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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 (pre)hypertrophic chondrocytes. Stimulation by the growth factors repressed the binding in proliferating chondrocytes; however, it enhanced it in (pre)hypertrophic 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 endochondoral ossification and intramembane 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. At 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_3 (2–4), and growth factors, such as fibroblast growth factor-2 (5), transforming growth factor-β (TGF-β[1] (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 *ccn1* (*cef-10/cyr61* (17, 18)), *ccn2* (*ctgf/hcs24/fisp12* (19–22)), *ccn3* (*nov* (23)), and several related genes such as *ccn4* (*elm-1/wisp-1* (24, 25)), *ccn5* (*ctgf-3/wisp-2/cop1* (25, 26), and *ccn6* (*wisp-3* (25)). CCN2 was initially isolated from angioendothelial cells as a growth factor related to PDGF and was shown to have PDGF-like mitogenic and chemotactic activities for fibroblasts (19, 27–30). Nowadays, however, CCN2 is no longer regarded as an accessory factor of PDGF but has been shown to be a multifunctional growth factor that regulates the growth and/or differentiation of various cells, including vascular endothelial cells (19, 31–33). Furthermore, we have reported that CCN2 stimulates both the growth and differentiation of chondrocytes and osteoblasts (22, 34, 35), indicating crucial roles of CCN2 in endochondral ossification.

These findings pointing to multiple physiological and/or pathological roles of CCN2 indicate that the gene expression of *ccn2* is regulated at multiple steps, such as transcriptional, post-transcriptional, and translational. However, as to the molecular regulatory mechanisms governing *ccn2* gene expression, although a few studies have been reported, most of these mechanisms still remain to be investigated. For instance, 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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[1] 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 minimum essential medium supplemented with 10% fetal bovine serum (FBS) in humidified air containing 5% CO_2 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 18-day-old chicken embryos and maintained in high-glucose Dulbecco's modified Eagle minimum essential medium supplemented with 10% FBS in humidified air containing 5% CO_2 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 α2(I), α1(II), and α1(X) collagen genes, β-actin gene, and 18 S rRNA gene were obtained by reverse transcription-mediated PCR, utilizing total cellular RNA of CEF cells (for α2(I) collagen gene) or US cells (for the other genes) as a template. The nucleotide sequences of the sense primer and antisense primer for α2(I) collagen (42) were 5′-TTA CTC CTC GCG ACT GTA TGC-3′ and 5′-GCT CAC CAG GAA CAC CTT GAA-3′, respectively. For α1(II) collagen (43), the sense and antisense primer sequences were 5′-GCA GAG ACC ATC AAC GGC GGT-3′ and 5′-CAG GCG CGA GGT CTT CTG CGA-3′, respectively; and those for α1(X) collagen (44), 5′-AAG GGG CCA CAC TTT CTA-3′ and 5′-TTC TCC AGG CTT CCC TAT CCC-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 CTA GAC TGT GGG GGA CTG-3′, respectively; those for chicken 18 S rRNA (47) were 5′-GAC TCC GGT TCT 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*, α2(I), α1(II), α1(X) collagen, and 18 S rRNA genes were utilized for the riboprobe preparation. The plasmids were linearlized 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 μCi of [α-^{32}P]CTP (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 RQ1 DNase (Amersham Biosciences) 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.

RNA Preparation and Northern Blot Analysis—Total cellular RNA was isolated by use of Isogen (Nippon Gene, Tokyo, Japan), according to the manufacturer's protocol.

Northern blot analysis was carried out as described previously (41). Total RNA was denatured by glyoxysal, 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 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), 40% glycerol, 5 mM $MgCl_2$, 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 2.5 mM), and 100 μCi of [α-^{32}P]UTP (3000 Ci/mmol, Amersham Biosciences) in a reaction buffer consisting of 10 mM Tris-HCl (pH 8.0), 5 mM $MgCl_2$, and 300 mM KCl. After digestion with RQ1 DNase 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 pGEM3Zf(+) (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 transcripts was hybridized to the blotted membranes at 42 °C for 72 h in 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% polyvinylpyrolidone, 0.02% Ficoll 400, and 0.02% 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 was isolated and used for the RNase protection assay.

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 1×10^4 cpm of *ccn2* or firefly luciferase probe in the presence of 1×10^3 cpm of 18 S rRNA 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 subjected to 6% PAGE in the presence of 7 M urea in 1× Tris borate-EDTA (TBE; 90 mM Tris, 90 mM borate, and 2 mM 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 (pGL3-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 transfection experiments to monitor the transfection efficiency. Plasmids pGL3L(+) and pGL3L(−) were described in a previous study (38); the luciferase-chicken *ccn2* 3′-UTR chimeric construct pGL3-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 follow: 5′-TCT AGA TGC CTG TAT CCT TGC AAA G-3′ for the sense primer of 3′-200, 5′-TCT AGA CTA TGG CTC 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′-CGG AAT TCC GAT ATA AAA AAT ATA AAT AC-3′ for the antisense primer of 3′-200, 3′-100, and 3′-50, and 5′-GAA TTC AAA CAT GTA ACT TTT GGT C-3′ for the antisense primer of 3′100/50. The sense and antisense primers contained an XbaI and an EcoRI site, respectively. The amplicons 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. 3*A*.

DNA Sequencing and Computer Analysis—The cDNA subcloned into each plasmid was sequenced by the dideoxy chain termination method

(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 optimized methodology (FuGENE6, 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 pGEM3Zf(+). *In vitro* transcription was carried out, as described in another subsection. For synthesis of each sense RNA, the plasmid was linealized by EcoRI and transcribed by Sp6 bacterial polymerase in the presence of 50 μCi of [α-^{32}P]CTP (3000 Ci/mmol). For synthesis of antisense RNA, the plasmid was linealized also by XbaI and transcribed by T7 bacteriophage polymerase in the presence of 50 μCi of [α-^{32}P]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.

