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Journal of Cellular Biochemistry 111:1607–1618 (2010)

Journal of Cellular Biochemistry

A Coding RNA Segment That Enhances the Ribosomal Recruitment of Chicken *ccn1* mRNA

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ABSTRACT

CCN1, a member of the CCN family of proteins, plays important physiological or pathological roles in a variety of tissues. In the present study, we initially found a highly guanine–cytosine (GC)-rich region of approximately 200 bp near the 5′-end of the open reading frame, which was always truncated by amplification of the corresponding cDNA region through the conventional polymerase chain reaction. An RNA in vitro folding assay and selective ribonuclease digestion of the corresponding segment of the *ccn1* mRNA confirmed the involvement of a stable secondary structure. Subsequent RNA electromobility-shift assays demonstrated the specific binding of some cytoplasmic factor(s) in chicken embryo fibroblasts to the RNA segment. Moreover, the corresponding cDNA fragment strongly enhanced the expression of the reporter gene in *cis* at the 5′-end, but did not do so at the 3′-end. According to the results of a ribosomal assembly test, the effect of the mRNA segment can predominantly be ascribed to the enhancement of transport and/or entry of the mRNA into the ribosome. Finally, the minimal GC-rich mRNA segment that was predicted and demonstrated to form a secondary structure was confirmed to be a functional regulatory element. Thus, we here uncover a novel dual-functionality of the mRNA segment in the *ccn1* open reading frame, which segment acts as a *cis*-element that mediates posttranscriptional gene regulation, while retaining the information for the amino acid sequence of the resultant protein. J. Cell. Biochem. 111: 1607–1618, 2010.

KEY WORDS: CCN FAMILY; CCN1; CYR61; POST-TRANSCRIPTIONAL REGULATION; RIBOSOME

CCN1 (Cyr61/CEF-10) is a cysteine-rich secretory protein, and a member of the "CCN family" [Lau and Lam, 1999; Brigstock et al., 2003; Perbal and Takigawa, 2005], which also includes CTGF/Fisp12 (CCN2) [Almendral et al., 1988; Bradham et al., 1991; Ryseck et al., 1991], Nov (CCN3) [Joliot et al., 1992], ELM-1/WISP-1 (CCN4) [Hashimoto et al., 1998; Pennica et al., 1998], CTGF-3/WISP-2/COP1 (CCN5) [Pennica et al., 1998; Zhang et al., 1998], and WISP-3 (CCN6) [Pennica et al., 1998]. Each of the CCN family proteins consists of four conserved modules, that is, the insulin-like growth factor-binding protein (IGFBP), von Willebrand factor type C repeat (VWC), thrombospondin type I repeat (TSP1), and carboxyl terminal (CT) modules. These modules are known to interact with a number of biomolecules to conduct extracellular signaling network that yields multiple effects on the development of a variety of tissues.

Cyr61, a human and mouse ortholog of CCN1, was first identified as a growth factor-inducible immediate–early response gene by differential hybridization screening of a cDNA library prepared from serum-stimulated mouse fibroblasts; [Lau and Nathans, 1985] and CEF-10, a chicken ortholog of CCN1 (cCCN1), was identified as a v-*src*-inducible gene in chicken embryo fibroblasts (CEF cells) [Simmons et al., 1989]. Thereafter, a number of biological functions of CCN1 were clarified. CCN1 enhances growth factor-stimulated cell migration [Kireeva et al., 1996, 1998; Babic et al., 1998] and mediates cell adhesion [Kireeva et al., 1996, 1997, 1998; Jedsadayanmata et al., 1999; Chen et al., 2001]. Recent reports revealed that CCN1 plays important roles in tumorigenesis or tumor suppression. Namely, CCN1 stimulates tumor progression of breast cancer [Tsai et al., 2000; Xie et al., 2001] and enhances the

Grant sponsor: Japan Society for the Promotion of Science; Grant numbers: 19109008, 21592360.

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Received 23 February 2010; Accepted 21 September 2010 • DOI 10.1002/jcb.22894 • © 2010 Wiley-Liss, Inc.
Published online 4 November 2010 in Wiley Online Library (wileyonlinelibrary.com).

malignant phenotype of a gastric adenocarcinoma cell line [Babic et al., 1998]. By contrast, it was also reported that expression of CCN1 is down-regulated in prostate cancer [Pilarsky et al., 1998] and leiomyoma [Sampath et al., 2001] and that overexpression of CCN1 suppresses cell proliferation associated with enhanced expression of p53 and $p21^{waf1}$ in non-small cell lung cancer cells [Tong et al., 2001]. Of note, the involvement of CCN1 in the induction of apoptosis has been described as well [Juric et al., 2009]. CCN1 also plays important roles in skeletal formation and acquisition of vascular integrity during fetal development [O'Brien and Lau, 1992]. Expression of *ccn1* is observed in developing cartilage and the circulatory system during embryogenesis [O'Brien and Lau, 1992], and CCN1 promotes the differentiation of murine limb bud mesenchymal cells into chondrocytes [Wong et al., 1997]. Finally, *ccn1*-null mice suffer embryonic death due to a failure of chorioallantonic fusion or placental vascular insufficiency and compromised vessel integrity [Mo et al., 2002]. As such, CCN1 displays various functions in physiological and/or pathological processes; however, the molecular mechanism of its gene expression, particularly that at the post-transcriptional level, still remains unclear.

Recent studies have demonstrated that post-transcriptional regulation of mRNA plays important roles in gene expression, as well as in transcriptional regulation and that *cis*-elements on mRNA and *trans*-factors in the cytoplasm and/or nuclei are involved in the regulation. A variety of mRNA regulatory *cis*-elements, such as adenine–uridine-rich elements (ARE) [Bevilacqua et al., 2003] and micro RNA targets [Siomi and Siomi, 2009] in a number of transcripts mediate post-transcriptional and translational gene regulation during the transport of mRNA from the nucleus to ribosome in the cytosol [St Johnston, 1995; Moallem et al., 1998] via the regulation of the stability of mRNA [Moallem et al., 1998] and translation efficiency [Siomi and Siomi, 2009]. Our recent studies also uncovered a *cis*-acting element of structure-anchored repression (CAESAR) and the 3′-100/50 element in the *ccn2* gene, which is another member of the CCN family [Kubota et al., 2000, 2005; Mukudai et al., 2005, 2008]. Most of these elements are reported to be present in 5′- and 3′-untranslated regions (UTRs) [Kubota et al., 1999]. However, certain RNA *cis*-elements are also present in the open reading frame (ORF) of several genes, such as those for interleukin 2 [Chen et al., 1998], thymidylate synthase [Lin et al., 2000], and manganese superoxide dismutase [Davis et al., 2001].

In the present study, we focused on a secondary-structured GC-rich region in the ORF of the chicken *ccn1* mRNA, and found that the region functions as a positive post-transcriptional *cis*-regulatory element of gene expression, by promoting the ribosomal entry of the *cis*-linked mRNA in a location-dependent manner.

MATERIALS AND METHODS

CELL CULTURE

CEF cells were isolated from a day-10 chicken embryo by trypsinization, and maintained in Dulbecco's modified Eagle's minimum essential medium supplemented with 10% fetal bovine serum in humidified air containing 5% CO_2 at 37°C, as described previously [Mukudai et al., 2003].

PURIFICATION OF TOTAL CELLULAR RNA AND REVERSE TRANSCRIPTASE-MEDIATED POLYMERASE CHAIN REACTION (RT-PCR)

Total RNA was isolated from sub-confluent CEF cells according to the method of Chomczynski and Sacchi [1987], and was treated with 250 μg/ml of protease K (Invitrogen, Carlsbad, CA) for 16 h in the final step of RNA preparation. Reverse transcription by avian myeloblastosis virus (AMV) reverse transcriptase was conducted by using a commercially available kit (Takara, Tokyo, Japan) with 1 μg of total RNA and random primers. The polymerase chain reaction (PCR) was carried out with a recombinant *Taq* DNA polymerase (Invitrogen), according to the manufacturer's protocol, in the presence or absence of "PCRx Enhancer Solution" attached as a supplement of the polymerase. Nucleotide sequences of the primers for amplification of the 5′-region of *ccn1* were 5′-CGC TAA GAC ATG GGC TC-3′ for the sense, and 5′-CCT CAG AAG CGT CCA GA-3′ for the anti-sense. These primers were designated "S-1" and "AS-1," respectively. The amplification cycle consisted of 30 s at 95°C, 30 s at 70°C, and 1 min at 72°C. After 35 cycles of chain reaction and subsequent incubation at 72°C for 5 min, the PCR products were analyzed by conducting 1% agarose gel electrophoresis.

