Identification of a Promoter for the Human C1q-Tumor Necrosis Factor–Related Protein-5 Gene Associated with Late-Onset Retinal Degeneration

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Purpose. The Complement-1q tumor necrosis factor-related protein 5 (*C1QTNF5/CTRP5*) gene is located in the 3′ untranslated region of the Membrane Frizzled Related Protein (*MFRP*) gene, and these two genes are reported to be dicistronic. The authors examined the 5′ upstream sequence of *CTRP5* for the presence of a promoter regulating the expression of this gene.

Methods. The sequence upstream of the translational start site of human *CTRP5* (*hCTRP5*) was analyzed by Promoter Inspector software. A series of plasmids containing segments of *hCTRP5* putative promoter sequence (−29 bp to −3.6 kb) upstream of the luciferase gene were generated. Cells were transiently transfected with these plasmids, and luciferase activity was measured. 5′ RACE analysis was performed to determine the functional transcription start site. V5 tagged-pig *CTRP5* (*pCTRP5*) gene, cloned downstream of the *hCTRP5* putative promoter, was expressed in a human retinal cell line (ARPE-19) and a Chinese hamster ovary cell line (CHO-K1) to study the functionality of the putative promoter.

Results. Bioinformatic analysis identified a putative promoter region between nt −1322 and +1 sequence of *hCTRP5*. 5′ RACE analysis revealed the presence of the transcriptional start site (TSS) at 62 bp upstream of the start codon in the *CTRP5*. The 1.3-kb sequence of the *hCTRP5* predicted promoter produced higher levels of luciferase activity, indicating the strength of the cloned *CTRP5* promoter. The promoter sequence between nt −1322 bp to −29 bp upstream of the first ATG of *CTRP5* was found to be essential for this promoter activity. The predicted *hCTRP5* promoter was found to control the expression of V5-tagged *pCTRP5* and nuclear GFP, indicating that the promoter was functional.

Conclusions. This study revealed the presence of a functional promoter for the *CTRP5* gene located 5′ of its start site. Understanding the regulation of *CTRP5* gene transcription may provide insights into the possible role of CTRP5 in the retina and the pathology underlying late-onset retinal degeneration caused by mutations in this gene. In addition, these studies will determine whether *CTRP5* and *MFRP* are functionally dicistronic. (*Invest Ophthalmol Vis Sci.* 2010;51:5499–5507) DOI:10.1167/iovs.10-5543

Several forms of late-onset retinal degeneration, including age-related macular degeneration (AMD), have been described in patients. AMD is a complex disorder involving genetic, environmental, and nutritional factors that contribute to the disease. Approximately 1.7 million Americans older than 65 are affected with AMD.[1] At least 11 genes associated with AMD have been identified.[2,3] Among these genes, a significant number implicate alterations in the complement pathway or the immune response pathway as the cause of retinal degeneration. In addition to the complex phenotype of AMD, monogenic late-onset retinal degenerations have also been described.[4–10] The late-onset retinal degeneration caused by a Ser163Arg mutation in the Complement 1q-tumor necrosis factor related protein-5 is one of the Mendelian diseases with a phenotype similar to that of AMD.[10,11] Clinical symptoms of late-onset retinal degeneration (LORD) include drusen at early stages of the disease and neovascularization at late stages. In addition, patients also develop abnormal anterior lens zonules at a young age.[12–15] The C1QTNF5/CTRP5 protein has been shown to interact with complementary factor H, which has been reported as a major genetic factor associated with AMD and an early-onset recessive drusen phenotype.[15] Understanding the biological function of *C1QTNF5/CTRP5* and regulation of its expression may provide insight into understanding the role of this gene in the normal retina and in the pathology of retinal degenerations including late-onset retinal degeneration and AMD.

The CTRP5 protein is a glycoprotein that contains a globular C1q domain and a short-chain collagen sequence. It exists in both membrane-bound and secreted forms and is expressed predominantly in the retinal pigment epithelium (RPE), lens, and ciliary body in ocular tissue; several other tissues also express low amounts of this gene transcript.[16] A high amount of CTRP5 expression is also found in adipose tissue.[17] It has been recently reported that the expression levels of CTRP5 increase in myocytes with depleted mitochondria, which, in turn, stimulates adenosine monophosphate (AMP)–activated protein kinase.[18] Furthermore, serum levels of CTRP5 were found to be significantly higher in obese/diabetic animals than in normal controls.[18] Understanding the regulation of CTRP5 expression may reveal the potential function of CTRP5 in different physiological conditions.