RNA molecular size standards were produced via *in vitro* transcription of a mixture of DNA templates (Century Marker, Ambion) in the presence of [α-^{32}P]CTP. The radiolabeled RNA transcripts were subjected to spin-column purification, and 1.5×10^4 cpm was used as an RNA molecular standard in each experiment.

Preparation of Nuclear and Cytoplasmic Extraction—The nuclear and cytoplasmic extracts were prepared by using a CelLytic NuCLEAR Extraction Kit (Sigma Aldrich), according to the manufacturer's protocol. The protein concentrations of both fractions were determined with a BCA protein assay kit (Pierce), utilizing bovine serum albumin as a standard (50).

RNA Electromobility Shift Assay (REMSA)—REMSA was carried out according to the method described previously (51, 52), with a slight modification. The nuclear or the cytoplasmic extract containing 0–10 μg of protein was incubated at 25 °C for 30 min with 5×10^4 cpm of radiolabeled RNA in 19 μl of binding buffer (5 mM HEPES (pH 7.9), 7.5 mM KCl, 0.5 mM $MgCl_2$, 0.1 mM EDTA, 0.5 mM dithiothreitol, 0.1 mg/ml yeast tRNA, and 0.1 mg/ml bovine serum albumin). Then, the binding mixture was incubated with 1 μl of 1/100 diluted RNase mixture (0.5 unit/μl RNase A and 20 units/μl RNase T1, Ambion) for a further 10 min at 37 °C. The RNA-protein complex was subjected to 6% native PAGE in 0.5× TBE buffer. The gels were subsequently dried and autoradiographed.

For competition experiments, the extracts were preincubated with 20 ng of unlabeled competitor RNA for 30 min at 25 °C, followed by incubation with the radiolabeled RNA for another 30 min at 25 °C.

UV Cross-linking Assay—RNA-protein binding reactions were carried out as described in the subsection on REMSA. After RNase digestion, the protein-RNA complexes were put on ice and UV-irritated for 10 min by a UV cross-linker (Amersham Biosciences). Then, the samples were heated at 95 °C for 5 min in an SDS sample buffer (Sigma Aldrich) in the presence of 5% 2-mercaptoethanol and separated by 12.5% or 4–20% gradient SDS-PAGE. The gels were subsequently dried and autoradiographed.

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-α-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.

Statistical Analysis—Unless otherwise specified, all experiments were repeated at least twice, and similar results were obtained in the repeated experiments. Statistical analysis was carried out by Student's *t* test if necessary. Data are expressed as the mean ± S.D. $p < 0.05$ was considered significant.

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. 1*A*) revealed that *ccn2* mRNA was expressed faintly in CEF cells, in which only α2(I) 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 strongest expression of 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. 1*B*) 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. 1*B*) 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 not fully support the increased amounts of steady-state mRNA. The results of RNA degradation analysis (Fig. 1*C*) accounted for this discrepancy. The stability of *ccn2* mRNA in the LS cells was almost the same as that in CEF cells ($t_{1/2}$ = 1.0 h). However, in US cells, the *ccn2* mRNA was more stable ($t_{1/2}$ = 1.5 h) than in CEF or LS cells. These results suggest that *ccn2* mRNA should be regulated not only at the transcriptional stage, but also at the post-transcriptional stage, particularly by the regulation of the stability of mRNA in chondrocytes. As such, post-transcriptional regulation of *ccn2* may play an important role in the regulation of CCN2 protein production during maturation and differentiation of chondrocytes.

Differential Response of ccn2 mRNA Stability to Growth Factors during Differentiation of Chondrocytes—It is reported that

