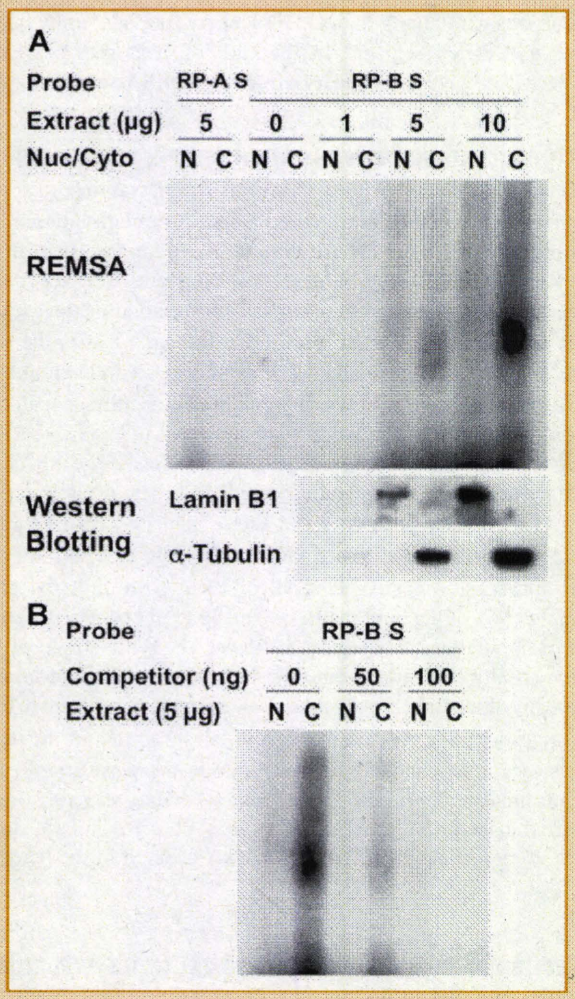


Fig. 3. Binding profile of chicken nuclear or cytosolic factors to the *ccn1* RNA segments, as evaluated by RNA gel electromobility shift assays (REMSA). A: Binding analysis of the *ccn1* mRNA segments to nuclear or cytosolic protein. The radio-labeled and folded RNA probes (RP-A S or RP-B S) were incubated 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 [32,39,40Q4]. 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.

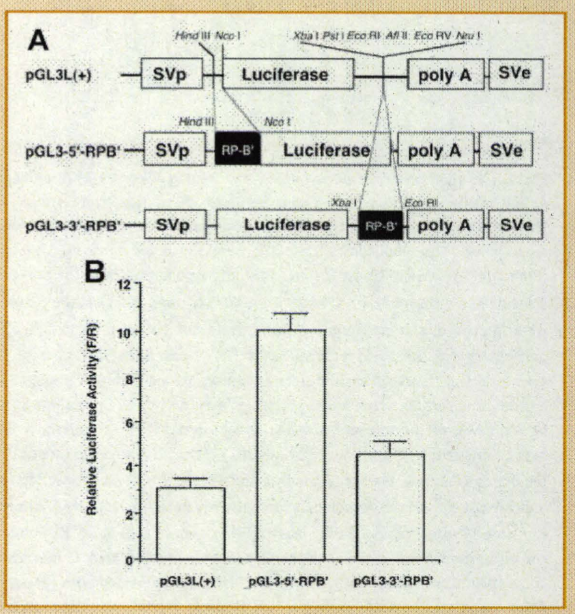


Fig. 4. *cis*-enhancing activity of RP-B S on reporter gene expression. A: Structures of the plasmids utilized in this evaluation. The pGL3L(+) was derived from pGL3-control vector (Promega), as described previously ([35,43,44]) and contains restriction enzyme cleavage sites as indicated. Both pGL3-5′-RPB′ 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 polyadenylation 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.