The amplicons were subcloned into pGEM T-Easy (Promega, Madison, WI) by a TA-cloning method and sequenced. Among the clones, those yielding sense transcripts from the T7 bacteriophage promoter in the plasmid were utilized for subsequent in vitro transcription experiments.

DNA SEQUENCING AND COMPUTER ANALYSIS

The cDNAs subcloned into the respective plasmids were sequenced with a Big Dye Terminator Cycle Sequencing Ready Reaction Kit ver. 2.0 (Applied Biosystems, Foster City, CA), 1× SEQUENCERx Enhancer Solution A (Invitrogen) as a supplement, and an ABI PRISM 310 Genetic Analyzer (Applied Biosystems). DNA sequence alignments and RNA secondary structure predictions were made by using a commercial computer software package, GENETYX-MAC ver. 11, or GENETYX ver. 6.1.2 (Software Development, Tokyo, Japan).

PREPARATION OF NUCLEAR AND CYTOPLASMIC EXTRACTS (S-100)

The nuclear fraction and cytoplasmic fraction (S-100) of CEF cells were prepared according to a previously described protocol [Glickman and Ripley, 1984] with a slight modification. Sub-confluent CEF cells in 10-cm dishes were washed with phosphate-buffered saline (PBS), collected into 1 ml per dish of hypotonic buffer [10 mM HEPES (pH 7.9), 1.5 mM $MgCl_2$, 10 mM KCl, 1 μg/ml leupeptin (Sigma–Aldrich, St. Louis, MO), 1 μg/ml aprotinin (Sigma–Aldrich), and 0.5 mM ditiothreitol (DTT)], and chilled on ice for 10 min. Then, the cells were homogenized by 10 strokes in a Dounce homogenizer on ice, and centrifuged at 3,000*g* for 15 min at 4°C to obtain the crude cytoplasmic and nuclear fractions, respectively.

For purification of the nuclear fraction, the crude nuclear fraction was re-suspended in the same volume of a low-salt buffer [20 mM HEPES (pH 7.9), 25% glycerol, 1.5 mM $MgCl_2$, 0.02 mM KCl, 0.2 mM EDTA, 1 μg/ml leupeptin, and 1 μg/ml aprotinin], and was gently shaken for 10 min at 4°C. Afterwards, the same volume of a high-salt

buffer [20 mM HEPES (pH 7.9), 25% glycerol, 1.5 mM $MgCl_2$, 1.2 M KCl, 0.2 mM EDTA, 1 μg/ml leupeptin, and 1 μg/ml aprotinin] was added drop wise into the swollen nuclear suspension; and the suspension was gently shaken for an additional 30 min at 4°C. The soluble nuclear fraction was obtained as the supernatant after centrifugation at 25,000*g* for 30 min at 4°C. Finally, it was dialyzed against a moderate salt buffer [20 mM HEPES (pH 7.9), 20% glycerol, 0.2 mM KCl, and 0.5 mM EDTA] for 16 h at 4°C, and then stored at −80°C until used.

For preparation of the cytoplasmic fraction, a 0.11 volume of 10× cytoplasmic buffer [0.3 mM HEPES (pH7.9), 1.4 M KCl, and 30 mM $MgCl_2$] was added to the crude cytoplasmic fraction, and centrifuged at 10,000*g* for 1 h at 4°C. The supernatant gave the cytoplasmic fraction (S-100), which was dialyzed and frozen in the same way as the soluble nuclear fraction.

The protein concentrations of both fractions were determined with a BCA protein assay kit (Pierce, Rockford, IL), utilizing bovine serum albumin (BSA; Sigma–Aldrich) as a standard.

PREPARATION OF RNA IN RIBOSOMAL FRACTION

The ribosomal fraction of CEF cells was prepared as described earlier [Canceill and Ehrlich, 1996] with slight modification. Sub-confluent CEF cells were washed with PBS, collected in PBS, and centrifuged at 500*g* for 5 min at 4°C. The cell pellet was re-suspended in a lysis buffer [50 mM Tris–HCl (pH 7.4), 0.25 M sucrose, 25 mM KCl, 5 mM $MgCl_2$, 2 mM DTT, 100 units/ml RNase inhibitor (Takara), and 0.7% NP-40], chilled on ice for 4°C, and centrifuged at 750*g* for 10 min at 4°C. The supernatant was re-centrifuged at 12,500*g* for 10 min at 4°C. The secondary supernatant was transferred to a new tube, and a 0.32 volume of high-KCl solution [50 mM Tris–HCl (pH 7.4), 0.25 M sucrose, 2 M KCl, 5 mM $MgCl_2$, 2 mM DTT, and 100 units/ml RNase inhibitor] was added, in order to adjust the KCl concentration to 0.5 M. The solution was layered onto 1 ml of a sucrose cushion solution [50 mM Tris–HCl (pH 7.4), 1 M sucrose, 0.5 M KCl, and 5 mM $MgCl_2$], and centrifuged at 245,000*g* for 6 h at 4°C. The pellet (ribosomal fraction) was composed of ribosomal proteins, ribosomal RNAs, and ribosome-associated messenger RNAs. Then, RNAs in the ribosomal fraction were purified by using the same method as used for the purification of total cellular RNA. Involvement of comparable quality and quantity of 28s and 18s rRNAs in each ribosomal fraction was confirmed by agarose gel electrophoresis.

IN VITRO TRANSCRIPTION OF RNA

The in vitro transcription reaction was carried out with a commercial kit, Riboprobe Combination System Sp6/T7 (Promega) according to the manufacturer's protocol. The plasmids described in another subsection were linearized by *Spe*I, *Pvu*II, or *Nco*I, transcribed by Sp6 or T7 bacteriophage RNA polymerase for 1 h at 37°C in the presence of alpha-[^{32}P]CTP (Amersham Pharmacia, Buckinghamshire, UK) or digoxigenin-11-UTP (Roche, Basel, Switzerland), and subjected to DNase digestion and spin-column (Amersham Pharmacia) purification [32]. Thereafter, the labeled transcripts were suspended in a Tris-borate EDTA (TBE)-urea sample buffer (45 mM Tris-borate, 45 mM boric acid, 1 mM EDTA, 2 M urea, 6% Ficoll, 0.005% bromophenol blue, and 0.005% xylene cyanol), heated at 95°C for 10 min, and cooled on ice. Then, the denatured RNAs were analyzed by conducting 6% polyacrylamide gel electrophoresis (PAGE) in the presence of 6 M urea in 1× TBE buffer. The gel was subsequently dried and autoradiographed, or processed for signal detection with a commercially available kit, following the manufacturer's indications (DIG Nucleic Acid Detection Kit; Roche). Unlabeled RNA transcripts were produced in a similar manner, but in the absence of labeled nucleotides. Schematic representations of the method and of the lengths of several transcripts are shown in Figure 2A.