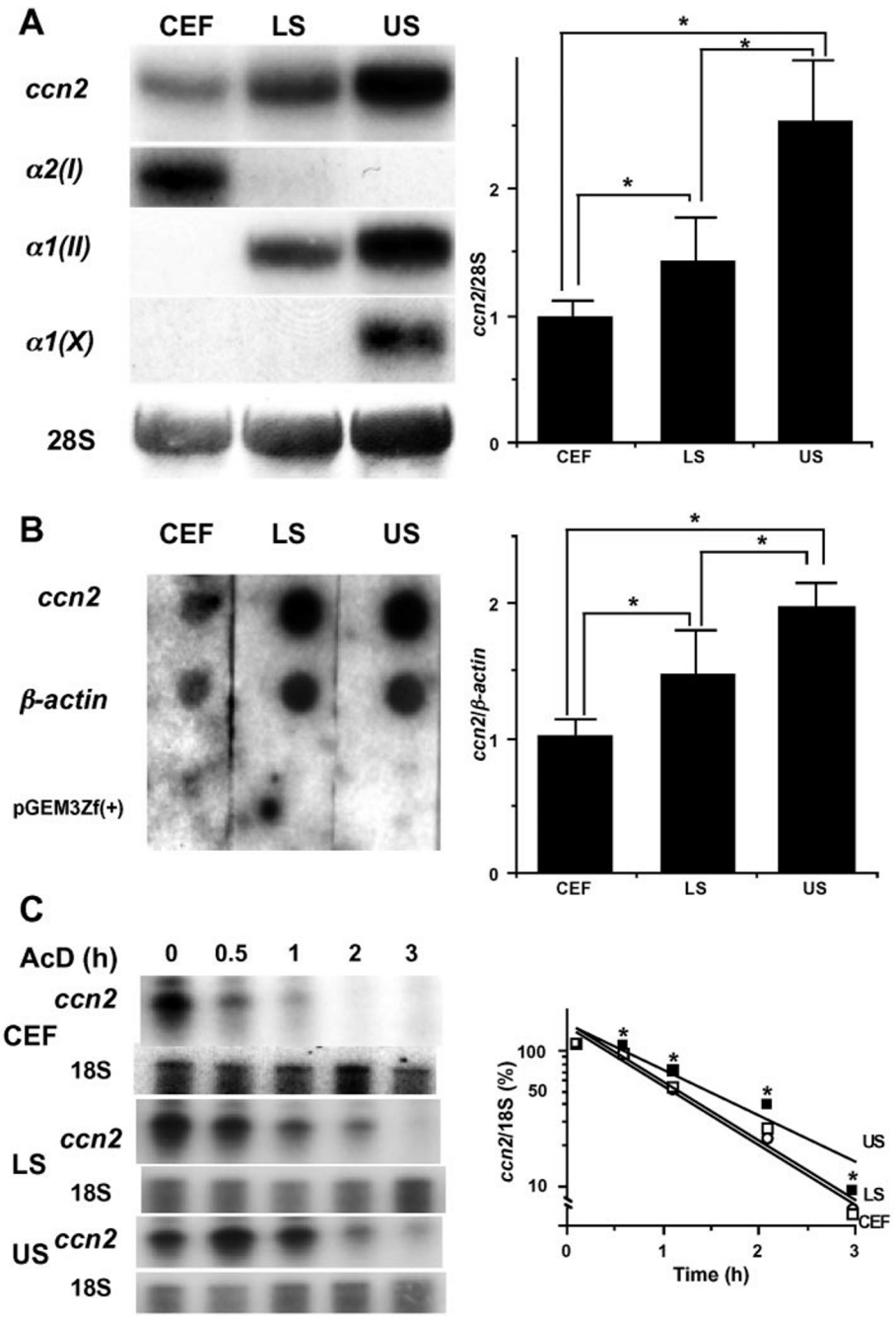

FIG. 1. **Steady-state level, transcription efficiency, and degradation pattern of *ccn2* mRNA in chicken fibroblasts and chondrocytes.** *A*, Northern blot analysis of *ccn2* and differentiation marker mRNAs. Ten micrograms of total RNA from each of CEF, LS, and US cells was denatured by glyoxysal, separated on 1% agarose gel, and blotted onto a nylon membrane. Prior to hybridization, the membrane was stained with methylene blue (*28S*) to show the equality of total RNA. Hybridization with *ccn2*, *α1* chain of type X collagen (*α1(X)*), α1 chain of type II collagen (*α1(II)*), and α2 chain of type I collagen (*α2(I)*) probes was sequentially carried out, after stripping the former probe. A representative result from three individual experiments is shown in the *left panel*. The relative mRNA level was normalized by the 28 S rRNA level, and the mean values of the three experiments are displayed in the *right panel* with the value for CEF cells set to unity and *error bars* indicating S.D. *, $p < 0.05$, significantly different between the cells. *B*, nuclear run-on assay of *ccn2*. Nuclear fraction from CEF, LS, and US cells was isolated and subjected to a nuclear run-on assay in the presence of [α-^{32}P]UTP. Autoradiograms of dot blot hybridization of *ccn2*, β-actin as an internal control, and pGEM3Zf(+) as a negative control, are shown. A representative result from three individual experiments is shown in the *left panel*, and the relative value of *ccn2* transcripts normalized by the β-actin level is displayed with *error bars* indicating S.D. in the *right panel*. *, $p < 0.05$, significantly different between the cells. *C*, RNA degradation analysis. 10 μg/ml actinomycin D (*AcD*) was added to cultures of CEF, LS, and US cells to terminate *de novo* RNA transcription. After 0.5, 1, 2, and 3 h, total mRNAs were isolated and subjected to the RNase protection assay for *ccn2* mRNA and 18 S rRNA (*18S*). For the *right panel*, the relative value of *ccn2* mRNA normalized by 18 S was first computed, and the obtained value was standardized against the one for time 0 of each cell (*open circle*, CEF; *open box*, LS; and *closed box*, US). Results representative of two individual experiments are shown in the *left panel*, and the relative values of *ccn2* transcripts normalized by 18 S rRNA are displayed on the semi-logarithm graph in the *right panel*. *, $p < 0.05$, significantly different from CEF cells.

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 had no significant effect on the transcriptional efficiency of the *ccn2* gene in the nuclear run-on assay (Fig. 2*A*). However, RNA degradation analysis (Fig. 2*B*) demonstrated that these growth factors changed the stability of *ccn2* mRNA differently, depending on the differentiation stage of the chondrocytes. In LS cells without growth factor stimulation, the half-life of *ccn2* mRNA was ~60 min. However, the cells stimulated by the growth factors uniformly showed significant increases in 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*