The *CTRP5* gene is reported to be a dicistronic partner of a membrane-type frizzled related protein (*MFRP*).[10] Studies on the *CTRP5* gene revealed that the open-reading frame of the human and mouse *CTRP5* gene is located in the 3′-untranslated region of the *MFRP*. The *MFRP* gene encodes a glycosylated transmembrane protein with an extracellular Frizzled-related cysteine-rich domain.[19] It is specifically expressed in the RPE and ciliary body.[16] A recessive mutation in the *MFRP* gene

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causes retinal degeneration in the rd6 mouse model.[20] In addition, in humans mutations in the *MFRP* gene are associated with nanophthalmos, retinitis pigmentosa, foveoschisis, optic disc drusen, and hyperopia.

Studies on MFRP and CTRP5[21,22] suggested the possible expression of CTRP5 independently of MFRP.[20] In this study, we evaluated the potential promoter activity of the 5′ upstream sequence of *CTRP5* and identified a putative promoter sequence that may regulate the expression of the *CTRP5* gene independently of *MFRP*. Additional studies on the *CTRP5* promoter will help us understand the regulation of *CTRP5* expression and its potential role in the pathology of late-onset retinal degeneration.

Materials and Methods

Antibodies

We raised rabbit anti–CTRP5 polyclonal antibodies; purification and characterization of these antibodies were described elsewhere.[16] Anti–rabbit and anti–mouse secondary antibodies conjugated to horseradish peroxidase (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), and anti–rabbit AlexaFluor-555 (1:2500 dilution; Invitrogen-Molecular Probes, Carlsbad, CA) were obtained from the commercial sources indicated.

Prediction of Core Promoter, Transcription Factor Binding Sites, and Insulators in the Upstream Region to *CTRP5*

A 4-kb sequence upstream from the transcription start site of the *CTRP5* gene was analyzed using genomic analysis software (Promoter Inspector; Genomatix, Munich, Germany).[23] The predicted promoter region was further confirmed with promoter prediction software (Gene2Promoter; Genomatix), which predicts the genomic context of eukaryotic polymerase II promoter regions with high specificity in mammalian genomic sequences, based on equivalence classes of International Union of Pure and Applied Chemistry words. The sequence containing *CTRP5* and its 5′ upstream sequence was used as input for this analysis. The identified region was marked as a true positive if a transcription start site was located within or up to 200 bp downstream of the predicted promoter region. Vertebrate transcription factor binding sites were identified with transcription factor analysis software (MatInspector; Genomatix).[24] The putative *hCTRP5* promoter sequence was compared for homology with its orthologous sequences using T-coffee multiple sequence alignment program (http://www.ebi.ac.uk/Tools/t-coffee/). We also checked for the presence of potential insulator regions between the *MFRP* and *CTRP5* genes by screening both MFRP- and CTRP5-encoding genomic DNA sequences using an Insulator database (http://insulatordb.utmem.edu/).

Cloning of the *CTRP5* Promoter and Luciferase Reporter Constructs

PCR primers (5′-GCTCCTGTGTGGGAAGCTTG-3′ and 5′-GGGTCCTCTCGCAGTCTGTG-3′) were used to amplify 1309 bp of the predicted *hCTRP5* promoter. The *hCTRP5* promoter was cloned upstream of the wild-type pig *CTRP5* (*pCTRP5*) or pig *CTRP5* with S163R mutation into pEF6/V5-His TA expression (TOPO; Invitrogen). These constructs were used for transfections and immunocytochemistry analysis. For the luciferase assays, a set of five *CTRP5* promoter deletion constructs was made by amplifying selected fragments of the putative promoter region and cloning them into vector (pGl3-Enhancer Vector; Promega, Madison, WI) (see Fig. 2), as described. The reverse primer 5′-cacacaggagcaaggcgaat-3′ was used in combination with the primers 358 del (5′-ggagaagcgcgggggctggag-3′), 647 del (5′-actgccctgcctctcctctgg-3′), and 1003 del (5′-cagggaggggggaagagaagtc-3′) to produce the plasmids with 385 bp, 647 bp, and 1003 bp of promoter sequence and the primer set 3.6 CTRP5 forward primer (5′-gaattgctccacctatgcct-3′) and 3.6 CTRP5 reverse primer (5′-cggggtcctctcgcagtctgtg-3′) was used to clone a 3.6-kb region from a Bac clone (R1133466) into the vector (pGl3-Enhancer; Promega). A plasmid with nuclear EGFP reporter (phPRO-nEGFP) (a gift from Kevin D. Wells, University of Missouri, Columbia, MO) downstream of the *CTRP5* promoter (1309 bp) was also constructed.

