UTR of Chicken *ccn2* mRNA**—It is widely known that 3′-UTRs of many genes possess signal sequences within them that determine mRNA stability (53). Previous studies of ours revealed that there exists a strong *cis*-acting repressive element in the 3′-UTR of human (38–40) and murine (39) *ccn2* mRNAs. Furthermore, we recently obtained results (41) showing that the 400-base fragment at the 3′-end of 3′-UTR of chicken *ccn2*...
mRNA also contains a cis-acting repressive element. However, the precise location of the minimal element remained to be clarified. Therefore, we sought to identify this cis-element by employing a transient expression and evaluation system using firefly luciferase fusion constructs, as we had done in previous studies (38–41).

As denoted in Fig. 3A, six chimeric firefly luciferase genes were designed and constructed. These plasmids and parental pGL3L plasmids were used for transfection, with Renilla luciferase co-expression as an internal control, and pGEM3Zf(+) as a negative control are shown. A representative from three individual experiments is shown in the left panel; the relative values of ccn2 transcripts normalized by β-actin, and standardized against the one for the control (−) without growth factor, along with error bars indicating S.D., are displayed in the right panel. B, RNA degradation analysis. LS and US cells were stimulated with BMP2 (200 ng/ml), PDGF (10 ng/ml), or CCN2 (30 ng/ml) in the presence of 10% FBS. After 24 h, 10 μg/ml actinomycin D (AcD) was added. Then, after 0, 0.5, 1, and 2 h, total RNAs were isolated and subjected to the RNase protection assay for ccn2 mRNA and 18S rRNA (18S).

These results represent of two individual experiments are shown in the left panel, and the computed half-lives (t1/2) of ccn2 mRNA normalized by those of 18S are displayed in the right panel.

FIG. 2. Effects of growth factors on the synthesis and degradation of ccn2 mRNA in LS and US cells. A, nuclear run-on assay. LS and US cells were stimulated with BMP2 (200 ng/ml), PDGF (10 ng/ml), or CCN2 (30 ng/ml) for 24 h in the presence of 10% FBS. Then, nuclear fractions were isolated and subjected to the nuclear run-on assay in the presence of [α-32P]UTP. Autoradiograms of dot blot hybridization of ccn2, β-actin as an internal control, and pGEM3Zf(+) as a negative control are shown. A representative from three individual experiments is shown in the left panel; the relative values of ccn2 transcripts normalized by β-actin, and standardized against the one for the control (−) without growth factor, along with error bars indicating S.D., are displayed in the right panel. B, RNA degradation analysis. LS and US cells were stimulated with BMP2 (200 ng/ml), PDGF (10 ng/ml), or CCN2 (30 ng/ml) in the presence of 10% FBS. After 24 h, 10 μg/ml actinomycin D (AcD) was added. Then, after 0, 0.5, 1, and 2 h, total RNAs were isolated and subjected to the RNase protection assay for ccn2 mRNA and 18S rRNA (18S). The results represent of two individual experiments are shown in the left panel, and the computed half-lives (t1/2) of ccn2 mRNA normalized by those of 18S are displayed in the right panel.
mRNA contains one or more RNA elements that destabilize the mRNA in cis, probably within the area between 50 and 100 bases from the polyadenyl tail.

Secondary Structure Formation of RNA Fragments of the 3'-UTR of Chicken ccn2 mRNA—It is also recognized that the cis-acting sequences in various mRNAs are able to form compact secondary structures (40). In line with this general finding, the fragments of 3'-UTR of chicken ccn2 mRNA, which conferred cis-repressive effects on reporter gene expression, were very rich in adenosine-uridine (AU-rich, Fig. 5), and computer analysis predicted that the RNAs of 3'-100 and 3'-100/50 could form stable secondary structures (data not shown). Thus, an RNA in vitro folding assay was carried out. In vitro transcription of those template cDNAs in the presence of [α-32P]CTP provided high quality corresponding RNAs that gave single bands with the expected electrophoretic mobility in 6% urea-denatured PAGE gels (Fig. 6A). Thereafter, the RNAs were forwarded to extensive in vitro folding assay (Fig. 6B). After heat denaturation and re-naturation by gradual cooling in the folding buffer, structured forms of 3'-100(+) and 3'-100(−) RNA fragments were observed.