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

RNA IN VITRO FOLDING AND ANALYTICAL RNASE PROTECTION ASSAYS

The RNA folding assay was carried out as described previously [Dignam et al., 1983; Odelberg et al., 1995] with a slight modification. Forty-thousand cpm or 100 ng of labeled RNA was heated at 95°C for 5 min, gradually cooled to room temperature, and then chilled at 4°C for more than 2 days in an RNA folding buffer [10 mM HEPES (pH 7.9), 40 mM KCl, 3 mM $MgCl_2$, 1 mM DTT, 0.5 mg/ml yeast tRNA (Boehringer Mannheim, Mannheim, Germany) and 0.5 mg/ml BSA], in order to allow the RNA to form a secondary structure. The folded RNAs were then digested with 1 μl of 100-fold diluted RNase T1 solution (1,000 units/μl, Ambion) for 10 min at 37°C. After phenol/chloroform extraction, the digested RNAs were precipitated by 2.5 M ammonium acetate and ethanol at −30°C for 2 h. The RNA pellets were dissolved in a TBE-urea sample buffer and then subjected to 6% TBE-urea PAGE as described in another subsection.

RNA ELECTROMOBILITY SHIFT ASSAY (REMSA)

The nuclear or cytoplasmic extract containing 0–10 μg of protein was incubated at 25°C for 30 min with 40,000 cpm of radio-labeled RNA in 19 μl of a binding buffer [5 mM HEPES (pH 7.9), 7.5 mM KCl, 0.5 mM $MgCl_2$, 0.1 M EDTA, 0.5 mM DTT, and 0.1 mg/ml BSA] containing 0.1 mg/ml of yeast tRNA to rule out non-specific interaction. Then, the binding mixture was incubated with 1 μl of 100-fold diluted RNase Cocktail (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 gel was subsequently dried and autoradiographed.

For competition experiments, proteins were pre-incubated with 0–100 ng of unlabeled competitor RNA for 30 min at 25°C, followed by incubation with the radio-labeled RNA for another 30 min at 25°C.

WESTERN BLOTTING ANALYSIS

The nuclear and cytoplasmic proteins were separated in a 12% SDS–PAGE gel, and then transferred to polyvinylidene difluoride membrane (Hybond-P; Amersham Pharmacia). The blot was blocked with 5% skim milk in Tris-buffered saline (TBS) for 16 h at 4°C. The blot was next incubated with a 1/2,000 dilution of monoclonal anti-

alpha tubulin antibody (Sigma–Aldrich) or 1/1,000 dilution of monoclonal anti-lamin B1 antibody (Zymed, South San Francisco, CA) in TBS containing 0.05% Tween 20 (TBS-T) for 1 h at 37°C and, thereafter, incubated with a 1/20,000 dilution of peroxidase-conjugated goat anti-mouse IgG antibody (American Qualex, La Mirada, CA) in TBS-T for 1 h at 37°C. Subsequently, the immunosignals were visualized with an ECL Western Blotting Analysis System (Amersham Pharmacia).

PLASMID CONSTRUCTS

The SV40 promoter-driven firefly luciferase expression plasmid (pGL3; Promega) was modified by inserting multiple cloning sites at the downstream end of the luciferase gene, as described in our previous studies [Kubota et al., 1999, 2000]; and this plasmid, designated pGL3L(+), was used to elucidate the *cis*-acting effects of the *ccn1* fragment. A HSV-TK promoter-driven *Renilla*-luciferase expression plasmid (pRL-TK; Promega) was used as an internal control for transfection experiments to monitor the transfection efficiency. The 5′ cDNA fragment of *ccn1* was obtained by PCR. For ligation at the upstream end of the luciferase gene, a sense primer (5′-AAG CTT GGG CTC TGC GGG AGC TCG-3′) and anti-sense primer (5′-CCA TGG TCT CCC CTC AGA CTG TGC TC-3′) were prepared. The sense and anti-sense primers contained flanking *Hin*dIII and *Nco*I sites, respectively; hence the amplicon was double-digested with *Hin*dIII site and *Nco*I, purified, and subcloned between the corresponding sites in pGL3L(+). On the other hand, for the insertion of the fragment at the upstream end of the luciferase gene, the sense primer (5′-TCT AGA TGG GCT CTG CGG AGC C-3′) and anti-sense primer (5′-GAA TTC TCC CCT CAG ACT GTG CTC-3′) were designed to contain flanking *Xba*I and *Eco*RI sites, respectively. Thus, the amplicon was double-digested with these enzymes, purified, and subcloned between the corresponding sites in pGL3L(+). For the construction of the plasmids expressing the minimal structured segment of 209 bases fused to the luciferase mRNA, four long oligonucleotides were synthesized and assembled between the unique *Hin*dIII and *Nco*I sites in pGL3L(+), utilizing the internal *Pvu*II site in the *ccn1* cDNA fragment. On the way to the construction of this plasmid, pGL3-RPC, two derivatives were also obtained as intermediate products. These plasmids, designated pGL3-RPF and pGL3-RPL, contain the former *Hin*dIII–*Pvu*II and latter *Pvu*II–*Nco*I subfragments of the minimal segment, respectively. These short cDNA segments were also subcloned into pGEM3Zf(−) (Promega) for in vitro transcription of RNAs with SP6 or T7 RNA polymerase. The structures of the newly constructed plasmids were confirmed by restriction enzymatic digestion and nucleotide sequencing analyses.

DNA TRANSFECTION

Two-hundred thousand CEF cells were seeded into a 35-mm tissue culture dish 24 h before transfection. Cationic liposome-mediated DNA transfection was carried out with 1 μg of each pGL3L(+) derivative in combination with 0.5 μg of pRL-TK, according to the manufacturer's methodology (Lipofectamine; Invitrogen). Forty-eight hours after the transfection, the cells were lysed in 500 μl of a passive lysis buffer (Promega); and then the cell lysate was directly used for the luciferase assay.

LUCIFERASE ASSAY

The dual luciferase assay system (Promega) was applied for the sequential measurement of firefly (reporter) and *Renilla* (transfection efficiency standard) luciferase activities with specific substrates of beetle luciferin and coelenterazine, respectively. Quantification of both luciferase activities and calculation of relative ratios were carried out manually with a luminometer (TD20/20: Turner Designs, Sunnyvale, CA), as described in our earlier study [Kubota et al., 1999].

QUANTITATIVE RNASE PROTECTION ASSAY

For preparation of the probe for the firefly luciferase gene transcript, pGL3L(+) was double-digested with *Hin*dIII and *Xba*I. The resulting 1.7-kbp fragment containing the firefly luciferase gene was separated in 1% agarose gel, excised, purified, and subcloned between the corresponding sites in pGEM3Zf(+) (Promega). The plasmid was linearized by *Hin*cII, and in vitro transcription was carried out by using T7 bacteriophage RNA polymerase, as described in another subsection. The length of the probe was approximately 400 nt.

RNase protection assays were carried out with a commercial kit (RPA II kit, Ambion), according to the manufacturer's protocol. Twenty-four hours after the transfection of CEF cells, total cellular or ribosome-fractional RNA was prepared, as described in another subsection. Two micrograms of RNAs was hybridized with the radio-labeled firefly luciferase probe, digested by the RNase cocktail, and precipitated in ethanol. The recollected RNA was subjected to 6% TBE-urea PAGE. Thereafter, the gels were dried and autoradiographed.

RESULTS

A HIGHLY GC-RICH SEGMENT IN THE ORF OF THE CCN1 cDNA AS INDICATED BY TRUNCATION DURING PCR

Initially, RT-PCR was carried out with CEF RNA, in order to simply obtain a cDNA fragment corresponding to the upstream end of the ORF of *ccn1*, which was predicted to be 600-bp in length (Fig. 1). However, to our surprise, the length of the resultant amplicon was

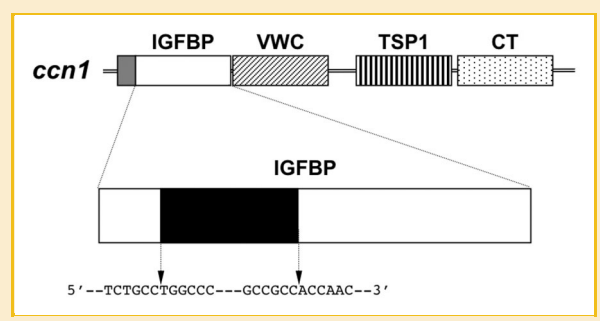

Fig. 1. Schematic representation of the cDNA structure of chicken *ccn1* and the truncated area. The entire cDNA structure that is composed of a signal peptide-encoding region (gray box) and module-encoding regions are shown at the top. An enlarged illustration is also provided for the IGFBP region to specify the approximate location of the truncated region of 209 bp and the nucleotide sequences at the boundaries.