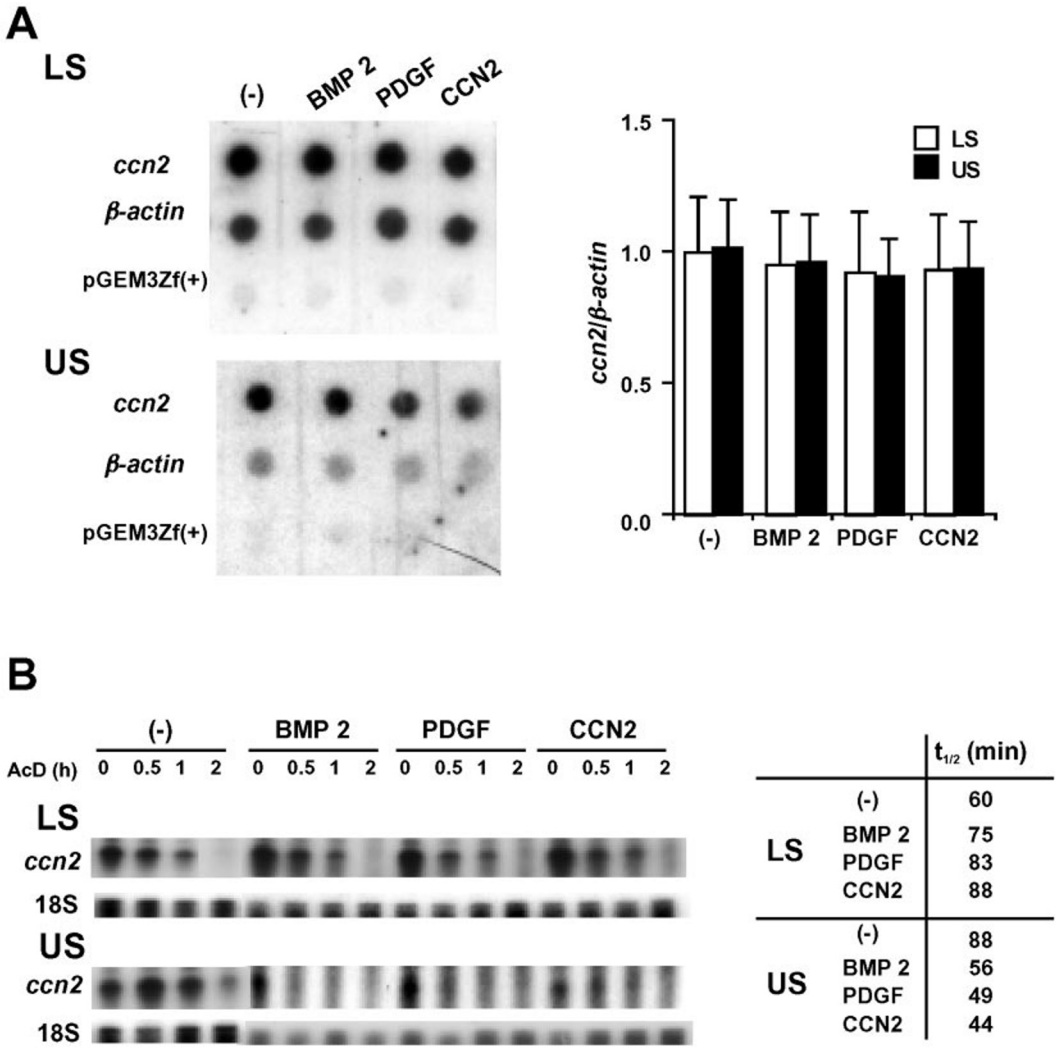

FIG. 2. **Effects of growth factors on the synthesis and degradation of *ccn2* mRNA in LS and US cells.** *A*, nuclear run-on assay. LS and US cells were stimulated with BMP2 (200 ng/ml), PDGF (10 ng/ml), or CCN2 (30 ng/ml) for 24 h in the presence of 10% FBS. Then, nuclear fractions were isolated and subjected to the nuclear run-on assay in the presence of [α-^{32}P]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 18 S rRNA (*18S*). The results representative of two individual experiments are shown in the *left panel*, and the computed half-lives ($t_{1/2}$) of *ccn2* mRNA normalized by those of 18 S are displayed in the *right panel*.

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. 3*A*, 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, of CEF, LS, and US cells, and a dual luciferase assay was then carried out (Fig. 3*B*). In all three cell types, three deletion mutants, *i.e.* 3′-200(+), 3′-100(+), and 3′-100/50(+) repressed luciferase gene expression in *cis*. FULL(+) did so as well. However, 3′-50(+) demonstrated much weaker *cis*-repressive effects than did the other three deletion genes and FULL(+). Of note, an antisense construct, 3′-100(−), also showed a weak repressive effect. Furthermore, the repressive effects of all of the fragments were shown to be the strongest in CEF cells, moderate in LS cells, and relatively weak in US cells. These results indicate that the minimal element of chicken *ccn2* 3′-UTR for the *cis*-repressive effect on gene expression was located within the region of 3′-100/50 in an orientation- and cell type-dependent manner.

Accelerated Degradation of a Reporter RNA by Repressive Elements in the 3′-UTR of Chicken ccn2 mRNA in cis—As described in a previous subsection, chicken *ccn2* 3′-UTR repressed the expression of the reporter gene in *cis*. So next we investigated the effect of the fragments of 3′-UTR on the stability of *cis*-linked mRNA, utilizing RNA degradation analysis for the luciferase gene (Fig. 4). In all transfectants, luciferase mRNA was highly expressed, because SV40 is a very strong promoter. In the control, the half-life ($t_{1/2}$) of the luciferase mRNA was ~45 min. In contrast, the mRNA containing the 3′-100(+) or 3′-100/50(+) 3′-UTR fragment was rapidly degraded ($t_{1/2} < 15$ min). The 3′-50(+) chimeric mRNA was also degraded more rapidly than the control; however, its effect was quite modest. These results are consistent with those of the luciferase assay and suggest that the 3′-UTR of chicken *ccn2*