![Diagram of mRNA and RNA Fragments](image-url)
of 3'-100/50 fragments in binding to nuclear and cytoplasmic proteins. These results indicate that the minimal element in the 3'-UTR of chicken ccn2 mRNA for binding to the putative 40 kDa-trans-factor is present within the 3'-100/50 portion.

Profile of Binding of cis-Element in 3'-UTR of ccn2 mRNA to the Putative trans-Factor during Differentiation of Chondrocytes—The results in previous subsections showed that, depending on the differentiation stage of chondrocytes, the putative chicken ccn2 mRNA undergoes post-transcriptional regulation through its mRNA stability and suggested the involvement of a repressive regulatory system enabled by the cis-element and putative trans-factor. These findings prompted us to examine whether the binding of the cis-element to the putative trans-factor protein is dependent on the differentiation stage of chondrocytes or not. Therefore, utilizing the nuclear and cytoplasmic extracts of CEF, LS, and US cells, we conducted extensive REMSA and UV cross-linking analysis. As shown in Fig. 8A, a gel-shift was observed for radiolabeled 3'-100 RNA (left panel) or 3'-100/50 RNA (right panel) incubated with nuclear or cytoplasmic protein. Overall, it is clear that the signals indicating RNA-protein interaction were much stronger with proteins from LS cells than with those from US cells. Interestingly, incubating the probe with the cytoplasmic protein of LS cells gave the most prominent shifted bands. More interesting, the results of the gel-shift analysis revealed quite opposite patterns of the subcellular distribution of the binding protein between US and LS cells. The UV cross-linking assay (Fig. 8B) showed the same results as those of REMSA. By incubating the probe with the cytoplasmic protein of LS cells, a single band corresponding to the binding protein of putative 40-kDa trans-factor was observed most prominently. Incubation with the nuclear protein of LS cells also yielded a band of the same density. In contrast, in US cells, no signal was detected with the cytoplasmic protein, whereas the binding signal was still distinct with the nuclear protein. The results of Western blotting analysis (Fig. 8C) for lamin B1 (a nuclear protein) and a-tubulin (a cytoplasmic protein) clarified the quality and quantity of each protein fraction. As such, no remarkable differences were observed among the cells tested. Therefore, we suggest that the amount and/or the binding affinity of the putative trans-factor protein interacting with the cis-element of the 3'-UTR of ccn2 mRNA might be regulated differentially among cell types, particularly among differentiation stages of chondrocytes. Furthermore, we propose that regulation of binding between the cis-element and the putative trans-factor might play an important role in the differentiation and maturation of chondrocytes by regulating the stability of the ccn2 mRNA.