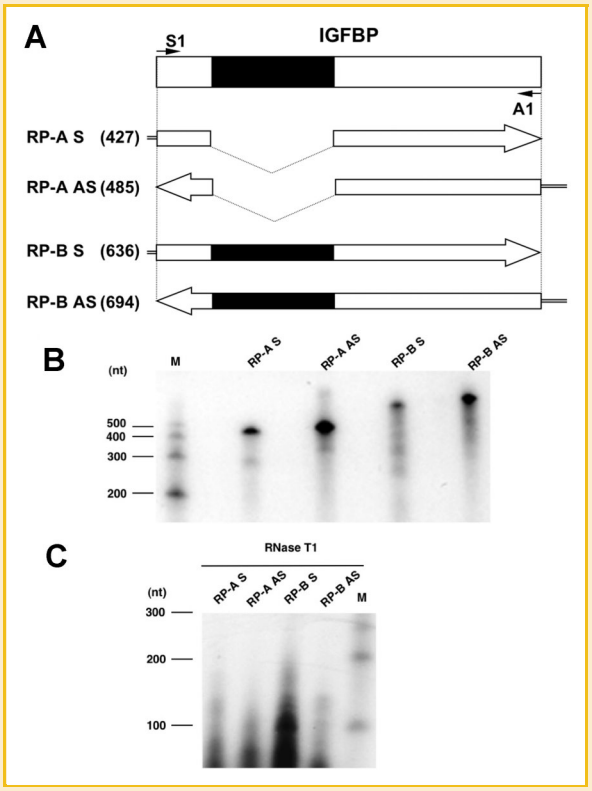

Fig. 2. In vitro transcription and analysis of folding of *ccn1* mRNA segments. A: Schema for preparation of radiolabeled RNAs in vitro. The structures of each transcription template and the resultant transcripts are shown as combined illustrations. The amplicons of RT-PCR were subcloned into pGEM T-Easy (Promega) for in vitro transcription of the corresponding RNAs. The names of transcripts are shown therein. Prior to the transcription reaction, the plasmids were linearized, and transcription was carried out by using T7 or SP6 RNA polymerase for sense (S) or anti-sense (As) transcripts, respectively. The orientation of transcription and sizes of transcripts are denoted. Double lines represent nucleotide sequences originating from the vector. B: Analysis by use of 6% PAGE of the transcribed RNAs shown in panel A. M represents RNA molecular size markers, produced by in vitro transcription of a mixture of RNA templates (Century Marker, Ambion), and the sizes of the transcripts are indicated at the left of the panel. C: Susceptibility of the *ccn1* mRNA fragments to RNase T1 after folding. Radio-labeled RNAs were heated at 95°C, gradually cooled to room temperature, and thereafter cooled further to 4°C, in order to allow them to self-fold. Then, the folded RNAs were digested with RNase T1. After digestion, the RNAs were purified through phenol extraction, precipitated with ethanol and ammonium sulfate, and separated by denaturing 6% PAGE. M refers to RNA molecular size markers, with sizes indicated at the left of the panel. The data in panel B and C are representative of two separate experiments with similar results.

approximately 400 bp, which was 200 bp shorter than that expected. In contrast, in the presence of PCRx Enhancer Solution, which is a reagent that facilitates the amplification of GC-rich sequences, an amplicon with the expected length was obtained.

To confirm their identity, these 400 and 600-bp amplicons, which were designated RP-A and RP-B, respectively, were subcloned into pGEM T-Easy vector by a TA-cloning method, and sequenced (Fig. 1). The nucleotide sequence of RP-B was exactly the same as that of the *ccn1* reported in GenBank (accession number: J04496) by Simmons et al. In contrast, the nucleotide sequence of RP-A lacked an internal 209-bp portion, and the GC-content of the deleted region was particularly high (73.5%). These findings firmly indicate a novel GC-rich segment in the ORF of *ccn1* cDNA, which is suspected to yield a secondary-structured mRNA segment.

SECONDARY STRUCTURE FORMATION OF RP-B RNA IN VITRO

In order to examine whether or not this region was able to actually form a stable secondary structure on mRNA through internal base-pairing, we carried out RNA in vitro transcription for use in an RNA in vitro folding assay. Figure 2A illustrates the length and orientation of the radio-labeled RNA corresponding to each RT-PCR amplicon (RP-A or RP-B). On 6% TBE-urea PAGE, the radio-labeled transcripts gave single bands of expected electromobility, as shown in Figure 2B, indicating proper RNA synthesis in all of the samples. Thereafter, the RNAs were subjected to extensive RNA in vitro folding assays using digestion with RNase. The RNAs were heat-denatured, and gradually cooled, in order to allow them to form a secondary structure, and then, incubated with RNase T1, which digests single-stranded RNA at "G" (Fig. 2C). Both the sense and anti-sense (negative control) of RP-A were digested almost completely, leaving only small ($<$50 nt) and faint bands corresponding to the undigestable region. However, the sense strand of RP-B was partially protected from digestion, leaving discrete bands of $>$100 nt, which indicates double-stranded regions resistant to RNase T1 digestion. Interestingly, the anti-sense strand of RP-B was digested almost completely, in contrast to the sense strand. These results indicate the existence of a stable secondary structure through internal base-pairing in the 5′ region of *ccn1* mRNA, further suggesting a capability of acting as a *cis*-element in the regulation of *ccn1*.

DIRECT BINDING OF CYTOSOLIC FACTOR(S) TO THE STRUCTURED REGION OF CCN1 mRNA

In general, protein counterparts are required for an element to regulate gene expression. Therefore, by utilizing RNA electromobility shift assay (REMSA) methodology, we next investigated the existence of a possible *trans*-factor protein(s) that could bind to this putative *cis*-element. As shown in Figure 3A, incubation of the folded sense-strand RNA of the corresponding region (RP-B S) with 5–10 μg of the cytoplasmic extract from CEF cells resulted in the retardation of the RNA in the gel. In contrast, no shifted band was observed after the incubation of the sense-strand RNA from the cDNA region with the internal 209-nt deletion (RP-A S), either with the nuclear or cytoplasmic extract, indicating that the interaction depended upon the 209-nt region. Western blotting analysis (Fig. 3A) revealed that lamin B1 (a marker of the nuclear fraction) and alpha-tubulin (a marker of the cytoplasmic fraction) were present only in the nuclear and cytoplasmic extract, respectively, confirming no cross contamination between each fraction. Furthermore, pre-incubation with unlabeled folded competitor RNA abolished the formation of a complex between RP-B S and cytoplasmic protein (Fig. 3B). Together with the fact that all binding reactions were carried out in the presence of 190 ng of non-specific tRNA, this result indicates that the interaction of the cytoplasmic protein with RP-B S RNA fragment was specific.