Dual Luciferase Reporter Assay

The *hCTRP5* promoter luciferase reporter plasmids, along with the internal control plasmid pRL-TK, were cotransfected into CHO-K1 cells. The luminescence in lysates of harvested cells was measured 48 hours after transfection with the dual luciferase reporter assay protocol as recommended by Promega. For luciferase assays, cells were grown in 24-well plates and washed twice with PBS, and lysates were prepared by incubation with lysis buffer (Passive Lysis Buffer; Promega) for at least 10 minutes. Twenty microliters of cell lysate was transferred into a 96-well flat-bottom plate (Costar; Corning, Corning, NY) containing 100 μL luciferase assay reagent. Firefly luciferase activity (M1) was measured first, and then Renilla luciferase activity (M2) was measured after the addition of 100 μL reagent (Stop & Glo; Promega). The ratio of M1/M2 was the relative luciferase activity of the experimental plasmid (pGl3-Enhancer; Promega). Three replicates were analyzed for each transfection set, and experiments were performed at least three times. Results are shown as mean ± SD fold induction of luciferase in at least three independent experiments.

RNA Ligase-Mediated Rapid Amplification of 5′ cDNA Ends (5′RLM-RACE)

The transcription start site of the *hCTRP5* promoter was determined using 5′ RLM-RACE with an amplification kit (GeneRacer; Invitrogen) according to the manufacturer's protocol. Briefly, total RNA (6 μg) isolated from CHO-K1 cells transfected with 1309 bp vector (pGl3-hCTRP5 Enhancer; Promega) was treated with calf intestinal alkaline phosphatase to remove the 5′ phosphates from truncated mRNA. The dephosphorylated RNA was then treated with tobacco acid pyrophosphatase to remove the 5′ CAP structure from the intact, full-length mRNA. This treatment leaves a 5′ phosphate required for ligation. An RNA oligonucleotide (5′-CGACUGGAGCACGAGGACACUGACAUGGACUGAAGGAGUAGAAA-3′) (GeneRacer; Invitrogen) was ligated to the 5′ end of the mRNA using T4 RNA ligase. Subsequently, reverse transcription was performed using reverse transcriptase (SuperScript II) and oligo dT primer (5′-GCTGTCAACGATACGCTACGTAACGGCATGACAGTG[T18]-3′) (GeneRacer; Invitrogen). The cDNA from reverse transcription was amplified by PCR using the 5′ primer (5′-GCACGAGGACACUGACAUGGACUG-3′) and the 5′ luc primer (5′-CATACTGTTGAGCAATTCACG-3′) (GeneRacer; Invitrogen). The amplicon generated from the final PCR product was cloned into the cloning vector (PCR2.1 TOPO; Invitrogen) and sequenced.

Transfection of CHO-K1 and ARPE-19 Cells

Cells were transiently transfected with the expression constructs using reagent (Lipofectamine 2000; Invitrogen) according to the manufacturer's instruction. Transfection was performed in serum-free medium (Opti-MEM I; Invitrogen) with reagent (Lipofectamine 2000; Invitrogen). Four hours after transfection, the medium is replaced with serum-containing medium and incubated 36 hours before the experiments were performed. Mock transfections were performed using transfection reagents only. After 36 hours of transfection, cells were harvested for protein isolation.[25]

Western Blot Analysis

Cell lysates were prepared in lysis buffer (50 mM Tris-HCl [pH 7.4], 0.15 M NaCl, 1 mM EDTA, 0.1% Triton X-100, and 0.1% [wt/vol] SDS), containing protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO).