Profile of binding of cis-Element of ccn2 mRNA to Putative 40-kDa trans-Factor Is Regulated by Growth Factors during Maturation of Chondrocytes—Finally, we investigated whether or not the growth factors that were shown to affect the stability of ccn2 mRNA, as described in a previous subsection, would also affect the binding between the cis-element and putative trans-factor. So, we carried out a UV cross-linking assay (Fig. 9A), utilizing the nuclear and cytoplasmic fraction of LS and US cells stimulated with BMP2 (200 ng/ml), PDGF (10 ng/ml), or CCN2 (30 ng/ml) for 24 h in the presence of 10% FBS. In LS cells (left panel), these growth factors decreased the binding of the probe to the putative 40-kDa nuclear trans-factor. Furthermore, all of the growth factors abolished the binding of the probe to the cytoplasmic protein almost completely. On the contrary, in US cells (right panel), all of the growth factors increased the binding of the probe to the 40-kDa nuclear puta-
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In the present study, we demonstrated that chicken ccn2 mRNA was expressed moderately in LS cells, in which the phenotypes of proliferating chondrocytes were expressed, and prominently in US cells, in which the phenotypes of prehypertrophic-hypertrophic chondrocytes were expressed (Fig. 1A). The results of the nuclear run-on assay (Fig. 1B) revealed that the transcriptional activity of the ccn2 gene was almost correlated with the mRNA level. Indeed, the stability of mRNA (Fig. 1C) of LS cells was the same as that of CEF cells \( t_{1/2} = 1.0 \) h. However, importantly, in US cells, the mRNA was the most stable \( t_{1/2} = 1.5 \) h, suggesting post-transcriptional regulation of mRNA stability in the ccn2 gene regulatory system. In cultured rabbit growth plate chondrocytes, ccn2 mRNA expression was also observed at a peak level at the hypertrophic stage (22). Furthermore, in situ hybridization revealed that its expression was predominant in hypertrophic chondrocytes of the mouse growth plate (22); and recombinant CCN2 protein stimulated the proliferation, proteoglycan synthesis, expression of type II and type X collagen genes, and alkaline phosphatase activity of rabbit growth plate chondrocytes (22, 34, 54). Therefore, these studies together suggest that regulation of the transcription activity producing ccn2 mRNA and that of its stability may control the proper growth and differentiation of chondrocytes during endochondral ossification.

It has been reported that ccn2 mRNA was induced in various cells by various growth factors, such as BMP 2 (22), PDGF (28), and CCN2 itself (22). These growth factors have also been reported to play an important role in growth and differentiation of chondrocytes (8–11). Nonetheless, in the present study, the results of the nuclear run-on assay (Fig. 2A) demonstrated that 24-h stimulation with BMP2, PDGF, or CCN2 had little effect on the de novo synthesis of ccn2 mRNA in chicken chondrocytes. This is partly because the cells were exposed to the growth factors for only a short time (24 h) and 10% FBS was present in the medium. In the presence of FBS, which contains a variety of factors, some stimulatory and some inhibitory toward growth and differentiation, the response of de novo synthesis of ccn2 mRNA to the growth factors might have been masked. For drastic enhancement of de novo synthesis of ccn2 mRNA, continuous stimulation for a long period of time might be required as well.

**FIG. 5. Nucleotide sequences and orientation of radiolabeled transcripts.** Four \(^{32}P\)-labeled ccn2 RNAs were prepared for in vitro folding analysis, REMSA, and UV-cross linking assay. The 3'-100, 3'-50, and 3'100/50 cDNA fragments of ccn2 3'-UTR shown in Fig. 3A were double-digested with EcoRI and XbaI and subcloned between the corresponding sites of pGEMZf(+) ). These plasmids were linearized by digestion with EcoRI or XbaI, and in vitro transcription by bacteriophage T7 or Sp6 RNA polymerase was carried out in the presence of \(^{32}P\)(CTP. The three transcripts designated 3'-100(+), 3'-50(+), and 3'-100/50(+) were sense transcripts of the 3'-UTR with the sequence denoted at the top of the panel. In contrast, the transcript named 3'-100(−) possessed an antisense strand sequence of the corresponding region (see "Experimental Procedure"). The AUUUA and AUUUA-like sequences, which are regarded as destabilizing mRNA cis-elements, are indicated by underlined boldface type.

**FIG. 6. Preparation and in vitro folding of radiolabeled RNA fragments of ccn2 3'-UTR.** A, analysis of the synthesized RNAs in urea-denatured 6% PAGE. Four \(^{32}P\)-labeled RNAs were prepared. RNA size markers (Century Markers; Ambion) are shown at the left side of the panel. B, in vitro folding assay of the radiolabeled RNAs. The RNAs were heated at 95 °C and gradually cooled to room temperature to allow them to form a secondary structure. Then, the RNAs were analyzed by electrophoresis through 6% native PAGE gels. Arrowheads indicate secondary-structured RNA.