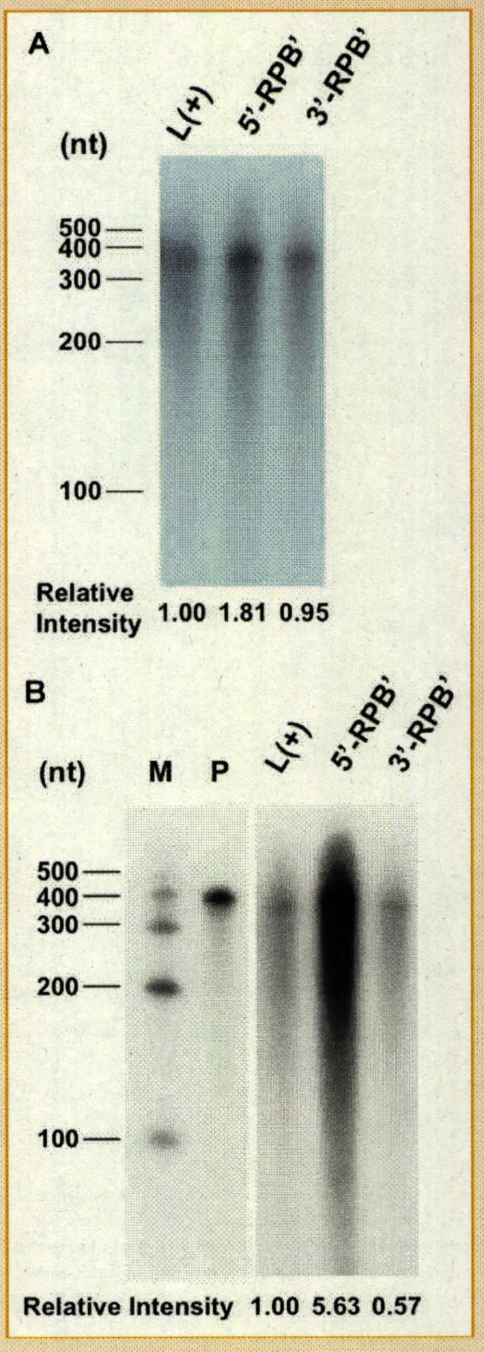


Fig. 5. RNase protection analysis of the luciferase mRNAs with or without RP-B expressed in CEF cells. CEF cells were transfected with each plasmid shown in Figure 4. After 2 days, total RNA (A) and the ribosomal fraction RNA (B) were collected and purified (see Materials and Methods section). Two micrograms 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.