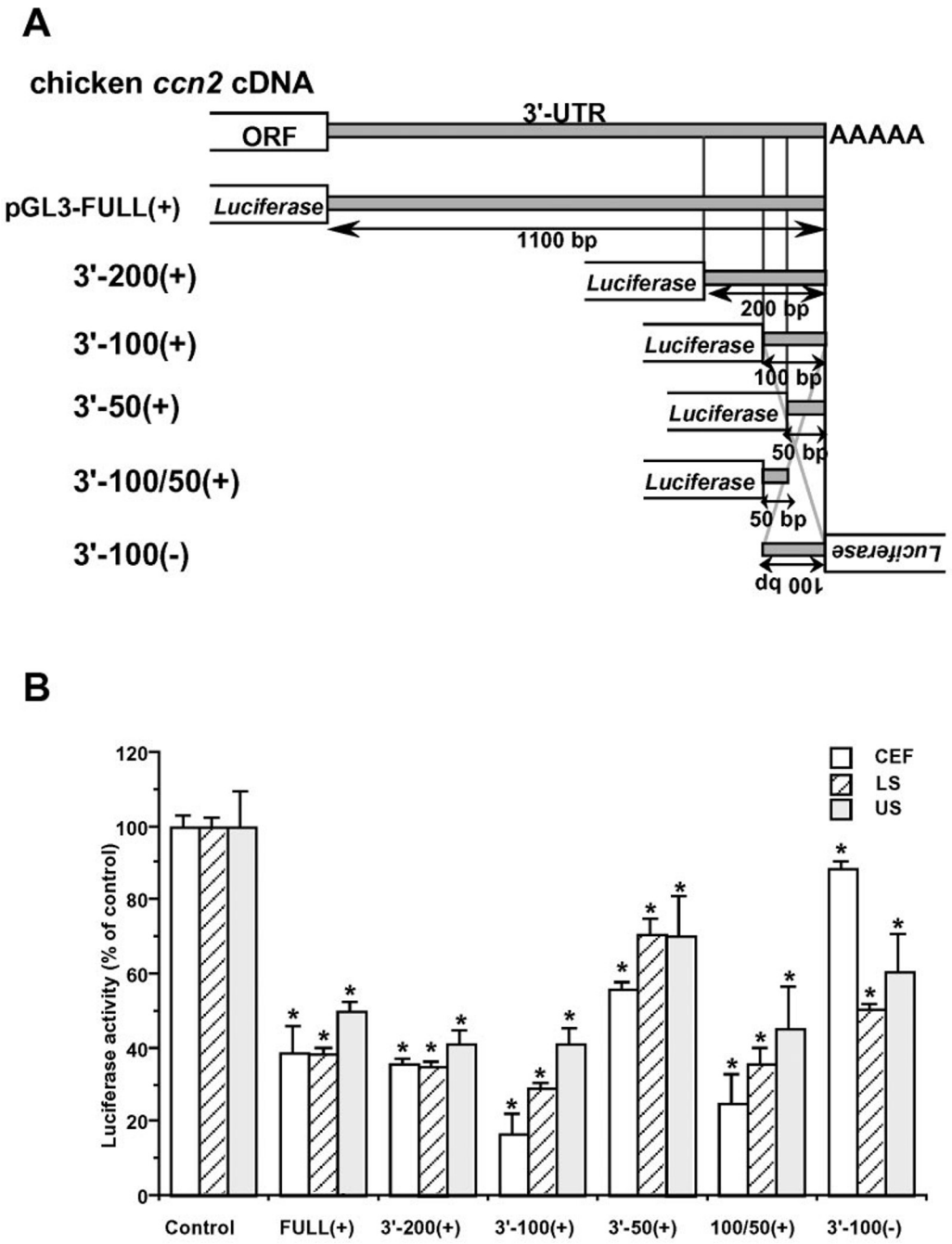

FIG. 3. **Identification of minimum functional *cis*-acting element in the chicken *ccn2* 3′-UTR for gene expression.** *A*, schematic representation of the structures of the plasmids utilized in this study. The 3′-half of the *ccn2* mRNA, including the 3′-UTR is illustrated at the *top*, and the full-length and deletion mutant fusion constructs are displayed. Full-length and deletion mutants of *ccn2* 3′-UTR were connected in the sense direction to the 3′-end of the firefly luciferase gene of parental pGL3L(+). Only in 3′-100(−) (*bottom* of the *panel*) was the 3′-UTR fragment placed in the antisense direction (see "Experimental Procedures"). *B*, relative firefly luciferase activities from the chimeric luciferase genes. The chimeric firefly luciferase constructs in *panel A* and pRL-TK containing *Renilla* luciferase gene (internal control) were used to co-transfect CEF, LS, and US cells. After 48 h, the cells were lysed, and the dual-luciferase assay was carried out. Activity is presented as a relative value of the measured luminescence of firefly luciferase *versus Renilla* luciferase, which was standardized against the parental control (100%) for each cell type. Mean values of the results of three experiments are displayed with *error bars* of S.D. *, $p < 0.05$, significantly different from each control.

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-^{32}P]CTP provided high quality corresponding RNAs that gave single bands with the expected electrophoretic mobility in 6% urea-denatured PAGE gels (Fig. 6*A*). Thereafter, the RNAs were forwarded to extensive *in vitro* folding assay (Fig. 6*B*). 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,

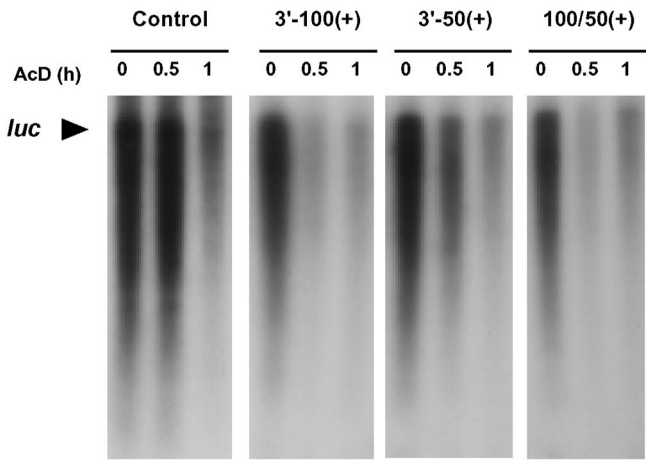

FIG. 4. **Effects of *cis*-acting element in the chicken *ccn2* 3′-UTR on RNA stability.** CEF cells were transfected with the chimeric firefly luciferase constructs (Full(+), 3′-100(+), 3′-50(+), and 3′100/50+(+)) illustrated in Fig. 3*A*. After 48 h, 10 μg/ml actinomycin D (*AcD*) was added. Then, after 0, 0.5, and 1 h, total RNAs were isolated and subjected to RNase protection assay for firefly luciferase mRNA (*luc*). The results representative of two individual experiments are shown.

which confer higher electromobility than the unfolded form, in native 6% PAGE gels. A similar mobility-shift was also distinctively observed with 3′-100/50(+) RNA, although the change in electrophoretic mobility itself was modest. In contrast, no folded form was observed in the case of the 3′-50(+) RNA fragment under the same conditions. These results demonstrate that the folding efficiency of RNAs *in vitro* is consistent with the repressive effects on reporter gene expression *in vivo*, suggesting that the secondary structure formation of the corresponding fragment of 3′-UTR of *ccn2* mRNA may be an important determinant for the repressive post-transcriptional regulation of gene expression.