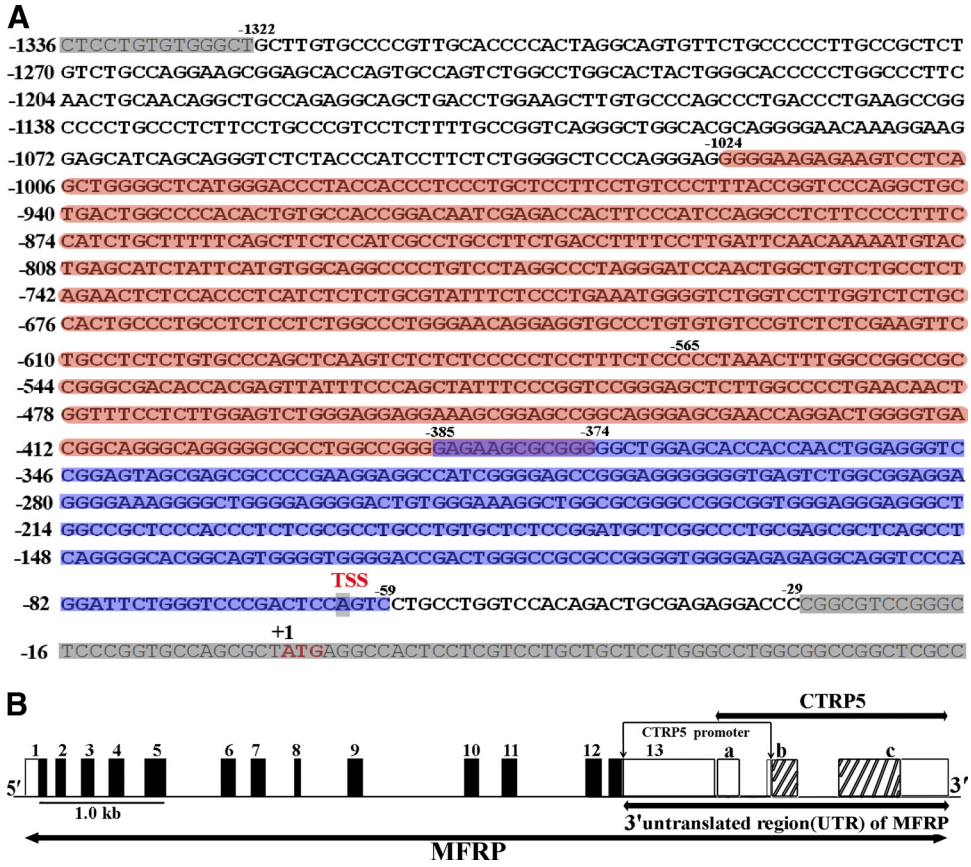

FIGURE 1. (**A**) The nucleotide sequence of the putative promoter region for the human *CTRP5* gene. *Top*: the 1.3-kb fragment (−1322 bp to −29 bp) of the 5′ region of the human *CTRP5* gene is shown. The sequence we cloned for the promoter analysis is shown in *bold*. The TSS, deduced from the 5′ RACE analysis, is located at −62 bp from the start of translation (ATG in *red* at +1). Predicted promoter regions −1024 bp to −374 bp (*pink*) and −385 bp to −59 bp (*blue*). (**B**) Schematic presentation of the *CTRP5* gene located in the 3′ UTR region of *MFRP* gene. The genomic structure of the *MFRP* and *CTRP5* genes is shown as reported earlier.[17] Exons corresponding to ORF are indicated by *closed boxes* (1–13) or *hatched boxes* (b–c), and exons corresponding to UTRs are shown as *open boxes*. Regions between the exons correspond to introns. The promoter of the *CTRP5* is located in its 5′ region.

All Western blot analyses were performed with denatured proteins (by adding 0.5% β-mercaptoethanol [βME] and 5 mM dithiothreitol [DTT] in SDS sample buffer) separated on 10% Tris-glycine gels.

Immunofluorescence Labeling of Cells

Immunostaining for CTRP5 protein and V5 epitope were performed according to published procedures using anti–CTRP5 antibody (1:2000), anti–V5 antibody (1:250), and Alexa Fluor-555 conjugated goat anti–mouse IgG (Molecular Probes, Eugene, OR).[26] Transfected CHO-K1 were used for the experiment as described.[25] Images were captured using a confocal microscope (LSM510; Carl Zeiss Meditec, Inc., Dublin, CA).