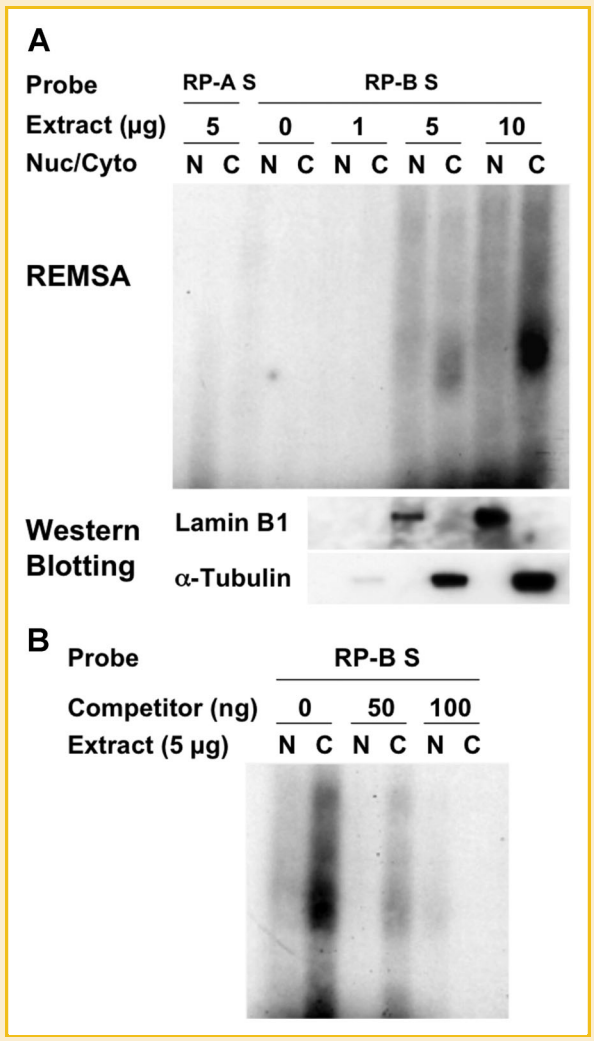

Fig. 3. Binding profile of chicken nuclear or cytosolic factors to the *ccn1* RNA segments, as evaluated by RNA gel electromobility shift assays (REMSA). A: Binding analysis of the *ccn1* mRNA segments to nuclear or cytosolic protein. The radio-labeled and folded RNA probes (RP-A S or RP-B S) were incubated with 0, 1, 5, or 10 μg of nuclear (N) or cytosolic (C) protein extract. After RNase digestion, the complex was subjected to 6% native PAGE. Western blotting analysis of the lamin or alpha-tubulin in the indicated amount of the extract was also performed to confirm successful subcellular fractionation in these experiments. B: Competition analysis to confirm the specificity of the interaction. Five micrograms of nuclear (N) or cytosolic (C) protein extract was pre-incubated with 0–100 ng of unlabeled RP-B S RNA as a competitor, followed by incubation with radio-labeled RNA probe (RP-B S). After RNase digestion, the complex was subjected to 6% native PAGE. The data in both panels are representative of two separate experiments, yielding comparable results.

THE RP-B FRAGMENT OF CCN1 cDNA-ENHANCED GENE EXPRESSION IN CIS

The results described in the previous subsection suggest the collaboration of the 5′-end ORF portion of *ccn1* mRNA and the cytoplasmic binding protein to exert post-transcriptional regulation of *ccn1*. Therefore, we evaluated the validity of this hypothesis by employing a system of chimeric firefly luciferase fusion gene constructs [Kubota et al., 1999, 2000; Mukkudai et al., 2003]. Initially, RP-B cDNA was minimally modified by deleting the "ATG" initiation codon to avoid translation interference and inserted in the sense direction at the 5′- or 3′-end of the firefly luciferase gene in a parental expression plasmid, pGL3L(+) (Fig. 4A). The resultant plasmid constructs were designated pGL3-5′-RPB′ and pGL3-3′-RPB′, respectively. The parental and two chimeric expression plasmids were subjected to a calibrated transient expression assay using CEF cells, with *Renilla* luciferase (pRL-TK) co-expression as an internal control. As demonstrated in Figure 4B, both pGL3-5′-RPB′ and pGL3-3′-RPB′ enhanced reporter gene activity in comparison with pGL3(+). However, importantly, the fragment RP-B located at the 5′-end of the reporter gene enhanced the reporter gene expression much more strongly (approximately 3.5-fold vs. control) than that at the 3′-end (approximately 1.5-fold vs. control). The difference in enhancing effects on the reporter gene expression between these two chimeric genes indicates not only that the 5′-end of the ORF of *ccn1* mRNA acted as an enhancing *cis*-element, but also that the effect was site dependent.

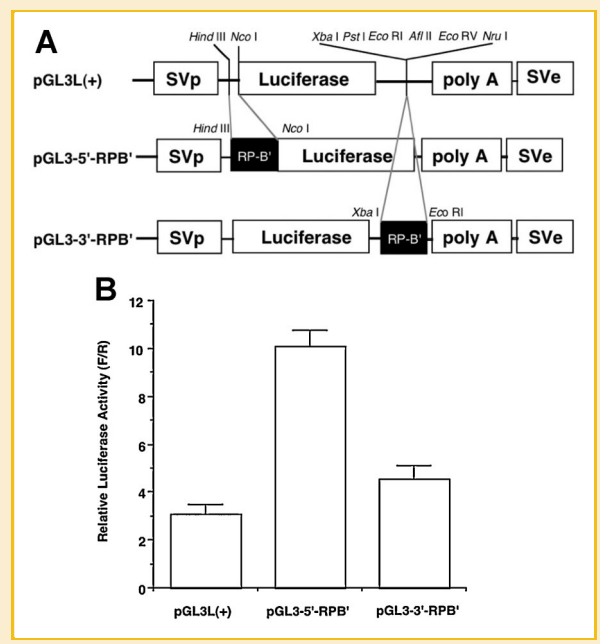

Fig. 4. *cis*-enhancing activity of RP-B S on reporter gene expression. A: Structures of the plasmids utilized in this evaluation. The pGL3L(+) was derived from pGL3-control vector (Promega), as described previously (Kubota et al., 1999, 2000; Mukudai et al., 2003) and contains restriction enzyme cleavage sites as indicated. Both pGL3-5′-RPB′ and pGL3-3′-RPB′ were derived from pGL3L(+), and thus, the basic structure of every plasmid was the same. In pGL3-5′-RPB′, RP-B cDNA was inserted between *Hin*dIII and *Nco*I sites upstream of the firefly luciferase gene. On the other hand, in pGL3-3′-RPB′, RP-B cDNA was inserted between *Xba*I and *Eco*RI sites, downstream of the firefly luciferase gene. Abbreviations: SVp, SV40 promoter; SVe, SV40 enhancer; polyA, SV40 poly-adenylation signal; Luciferase, firefly luciferase gene. B: Firefly luciferase activities from the plasmid in panel A in CEF cells. CEF cells were co-transfected with one of the plasmids in panel A and pRL-TK (Promega: as an internal control). After 2 days, a Dual Luciferase Assay (Promega) was carried out. Activity levels are represented as relative values of the measured luminescence of firefly luciferase versus *Renilla* luciferase. Mean values of the results of three experiments are displayed with error bars of standard deviations.

EFFECTS OF THE RP-B FRAGMENT ON THE RIBOSOMAL ASSEMBLY OF LINKED mRNA

It is now recognized that RNA *cis*-elements play important roles in post-transcriptional regulation in collaboration with nuclear and/or cytosolic *trans*-factor protein(s) at various stages, such as stabilization or destabilization of mRNA, transportation from the nucleus to the cytosol and ribosomal entry of mRNAs. Based on this knowledge and the results of the reporter gene assay (Fig. 4), we evaluated the effect of the fragment of *ccn1* mRNA on the ribosomal association of mRNA. The total RNA and ribosomal RNA fractions of the CEF cells, into which the chimeric constructs described in the previous subsection were transfected, were subjected to an RNA protection assay, with a 400-nt RNA of the firefly luciferase gene probe (Fig. 5). As a result, total luciferase mRNA level was significantly, but modestly (<2-fold) increased by the addition of the RP-B fragment at the 5′-end, whereas it enhanced the ribosomal association of the reporter gene mRNA much more strongly. Indeed, it was fivefold higher than the control value and was consistent with the result of the reporter gene assay. In contrast, RP-B fragment at the 3′-end conferred no significant effect on the ribosomal entry of the *cis*-lined luciferase RNA. These results indicate the RP-B segment to function as a post-transcriptional regulatory segment that predominantly enhances the ribosomal association of the mRNA when located *in cis* at the 5′-end.