Binding Profile of Nuclear and Cytoplasmic Protein(s) to 3′-UTR Fragments of ccn2 mRNA in Vitro—The results described in previous subsections suggest that these self-folded fragments might be able to bind specific nuclear and/or cytoplasmic protein(s). To explore this point further, we conducted REMSA and UV cross-linking assays. As shown in Fig. 7*A*, an RNA mobility shift was observed by incubating the 3′-100 fragment with the nuclear and cytoplasmic extracts of CEF cells. Interestingly, the shifted RNA band, which resulted from binding with the nuclear protein, was stronger than that with the cytoplasmic protein. The 3′-50 fragment, which was not able to form a secondary structure in the *in vitro* folding assay, bound to neither the nuclear protein nor the cytoplasmic protein. However, the 3′-100/50 fragment showed almost the same result as obtained for the 3′-100 fragment. The results of the UV cross-linking assay (Fig. 7*B*) clearly indicate that 3′-100 and 3′-100/50 fragments were able to bind not only to the protein in the cytoplasmic extract, but also to the protein in the nuclear extract, with the latter giving much stronger signals. These findings are in agreement with the results of REMSA. Furthermore, these results also suggest that the bound protein in each extract may be identical and indicate that the molecular mass of the protein was ~40 kDa.

Subsequent competition analysis confirmed the specificity of the observed interaction. Namely, co-incubation with an unlabeled competitor of the 3′-100 fragment completely abolished the RNA mobility-shift (Fig. 7*C*) and cross-linked signals (Fig. 7*D*) that resulted from the binding of 3′-100 and 3′-100/50 fragments to nuclear and cytoplasmic proteins. In the same way, an unlabeled competitor of the 3′-100/50 fragment also completely abolished the specific interaction. In contrast, the unlabeled 3′-50 fragment had no effect on the gel-shift or cross-linked signals, indicating that 3′-50 fragment was unable to compete with either 3′-100 or 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. 8*A*, 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. 8*B*) 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. 8*C*) for lamin B1 (a nuclear protein) and α-tublin (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. 9*A*), 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-

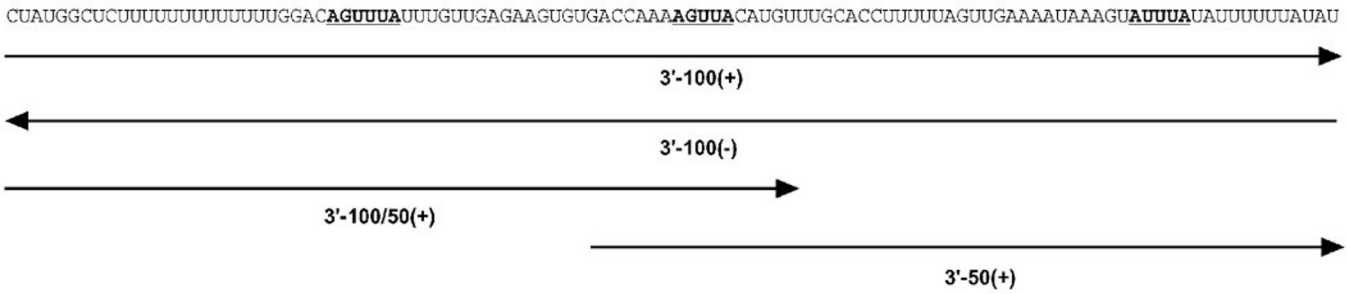

FIG. 5. **Nucleotide sequences and orientation of radiolabeled transcripts.** Four ^{32}P-labeled *ccn2* RNAs were prepared for *in vitro* folding analysis, REMSA, and UV-cross linking assay. The 3′-100, 3′-50, and 3′100/50 cDNA fragments of *ccn2* 3′-UTR shown in Fig. 3*A* were double-digested with EcoRI and XbaI and subcloned between the corresponding sites of pGEM3Zf(+). 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 [α-^{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*.

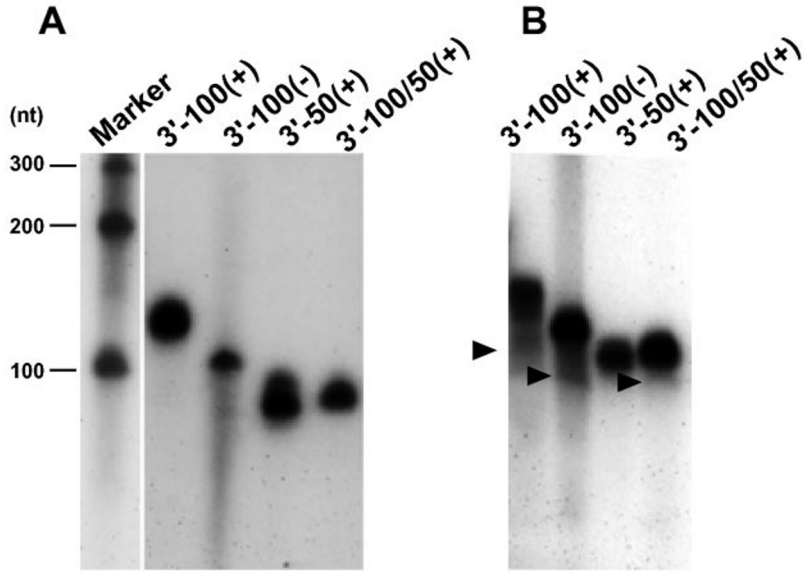

FIG. 6. **Preparation and *in vitro* folding of radiolabeled RNA fragments of *ccn2* 3′-UTR.** *A*, analysis of the synthesized RNAs in urea-denatured 6% PAGE. Four ^{32}P-labeled RNAs were prepared. RNA size markers (Century Marker; Ambion) are shown at the *left side* of the *panel*. *B*, *in vitro* 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. 9*B*) 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. 1*A*). The results of the nuclear run-on assay (Fig. 1*B*) revealed that the transcriptional activity of the *ccn2* gene was almost correlated with the mRNA level. Indeed, the stability of mRNA (Fig. 1*C*) 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. 2*A*) 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.