RESULTS

Identification of the Putative *CTRP5* Promoter

Although the *CTRP5* and *MFRP* genes are reported to be dicistronic, the presence of independent RNA transcripts for *CTRP5* was observed in the eye and brain indicating the possible expression of CTRP5 independent of MFRP.[20] We evaluated the upstream region of *CTRP5* for the presence of a potential promoter that can regulate the expression of CTRP5 independently of MFRP. The genomic sequence upstream of the human *CTRP5* gene was retrieved from the NCBI database (NW_925173.1). Analysis of the upstream 5′ region using software (Promoter Inspector) from the Genomatix suite predicted the presence of two transcription regulatory elements (−565 to −386, −205 to −117) and two predicted promoter regions (−1324 to −374 bp, −385 to −59 bp) (Fig. 1A). The genomic structure of the *CTRP5* and *MFRP* genes and the location of the putative *CTRP5* promoter are presented in Figure 1B.

To identify the core/minimal promoter region for the *CTRP5* gene, we generated a series of constructs containing parts of the predicted promoter sequence upstream of the luciferase gene. All these constructs were generated using pGl3 Basic or pGl3 Enhancer vectors, or both (Fig. 2). Measurement of luciferase activity in cell lysates transfected with these constructs revealed that the region between −29 bp to −3569 bp located 5′ to the *CTRP5* transcription start site had significant luciferase activity compared with the control (Fig. 3), whereas cells transfected with constructs containing smaller segments of the sequence between −386 bp to −29 bp, −675 bp to −29 bp, −1041 bp to −29 bp, −1322 bp to −922 bp, and −1322 bp to −1180 bp did not show significant luciferase activity. A minimal 1280-bp sequence from −1322 bp to −29 bp upstream of the *CTRP5* TSS showed the highest level of luciferase activity compared with the positive pGl3 control plasmid, which uses the strong SV40 viral promoter and all other constructs tested.

Characterization of the Human *CTRP5* Transcription Start Site

The reported sequence of the *CTRP5* gene contains three exons. Exon 1 is a noncoding exon. The start codon for *CTRP5* gene is present in exon 2, and the stop codon is present in exon 3. The entire sequence of CTRP5 is reported to be present in the 3′ UTR of MFRP.[10] Alignment of the protein sequence of human CTRP5 with its orthologues in mouse and pig showed that it has 98% amino acid homology with pig CTRP5 and 85% with mouse CTRP5.

To identify the functional promoter of *CTRP5*, we mapped the TSS of the human *CTRP5* gene. RLM-RACE was performed by transfecting CHO-K1 cells with the pGl3-

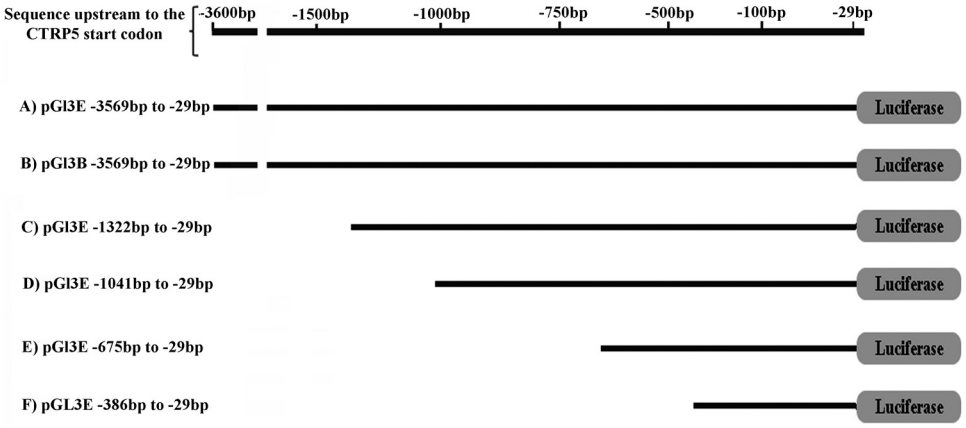

FIGURE 2. Schematic representation of the promoter deletion constructs generated in pGl3-Enhancer vector.

Enhancer plasmid, which contains the minimal *CTRP5* predicted promoter sequence (−1322 bp to −29 bp) driving the expression of luciferase. 5′ RACE performed on total RNA from the transfected CHO-K1 cells (Fig. 4, lane 2) with primers specific to the luciferase gene gave a single PCR product of the expected size (~500 bp). A total of six 5′RACE products were cloned and sequenced. These revealed a common 5′ transcription start site that is 62 nucleotides upstream of the start codon.