SECONDARY STRUCTURE OF THE GC-RICH REGION IN THE RP-B SEGMENT

In order to further confirm the secondary structure formation and to analyze the structure actually formed in solution, we performed an RNase T1 protection analysis of the RP-B segment. Unless forming a secondary structure, the *cis*-regulatory element was anticipated to highly susceptible to RNase T1 digestion, since it was characterized by quite high GC-content. As observed in Figure 6A, the anti-sense form of the RP-B segment was digested into small pieces by RNase T1. In contrast, three major RNA fragments of 50–100 bases in the RP-B sense transcript were resistant to RNase T1, whereas no longer than 22 base fragment can be expected without secondary structure. To gain more insight into the structure of the *cis*-regulatory element in the RP-B segment, we analyzed the region truncated through regular PCR in the initial experiments in silico. The computer program predicted that this region would form a stable secondary structure through internal base-pairing; indeed more than 65% of the bases in this region would be able to be paired by hydrogen bonds (Fig. 6B). Thus, it was strongly suspected that the protected RNA segments observed in Figure 6A could originate in this minimal GC-rich segment. In order to confirm this point, we repeated analytical RNase protection assay with the minimal RNA segment (RPC) and its subfragments (RPF and RPL; Fig. 6C). Consequently, an RNA segment in RPC was distinctly protected from RNase T1 digestion. In contrast, RPL, the latter-half subfragment of RPC was totally degraded by the RNase. It should be noted that an RNA segment in RPF, the former-half fragment was also protected. As such, these major bands were anticipated to originate in the two stem loops formed at the upstream side of the GC-rich region. These findings further provided a structural basis for this segment to function as an RNA *cis*-regulatory element.

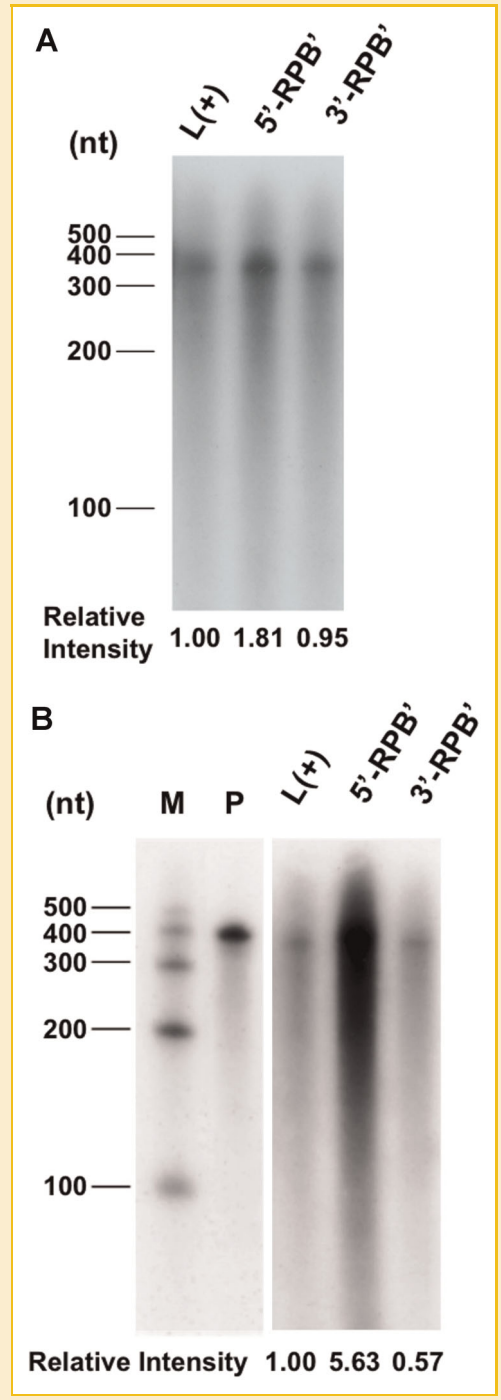

Fig. 5. RNase protection analysis of the luciferase mRNAs with or without RP-B expressed in CEF cells. CEF cells were transfected with each plasmid shown in Figure 4. After 2 days, total RNA (A) and the ribosomal fraction RNA (B) were collected and purified (see Materials and Methods section). Two micrograms of each RNA was subjected to the RNase protection assay. The free probe (P) and protected probe fragments of each sample were separated by denaturing 6% PAGE. M represents RNA molecular size markers, with the sizes indicated at the left of the panel. Relative ribosome-associated mRNA levels versus that of L(+) are shown below the autoradiogram. The data are representative of three separate experiments.

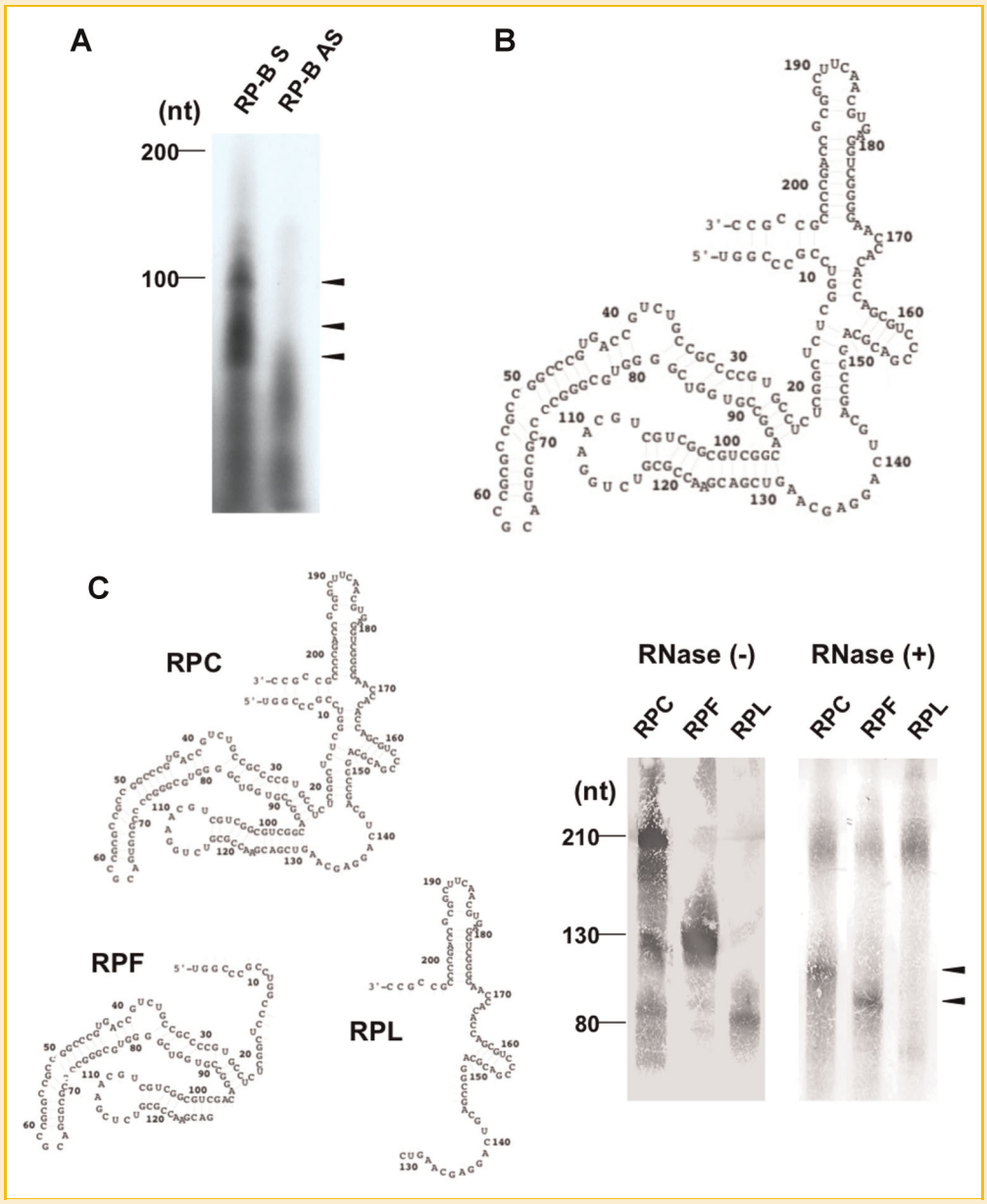

Fig. 6. Secondary structure of the GC-rich region. A: Analytical RNase protection assay of the folded RP-B RNA segment. Three major protected RNA fragments in denaturing gel electrophoresis are indicated by arrowheads (right). Positions of RNA chain-length markers are shown at the left. B: Predicted secondary structure of the 209-base region truncated during RT-PCR of the RP-B segment. Numbers are counted from the upstream boundary of the amplified and truncated areas. C: RNase protection assay of the minimal GC-rich region and its subfragments. Left panel represents the RNA structures of the entire 209-base region and its subfragments. Each in vitro transcribed RNA was folded and subjected to denaturing gel electrophoresis with or without RNase T1 digestion, which is displayed in the right panel. Approximate chain lengths of the undigested RNAs are given at the left, while protected bands are pointed by arrowhead at the right.