tive trans-factor protein. However, the growth factors had no effect on binding to the cytoplasmic protein, and thus no band was observed. Western blotting analysis (Fig. 9B) of fraction markers confirmed the quality and quantity of each protein fraction. Taken together, the observed differential effect of the growth factors on the binding profile between LS and US cells suggest that the regulatory system through binding of the cis-element of the 3'-UTR of ccn2 mRNA to the 40-kDa putative trans-factor protein might regulate the expression of ccn2 mRNA during differentiation of chondrocytes. In particular, this regulatory system might play an important role in chondrocyte differentiation through feedback regulation of the mRNA expression by autocrine CCN2 protein.

**DISCUSSION**

In the present study, we demonstrated that chicken ccn2 mRNA was expressed moderately in LS cells, in which the phenotypes of proliferating chondrocytes were expressed, and prominently in US cells, in which the phenotypes of prehypertrophic-hypertrophic chondrocytes were expressed (Fig. 1A). The results of the nuclear run-on assay (Fig. 1B) revealed that the transcriptional activity of the ccn2 gene was almost correlated with the mRNA level. Indeed, the stability of mRNA (Fig. 1C) of LS cells was the same as that of CEF cells \( t_{1/2} = 1.0 \) h. However, importantly, in US cells, the mRNA was the most stable \( t_{1/2} = 1.5 \) h, suggesting post-transcriptional regulation of mRNA stability in the ccn2 gene regulatory system. In cultured rabbit growth plate chondrocytes, ccn2 mRNA expression was also observed at a peak level at the hypertrophic stage (22). Furthermore, in situ hybridization revealed that its expression was predominant in hypertrophic chondrocytes of the mouse growth plate (22); and recombinant CCN2 protein stimulated the proliferation, proteoglycan synthesis, expression of type II and type X collagen genes, and alkaline phosphatase activity of rabbit growth plate chondrocytes (22, 34, 54). Therefore, these studies together suggest that regulation of the transcription activity producing ccn2 mRNA and that of its stability may control the proper growth and differentiation of chondrocytes during endochondral ossification.

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FIG. 7. Profile of binding of the nuclear or cytoplasmic protein(s) of CEF cells to the ccn2 3'-UTR RNA. A, REMSA of the RNA fragments of ccn2 3'-UTR. Three radiolabeled and folded RNA probes (3'-100, 3'-50, and 3'-100/50) corresponding to the ones shown in Fig. 5 were incubated with or without (→) 10 μg of nuclear extract (N) or cytoplasmic extract (C) of CEF cells. After RNase digestion, the mixtures were analyzed by electrophoresis through 6% native PAGE gels. The bottom of the gel, RNase-digested free probes are observed. B, UV cross-linking assay of the RNA fragments of ccn2 3'-UTR. The radiolabeled and folded RNA probes were incubated with the extract, and digestion with RNase was performed as for REMSA. Then, the mixtures were irradiated by UV on ice and analyzed by SDS-PAGE in a 4–20% gradient gel. The bottom of the gel, RNase-digested free probes are observed. C, competition analysis of the REMSA. Twenty nanograms of unlabeled RNAs was preincubated with the extracts. Then, radiolabeled probes were added, and REMSA was carried out as described above. D, competition analysis of the UV cross-linking assay. Twenty nanograms of unlabeled RNAs was preincubated with the extracts. Then, radiolabeled probes were added, and UV cross-linking assay was carried out as described above except that the SDS-PAGE was carried out in a 12.5% gel. Positions of molecular standards are shown at the left side of the panel. RNase-digested free probes are seen at the bottom of the gels.
On the other hand, RNA degradation analysis (Fig. 2C) revealed that the growth factors did affect the stability of ccn2 mRNA. Of note, the response of the stability of ccn2 mRNA to the growth factors was different between LS and US cells. Namely, by stimulation with the growth factors, the stability of mRNA was increased in LS cells and decreased in US cells, showing the exact opposite effects between two different stages (proliferating stage versus prehypertrophic-hypertrophic stage) of chondrocytes. Therefore, we were led to hypothesize that the gene expression of ccn2 might be regulated by a bi-directional feedback loop, changing from positive to negative during chondrocyte differentiation. In this regard, although gene expression of ccn2 in vivo has been shown to be the highest in hypertrophic chondrocytes (3, 22), detailed observation revealed that the ccn2 expression during the differentiation of growth cartilage cells was the highest in early hypertrophic (non-calcified hypertrophic) chondrocytes and then decreased in chondrocytes in the calcified zone (22). In addition, an in vitro culture system of rabbit growth cartilage cells showed that the up-regulated expression of ccn2 in hypertrophic chondrocytes decreased as the cells differentiated during the calcifying stage (22). Therefore, it is feasible that growth factors that promote the terminal differentiation (calcification followed by apoptosis) of chondrocytes, such as BMP-2 (55) and CCN2 (3, 54), would down-regulate the expression level of ccn2 in the US cells, which mainly consist of prehypertrophic and hypertrophic chondrocytes. So far, there has been no report on the effect of PDGF on the terminal differentiation of growth plate chondrocytes, but it would be interesting to investigate the effect of this factor on hypertrophic chondrocytes, because they have been reported to be stainable with anti-PDGF antibodies (56). The physiological
significance of the switch from positive to negative regulation is unknown. However, because forced overexpression of ccn2 has been shown to induce apoptosis of cells (57) and because the highest basal expression level was observed in the US cells (Fig. 1A), further stimulation by such factors may activate the negative feed-back loop of mRNA degradation to avoid overproduction of CCN2, which might cause early apoptosis before calcification.