Identification of Regulatory Elements in the Promoter Region

Analysis of the putative *CTRP5* promoter region (~1.3 kb) upstream of the start site of the *CTRP5* gene using transcription factor analysis software (MatInspector; Genomatix) identified several possible transcription-factor binding sites within the sequence, including E-box binding factors (−538 bp to −526 bp), Activator-, mediator and TBP-dependent core promoter elements for RNA polymerase II transcription for TATA-less promoters, core promoter 10 elements, and 5 sites for SP1/GC binding sites (Table 1). The presence of TATA box or GATA box was not detected. The putative human *CTRP5* promoter sequence shares significant homology with orthologous sequences in *Bos Taurus*, *Mus musculus*, *Pan troglodytes*, *Rattus norvegicus*, and *Sus scrofa* with alignment scores of 88%, 78%, 99%, 78%, and 87%, respectively, indicating the evolutionary conservation of this region (Fig. 5). The presence of these regulatory regions in the conserved sequences of the *CTRP5* promoter further supports the importance of these transcriptional factor binding sites in regulating its expression. Additional analysis is needed to validate the functionality of these putative regulatory elements.

Analysis of the 5′ upstream sequence of *CTRP5* using the insulator database (http://insulatordb.utmem.edu/) identified the presence of two CTCF-binding sites and an insulator with 1199 bp (INSUL_ZHAO12305). This sequence is located between the stop codon of *MFRP* and the translation start codon

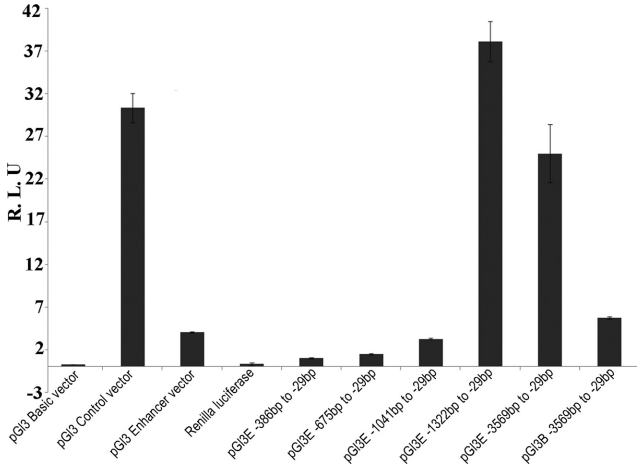

FIGURE 3. Deletion analysis of 5′ flanking region of the human *CTRP5* gene promoter. Luciferase assay was performed on the *CTRP5* promoter deletion constructs where −386 bp to −29 bp (358 bp), −675 bp to −29 bp (647 bp), −1041 bp to −29 bp (1013 bp), −1322 bp to −29 bp (1350 bp), and −3569 bp to −29 bp (3.5 kb) nucleotides upstream of the ATG start sites were cloned in the pGl3-Enhancer vector (pGl3E) and only −3569 bp to −29 bp (3.5 kb) was cloned in pGl3Basic (pGl3B) vectors. The promoterless plasmids pGl3Basic and pGl3 Enhancer were used as negative controls, and the plasmid pGl3Control containing the SV40 promoter was used as positive control for the experiment. The pRL-SV40 vector containing the SV40 early promoter upstream region of Renilla luciferase was used as an internal control for the normalization of transfection efficiency. Data represent mean ± SD from at least three independent experiments.

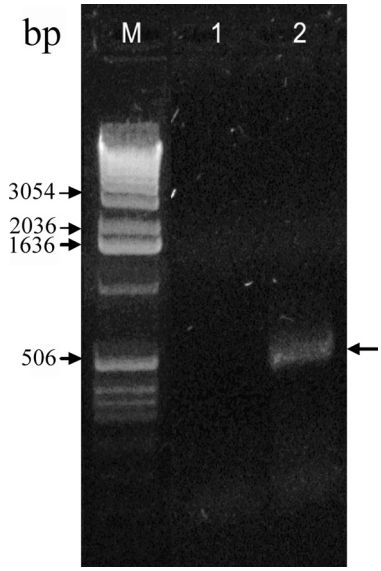

FIGURE 4. 5′ RLM-RACE analysis of the putative human *CTRP5* promoter. Agarose gel electrophoresis of 5′ RLM-RACE products using total RNA from CHO-K1 cells transfected with plasmids having *CTRP5* promoter upstream of the luciferase gene. *Lane 1*: product of the luciferase-specific primary PCR. *Lane 2*: product of the subsequent nested PCR reaction. A band of approximately 500 bp (*black arrow*, *lane 2*) was detected and used for sequencing. M, DNA marker lane.