EVOLUTIONARY CONSERVATION OF THE GC-RICH REGION AMONG VERTEBRATES

Generally, important nucleotide sequences are highly conserved among species that need the function of the corresponding regions. In this context, we compared the cDNA sequences of the GC-rich region in the *ccn1* gene among chick, mouse and human species. Alignment of the three sequences clearly indicated that the GC-rich region has been highly conserved during the evolution of vertebrates (Fig. 7A). This is partly because these sequences are involved in the ORF encoding CCN1. However, the results of maximum-matching analysis in comparison with those of the other ORF regions clearly indicated that the GC-rich region has been further conserved. Indeed, the matching score was 7–8% higher in the GC-rich regions than the others (Fig. 7B). These findings suggest the functional significance of the GC-rich segment in the natural context, at least in vertebrates.

FUNCTIONAL CONFIRMATION OF THE MINIMAL STRUCTURED REGION AS A REGULATORY ELEMENT

Finally, to examine if the GC-rich structured region of 209 bases was necessary and sufficient to enhance gene expression *per se*, the

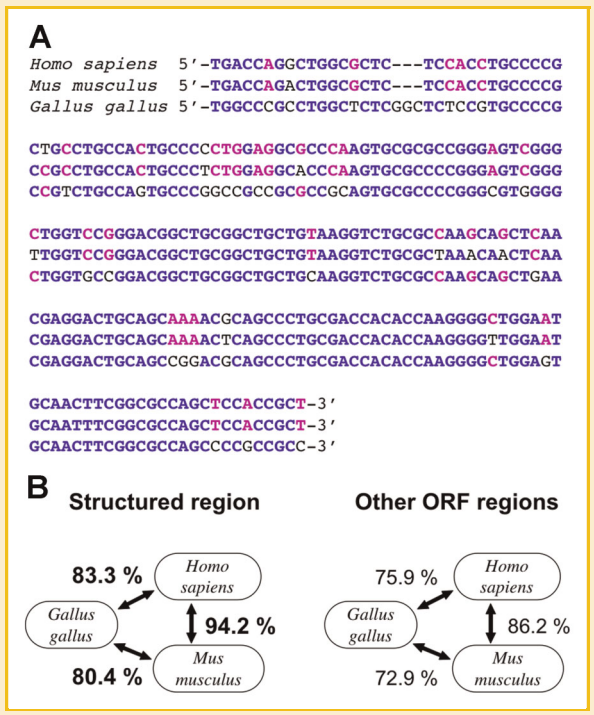

Fig. 7. Evolutionary conservation of the GC-rich region. A: Alignment of the cDNA sequences of the GC-rich regions in the *ccn1* genes from three vertebrates. Nucleotide sequences conserved among the three are shown in blue bold face letters, whereas those conserved between two species are shown in purple. NCBI accession numbers are; NM_001554.4 for *homo sapiens*, NM_010516.2 for *mus musculus*, and NM_001031563.1 for *gallus gallus*, respectively. B: Comparison of maximum matching scores between two species shown in panel A. The matching scores of the GC-rich structured region and those of the other ORF regions are shown at the left and right, respectively.

corresponding cDNA was constructed by assembling four synthetic oligonucleotides into a parental reporter construct at the upstream end of the luciferase gene. Two chimeric constructs containing the subfragments of the structured region were constructed as well (Fig. 8A). The results of DNA transfection and luciferase assay clearly revealed that the minimal element, RPC, was a positive *cis*-regulatory element *per se* (Fig. 8B). Interestingly, the former-half of the element, RPF, containing two upstream loops was found to be as potent as the entire element, whereas the latter-half, RPL showed no activity. These findings suggest the critical requirement of the two loops for the regulatory function.

DISCUSSION

The *ccn1* mRNA is approximately 1.8 kb-long, and its overall GC-content is <50%; whereas that of its 5′ region (1–500 bases from the initiation codon) of the ORF is nearly 70%. We initially found that the PCR products of the corresponding region were distinctly shorter than the expected, by approximately 200 bp (Fig. 1; RP-A). In such a case, involvement of alternative splicing products may be suspected. However, since the PCR product recovered the expected length (Fig. 1; RP-B) in the presence of PCRx Enhancer Solution, a PCR supplement for problematic and GC-rich templates, we concluded that the shortening to RP-A was not the result of an alternative splicing of mRNA, but of miselongation by DNA polymerase. This conclusion was further supported by the reproduction of the same results by the experiments with purified RP-B DNA (data not shown). Nucleotide sequencing of RP-A (Fig. 1A) demonstrated that this shortening arose from the consistent deletion of a central 209-bp segment with 73.5% GC-content. It was previously reported that DNA polymerases, including *Taq* DNA polymerase, skip a large region during the synthesis of template DNA, if the region has a stable secondary structure [Dignam et al., 1983; Hew et al., 1999, 2000; Kontoyiannis et al., 2001]. Hence, the observations above indicate a stable secondary structure in the 5′ region of the ORF of *ccn1* cDNA, over which the elongation of DNA continued, skipping the site of the secondary structure during PCR.

Next, the results of the analytical RNase protection assay of the folded RNA (Fig. 6) revealed that the radio-labeled RNAs corresponding to the 5′-region (RP-B) and the minimal structured region (RPC) were resistant to digestion with RNase, indicating the presence of a stable secondary structure in the GC-rich region on the RNA as well. Digestion of RPC as well as RP-B S with RNase T1 resulted in a residual product of 100-nt in length. These fragments are most likely to have arisen from the digestion of the structured region at the major loops that are assumed to be sensitive to RNase T1. Additionally, our other results showing that RNase T1 was able to completely digest both the sense and anti-sense strands of RP-A and the anti-sense strand of RP-B (Fig. 2C) confirmed the specific formation of a secondary structure in the GC-rich RP-B region of the ORF in the *ccn1* mRNA.

The stable secondary structure of the 5′-region of the ORF of *ccn1* mRNA suggests that the fragment may play a role in the regulation of gene expression as a *cis*-element. Therefore, REMSA (Fig. 3) was performed, and the results clearly demonstrated the specific binding of a cytoplasmic protein or multimolecular complex of proteins from CEF cells to the sense strand of the RP-B region. Since no such binding was observed for RP-A, we concluded that the secondary structure was required for the binding between the *ccn1* mRNA and the cytoplasmic protein(s). Of note, no binding protein was detected in the nuclear fraction, suggesting a cytoplasmic event, in which this mRNA segment might play a significant role (Fig. 3B).