It is widely known that mRNAs of a number of genes contain cis-elements in their 3'-UTRs to regulate their protein expression, through export from nuclei to ribosomes (58) and stability (59). Of note, both mammalian and avian ccn2 mRNAs possess 1.1-kbp-long 3'-UTRs, which contain strong cis-acting elements that repress on gene expression (38–41). Therefore, we sought to define the minimum cis-element in the 3'-UTR of chicken ccn2 mRNA by employing a transient expression and evaluation system with firefly luciferase fusion constructs. Thereby, we demonstrated that the element was located 100 bp upstream from the 3’-end of the mRNA and that the effect was orientation-dependent (Fig. 3). Importantly, the repressive effect of each potent fragment appeared weaker in CEF, moderate in LS, and more prominent in US cells. These findings suggest that the discrepancy in mRNA stability (Fig. 1C) between these cells might be the outcome of the negative regulation by the mRNA portion. Furthermore, RNA degradation analysis (Fig. 4) directly revealed that these fragments accelerated degradation of the reporter gene in cis and that the minimal fragment for repression of gene expression was required for the rapid RNA degradation. These results suggest that one or more cis-elements in the 3’-UTR of chicken ccn2 mRNA play an important role in the post-transcriptional regulation of ccn2 gene through RNA degradation.

Indeed, nucleotide sequence analysis of this portion (Fig. 5) revealed that this region was highly AU-rich and contained AUUUA and AUUUA-like sequences, which have been regarded as mRNA-destabilizing cis-elements interacting with trans-factors in mammalian species (59–62). Therefore, we regard the fragment “3’-100/50” as a major negative regulatory cis-element in the 3’-UTR of chicken ccn2 mRNA and consider it to be related to the destabilization of the mRNA.