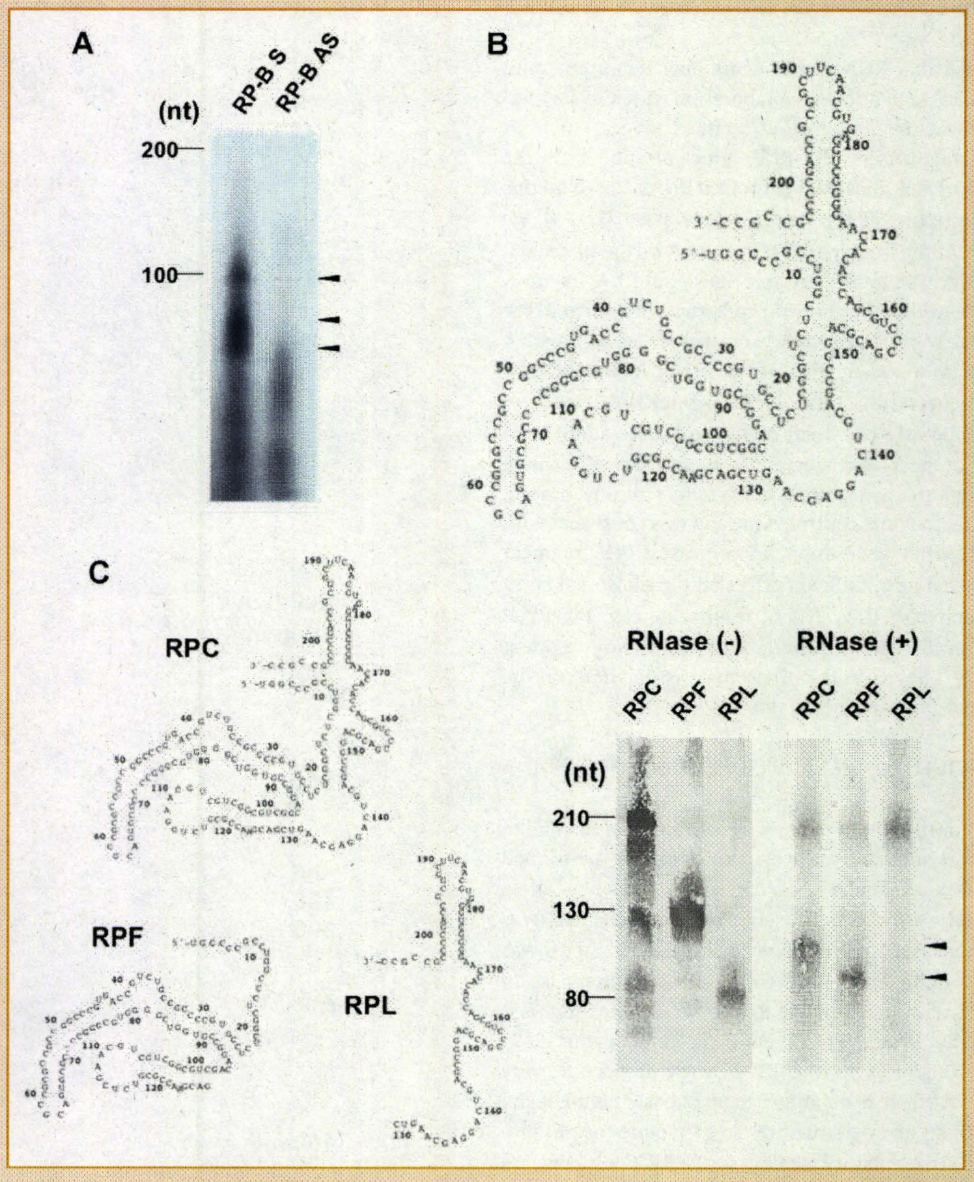


Fig. 6. Secondary structure of the GC-rich region. A: Analytical RNase protection assay of the folded RP-B RNA segment. Three major protected RNA fragments in denaturing gel electrophoresis are indicated by arrowheads (right). Positions of RNA chain-length markers are shown at the left. B: Predicted 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

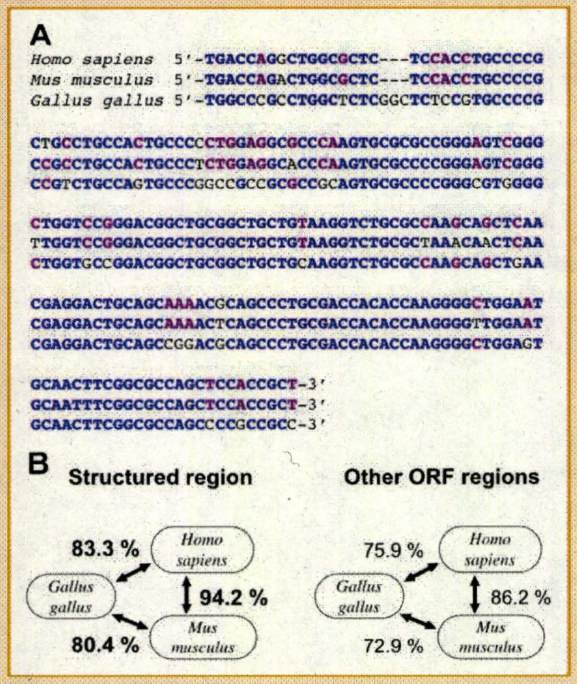


Fig. 7. Evolutionary conservation of the GC-rich region. A: Alignment of the cDNA sequences of the GC-rich regions in the *ccn1* genes from three vertebrates. Nucleotide sequences conserved among the three are shown in blue bold face letters, whereas those conserved between two species are shown in purple. 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