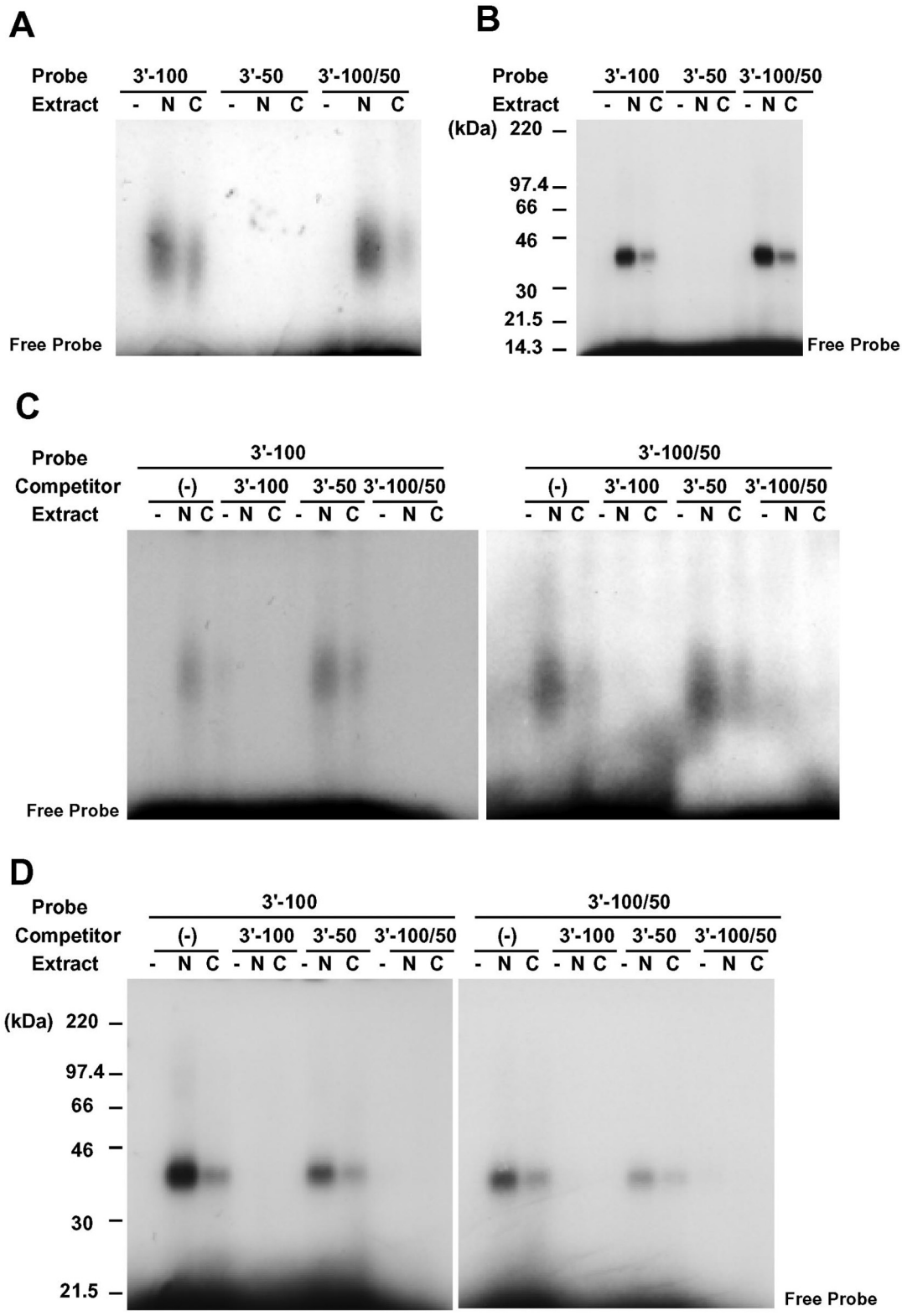

FIG. 7. **Profile of binding of the nuclear or cytoplasmic protein(s) of CEF cells to the *ccn2* 3′-UTR RNA.** *A*, REMSA of the RNA fragments of *ccn2* 3′-UTR. Three radiolabeled and folded RNA probes (3′-100, 3′-50, and 3′-100/50) corresponding to the ones shown in Fig. 5 were incubated with or without (−) 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. At 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. Positions of molecular standards (Rainbow Marker, Amersham Biosciences) are shown at the *left side* of the *panel*. At 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.