Finally, luciferase reporter gene analysis (Fig. 4) revealed an enhancing effect of the corresponding region (RP-B) on the gene expression. Importantly, the enhancing effect of RP-B′ was much stronger at the 5′-region of the reporter gene than at the 3′-region, indicating a location-dependent enhancing effect. Since RP-B is originally located in the 5′-region of the *ccn1* ORF, this segment ought to contribute to the enhancement of *ccn1* gene expression in the natural context. Indeed, nucleotide sequence alignment of the GC-rich regions from three vertebrate species showed that this region has been highly conserved during evolution (Fig. 7A). Of note, the matching scores representing evolutionary conservation were markedly higher in the GC-rich region than the other regions of the ORF (Fig. 7B). These findings indicate another indispensable function of the GC-rich region than encoding protein sequences. Consistent with this indication, the minimal structured segment was confirmed to be functional *per se*. However, the enhancing effect by

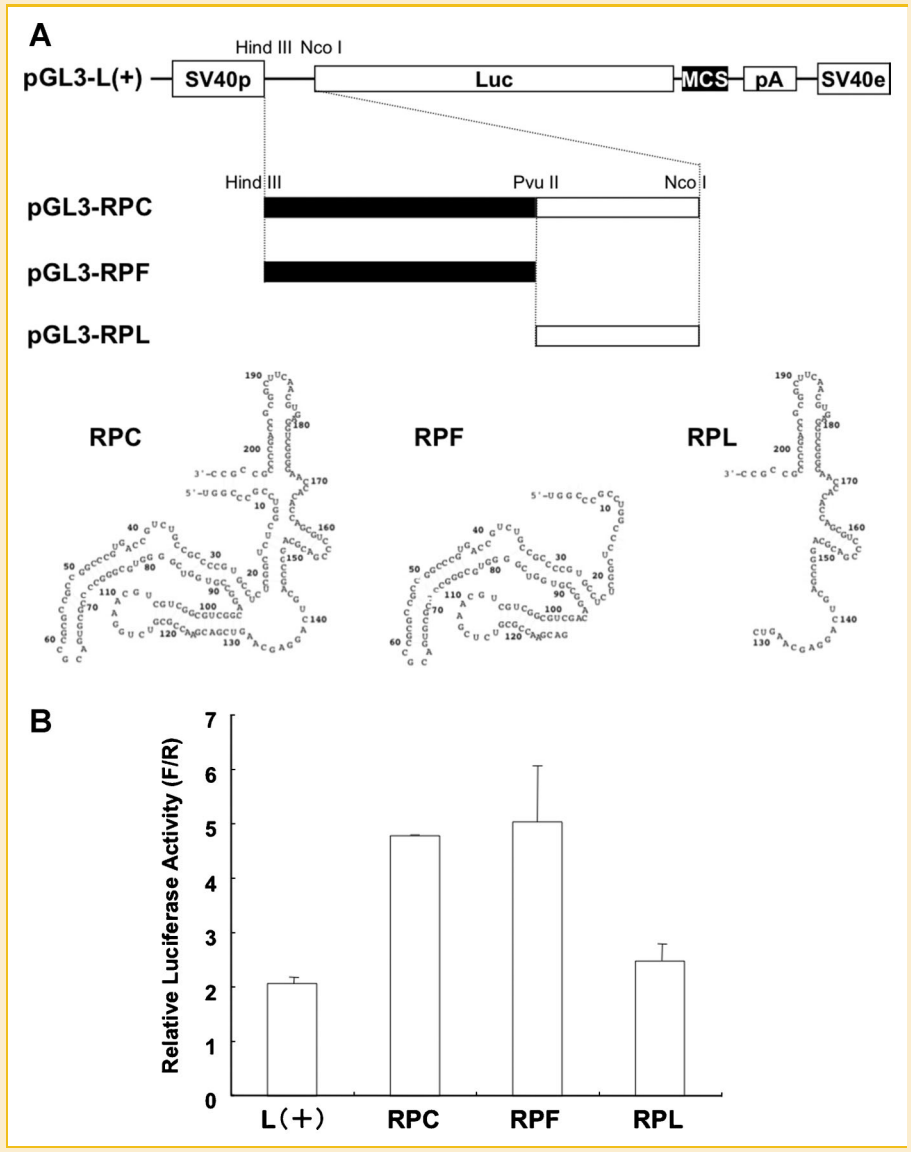

Fig. 8. Functional definition of the GC-rich region as a regulatory element. A: Construction of the plasmids for the evaluation of the GC-rich region and its subfragments. Structures of the parental plasmid and built-in *ccn1* fragments are displayed, together with the names of the resultant reporter plasmids. B: Firefly luciferase activities from the plasmids described in panel A. Experiments and computation of the data obtained were performed essentially as described in the legend to Figure 4. The data are representative of two sets of independent experiments.

this element, RPC, was not so strong as that by RP-B′, as yet suggesting a functional contribution of another segment in RP-B′.

Several reasons can be considered to account for the mechanism of the observed effect of the RP-B′ on gene expression in *cis*. It is widely recognized that a number of mRNA *cis* elements, especially those in the UTRs, possess signal sequences for mRNA export from the nucleus [St Johnston, 1995]. Also, a number of structured RNA elements that affect mRNA translation have been described. For example, in another CCN family member, CCN2, *cis*-acting element of structure-anchored repression was discovered [Kondo et al., 2000; Kubota et al., 2000, 2005]. Furthermore, our group recently clarified that chicken *ccn2* mRNA level is regulated by selective mRNA degradation under the collaboration of a structured mRNA element (5′–100/50) and nucleophosmin/B23 [Mukudai et al., 2008]. Additionally, regulation of *ccn2* gene by miR-18a *via* a target in the 3′-UTR has also been reported [Ohgawara et al., 2009]. In the present study, the RNase protection assay revealed that RP-B markedly increased the amount of the corresponding mRNA in the ribosomal fraction (5.6-fold vs. control: Fig. 5B), whereas it slightly enhanced the level of total mRNA of the reporter gene (1.8-fold vs. control: Fig. 5A). These results suggest that the enhancing effect of RP-B′ mainly resulted from the promotion of transportation and/or entry of mRNA into the ribosome, with modest up-regulation of the steady-state expression level of mRNA. Therefore, the structured segment might possess the ability to transport the mRNA from the nucleus into ribosome through interaction with cytoplasmic protein(s), as observed in the case of internal ribosomal entry site (IRES) of viral mRNAs. As such, regulation of the translation process

is strongly suspected as the major function of RP-B at the original location in the *ccn1* mRNA. Studies are currently ongoing in order to address these issues.

CCN1 plays important physiological roles in cell growth [Kireeva et al., 1996; Babic et al., 1998], migration [Kireeva et al., 1996; Babic et al., 1998], adhesion [Kireeva et al., 1996, 1997, 1998; Jedsadayanmata et al., 1999; Chen et al., 2001] and differentiation [O'Brien and Lau, 1992; Mo et al., 2002] of a variety of cells. Moreover, CCN1 is also involved in tumorigenesis, either positively or negatively [Kireeva et al., 1996; Tsai et al., 2000; Tong et al., 2001; Xie et al., 2001; Juric et al., 2009]. In executing such various functions, CCN1 is thought to be under complex regulation not only at the transcriptional level, but also at the post-transcriptional level. In the present study, we discovered that the RP-B region of the ORF of *ccn1* mRNA was able to regulate gene expression through interaction with as yet some unidentified cytoplasmic protein(s). Moreover, we also demonstrated that this RNA segment enhanced gene expression mainly by altering the transportation and/or entry of the mRNA into the ribosome. It should be noted that RP-B region itself encodes part of the amino acid sequence of CCN1 and thus undergoes translation process. Therefore, identification of this binding protein(s) and characterization of its (their) interaction with eukaryotic initiation and elongation factors will uncover the entire regulatory system mediated by the RP-B segment of *ccn1* mRNA.

ACKNOWLEDGMENTS

The authors wish to thank Drs. Tohru Nakanishi, Takashi Nishida, Gen Yosimichi, Kumiko Nawachi, Norifumi H. Moritani, Kyoji Nakao and Eiji Nakata for helpful suggestions; Ms. Kazumi Ohyama and Ms. Harumi Kawaki for technical assistance; and Ms. Yuki Nonami for secretarial assistance. This study was supported by Grants-in-aid for Scientific Research from Japan Society for the Promotion of Science 19109008 (for M.T.) and 21592360 (for S.K.).

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