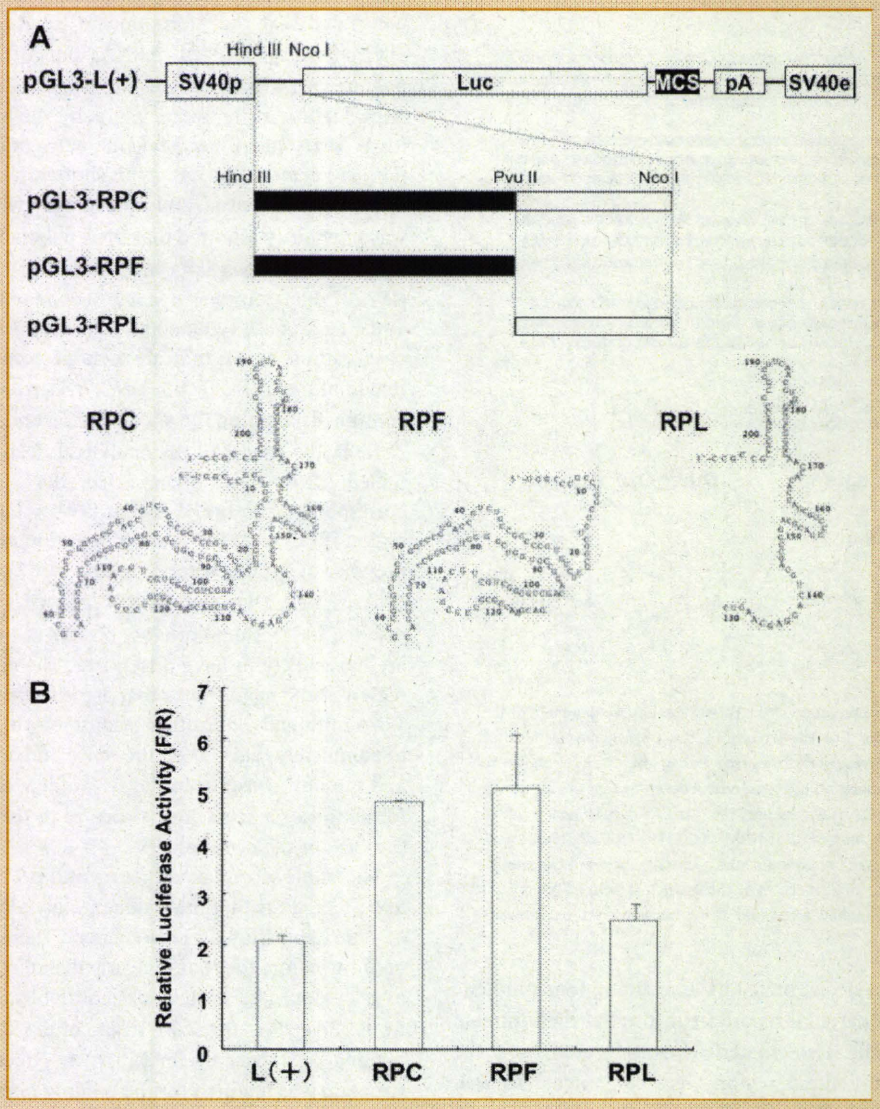


Fig. 8. Functional definition of the GC-rich region as a regulatory element. A: Construction of the plasmids for the evaluation of the GC-rich region and its subfragments. Structures of the parental plasmid and built-in *ccn1* fragments are displayed, together with the names of the resultant reporter 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, 1997[05]; 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, Seiji Kondo, 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 Japan Society for the Promotion of Science 19109008 (for M.T.) and 21592360 (for S.K.).

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