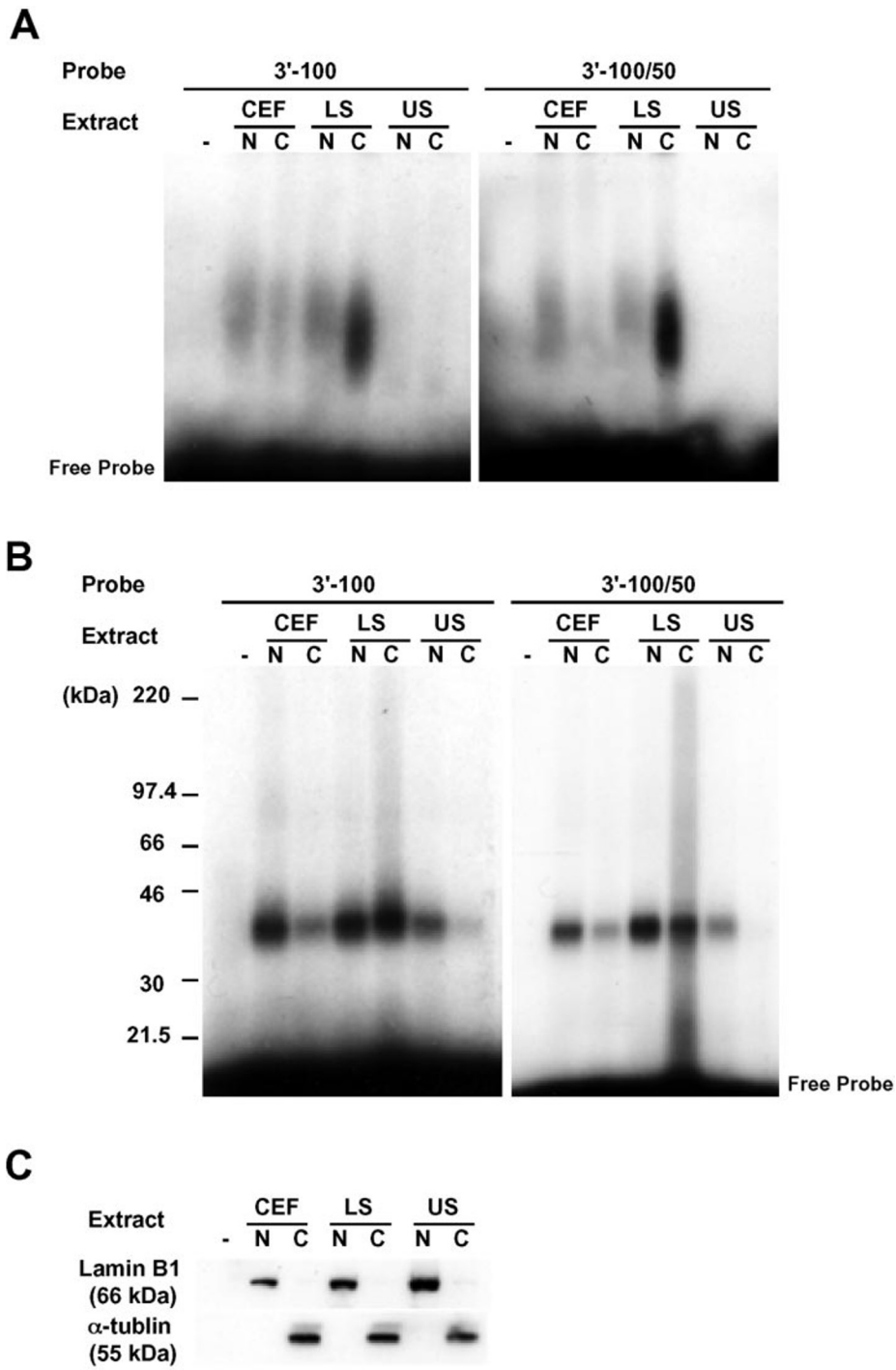

FIG. 8. **Different profiles of binding of the putative *trans*-factor to *ccn2* 3′-UTR among cell types.** *A*, REMSA of the *ccn2* 3′-UTR probes with nuclear or cytoplasmic extracts of CEF, LS, and US cells. The radiolabeled and folded probes (3′-100 and 3′-100/50) were incubated with or without (−) 10 μg of nuclear extract (*N*) or cytoplasmic extract (*C*) of CEF, LS, or US cells. After RNase digestion, the mixtures were analyzed by electrophoresis through 6% native PAGE gels. At the *bottom* of the gels, RNase-digested free probes are also visible. *B*, UV cross-linking assay. The radiolabeled and folded RNA probes were incubated with the extract and then digested with RNase as performed in REMSA. Then, the mixtures were irradiated by UV on ice and analyzed by SDS-PAGE in a 4–20% gradient gel. Positions of molecular weight standards are shown at the *left side* of the *panel*. At the *bottom* of the gels, RNase-digested free probes are also visible. *C*, Western blotting analysis. 10-μg proteins of each extract was subjected to 12.5% SDS-PAGE and blotted onto a polyvinylidene difluoride membrane. The blot was then incubated with anti-lamin B1 or anti-α-tublin antibody.

On the other hand, RNA degradation analysis (Fig. 2*C*) 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

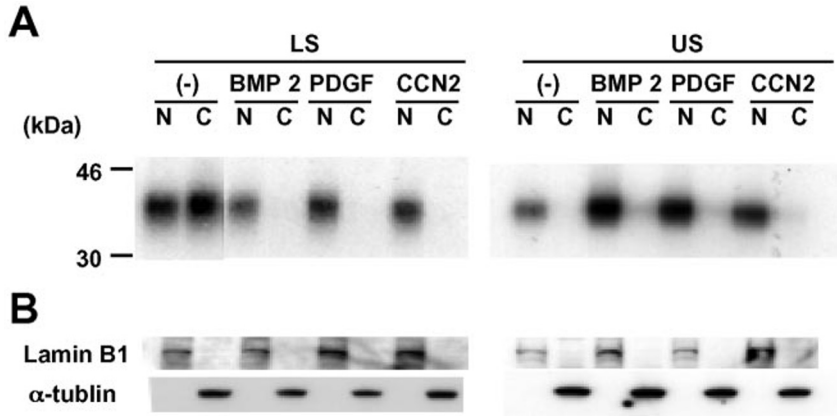

FIG. 9. **Growth factor modulation of the binding of the putative *trans*-factor in LS and US cells to *ccn2* 3′-UTR.** *A*, UV cross-linking assay of *ccn2* 3′-UTR probes with nuclear and cytoplasmic extracts of LS and US cells stimulated by growth factors. LS and US cells were stimulated with BMP2 (200 ng/ml), PDGF (10 ng/ml), or CCN2 (30 ng/ml) for 24 h in the presence of 10% FBS; and nuclear (*N*) and cytoplasmic (*C*) extracts were then prepared. The radiolabeled and folded probes were incubated with or without (−) 10 μg of the extracts. After RNase digestion, the mixtures were irradiated by UV and analyzed by 12.5% SDS-PAGE. Positions of molecular weight standards are shown at the *left side* of the *panel*. At the *bottom* of the gel, RNase-digested free probes are observed. *B*, Western blot analysis. 10-μg proteins of each extract was subjected to 12.5% SDS-PAGE and blotted onto a polyvinylidene difluoride membrane. The blot was then incubated with anti-lamin B1 or anti-α-tublin antibody.

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. 1*A*), 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 (53). 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. 1*C*) 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. 6*B*) 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. 7*A*) and UV cross-linking assay (Fig. 7*B*) 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. 8*A*) and UV cross-linking assays (Fig. 8*B*) 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. 1*C*), 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 disap-

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, *ccn2*-overexpressing transgenic mice presented a dwarfism phenotype and decreased bone density (66), whereas *ccn2-null* mice showed skeletal dysmorphisms as a result of impaired chondrocyte proliferation (67), showing the critical importance 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 chondroytes 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 nuclei 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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