Previous studies in other laboratories (60, 63, 64) showed that such AU-rich RNA sequences are capable of forming a secondary structure and that a unique stem-loop or bulge of 3’-UTR is characteristic of the trans-factor binding site for post-transcriptional regulation. Furthermore, AU-rich elements in 3’-UTR of several proto-oncogenes, such as c-myc (59), hormones, such as parathyroid hormone (62), and cytokines, such as granulocyte-macrophage colony stimulation factor (65), have been recognized as signal targets for rapid degradation, which contributes, in part, to the rapid turnover of their mRNAs. As to chicken ccn2 mRNA, computer analysis predicted that the corresponding RNA portions of 3’-100(+) and 3’-100/50(+) were capable of forming stable secondary structures. RNA in vitro folding analysis (Fig. 6B) actually revealed that these RNA fragments formed secondary structures; however, no folded form was observed in the case of the 3’-50(+) RNA fragment. These results, taken together with those of the reporter gene assay, thus suggest that the stable self-folding of the portion “3’-100/50” may contain the regulatory center that interacts with a putative trans-factor, playing the role of a negative regulator of gene expression, probably acting as an RNA destabilizer in cis.

Next, we investigated the putative trans-factor(s) that bound to the cis-element of the 3’-UTR of chicken ccn2 mRNA. REMSA (Fig. 7A) and UV cross-linking assay (Fig. 7B) revealed that 3’-100/50 fragment as well as 3’-100 fragment bound 40-kDa nuclear and cytoplasmic proteins of CEF cells. The competitor analysis of each experiment (Fig. 7, C and D) confirmed that the binding was probe-specific, suggesting the protein to be a putative trans-factor with the capacity to bind to the cis-element in the 3’-UTR of ccn2 mRNA. Hence, the 40-kDa protein that bound to the 3’-100 fragment is likely to be identical to the one that bound to the 3’-100/50 fragment.

Further REMSA (Fig. 8A) and UV cross-linking assays (Fig. 8B) revealed the distinct patterns of binding of the putative trans-factor to the radiolabeled probes between LS and US cells. Namely, the binding profile of the nuclear and cytoplasmic proteins of LS cells was opposite to that of US cells. In particular, unlike in the case of CEF and LS cells, only very faint binding was detected by incubation with the cytoplasmic protein of US cells. Considering the fact that ccn2 mRNA was more stable in US cells than in CEF and LS cells (Fig. 1C), the 40-kDa putative trans-factor may destabilize ccn2 mRNA by interacting with cis-element in the 3’-UTR. Indeed, the prominent binding between the cis-element and the factor in LS cells might induce rapid degradation of ccn2 mRNA, and the decreased binding in US cells might contribute to the strong expression of ccn2 mRNA in them. It is also of note that the binding signal for the cytoplasmic putative trans-factor disapp-
peared upon stimulation by the growth factors that extended the half-life of ccn2 mRNA in US cells (Fig. 9). These findings indicate that this regulated mRNA degradation is carried out mostly in the cytoplasm.

A number of laboratories, including ours, have reported important physiological or pathological roles of CCN2, such as promotion of cell growth and differentiation in development (22, 34), angiogenesis (32), and wound healing (28). In addition, promotion of cell growth and differentiation in development is currently in progress.

Transcriptional regulation system of chicken ccn2 among chondrocytes

Most of the strict regulation of CCN2 gene expression during endochondral ossification. Therefore, proper expression of ccn2 during the course of differentiation of chondrocytes in development should be regulated at a variety of stages, such as transcriptional, post-transcriptional, and translational. Indeed, previous studies of ours revealed that detectable expression of ccn2 mRNA was three-dimensionally highly restricted (22) and that the 1.1-kbp-long 3′-UTR contained cis-element that repressed gene expression (38–41). However, the precise role of the cis-element during differentiation of chondrocytes had remained unclear. In the present study, we first showed that the expression of chicken ccn2 mRNA was regulated not only at the transcriptional level, but also at the post-transcriptional level, and that the post-transcriptional regulation was based on the modulation of mRNA stability by the interaction between a cis-element in the 3′-UTR and a putative 40-kDa trans-factor in the nucleus and cytoplasm. Moreover, we also demonstrated that this interaction was differentially regulated through the differentiation stages of chondrocytes. It is of particular interest to us to pursue the minimal cis-element in the 3′-UTR and to identify the putative trans-factor, to define better the post-transcriptional regulation system of ccn2. Further investigation is currently in progress.

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