TABLE 1. Some Selected Transcription Factor Binding Sites within the 1.3-kb Promoter of the Human *CTRP5* Gene

Binding Site	Number	Position	Matrix Similarity
E-box binding factors	1	−538 to −526 bp	0.85
Activator-, mediator-, and TBP-dependent core promoter element for RNA polymerase II transcription from TATA-less promoters	1	−383 to −370 bp	0.823
Core promoter motif 10 elements	1	−343 to −323 bp	0.803
GC-box factors SP1/GC	5	−110 to −95 bp, −270 to −256 bp, −276 to −262 bp, −303 to −289 bp, −305 to −291 bp	0.849, 0.878, 0.884, 0.889, 0.895
RNA polymerase II transcription factor II B	2	−114 to −108 bp, −1086 to −1080 bp	1, 1
Nuclear factor κ B/c-rel	3	−111 to −99 bp, −295 to −283 bp, −1241 to −1229 bp	0.86, 0.84, 0.89
CCAAT binding factors	1	−393 to −379 bp	0.85

of *CTRP5* genes and may contribute to the independent regulation and expression of CTRP5 and MFRP. Another insulator, INSUL_PRE01140, with a binding region of 20 bp (chr11: 118716000–118716019) is located between the second and third exons of the *CTRP5* gene. The presence of this insulator within a gene does not necessarily indicate the functionality of this insulator, but it may function separately with alternative promoters within the same gene.[27]

We screened the *CTRP5* gene 5′ sequence for CpG islands using the CpG island searcher software (http://cpgislands.usc.edu/).[28] One strong CpG island of 504 bp from −60 to −563 was identified with 55% CG content. The observed CpG/expected CpG ratio in this region was greater than 0.65. This could play an important role in the regulation of CTRP5 transcription.

Expression of Genes Cloned Downstream of *CTRP5* Promoter

To determine the functionality of the *CTRP5* promoter in the RPE, ARPE-19 cells were transfected with a series of constructs (Fig. 6A) that contained either wild-type pig CTRP5 (*pCTRP5*) or *pCTRP5* with S163R mutation, or nuclear GFP was cloned downstream of the CTRP5 putative promoter. Expression of pig CTRP5 under the control of elongation factor (EF) promoter was used as a positive control. The expression of CTRP5 was analyzed using immunocytochemistry and Western blot analysis.

The pig CTRP5 cloned downstream of an EF promoter was found to be evenly distributed in the cytoplasm when tested with anti-CTRP5 or tag antibodies (Fig. 6BAii).

The expression patterns of the wild-type and S163R mutant *pCTRP5* under the control of putative *CTRP5* promoter were consistent with the patterns observed for the human wild-type and mutant *CTRP5* gene when transfected into cells (Fig. 6B), thus validating its functionality.[29] ARPE-19 cells transiently transfected with constructs containing the nuclear GFP cloned downstream of the human CTRP5 (1252 bp) putative promoter showed GFP fluorescence in the nucleus (Fig. 6BDii).

Western blot analysis also revealed that the genes cloned downstream of the putative *CTRP5* promoter were able to express high levels of protein, thus confirming the functionality of the putative promoter (Fig. 7). CHO-K1 cells transfected with plasmids containing mouse RPE-65 promoter used as a positive control and (phPRO)human CTRP5 predicted promoter. These cells also showed expression of CTRP5 fusion protein (~30 kDa) by Western blot analysis (Fig. 7).

DISCUSSION

This is the first report revealing the presence of an independent promoter for *CTRP5* located in its adjacent 5′ region. The 1.3-kb sequence upstream of the start codon of human and pig *CTRP5* revealed approximately 73% homology, indicating that this region is highly conserved. This 1.3-kb region exhibited promoter activity indicating this sequence as the potential core promoter region of the *CTRP5* gene. The promoter is observed to be functional in CHO-K1 cells and ARPE-19 cells, indicating that it is not specific to RPE even though highest amounts of CTRP5 among ocular tissues are expressed in the RPE.[16] Functionality of the *CTRP5* promoter in multiple tissues is consistent with the expression profile of CTRP5.

CTRP5 has been reported to be dicistronic with MFRP in the mouse.[10] Several polycistronic transcripts identified in prokaryotes encode proteins involved in the same functional pathway, thereby constituting an operon.[30] Several dicistronic transcripts have also been reported in mammals, and most of them encode functionally related proteins.[31–37] The CUB domains present in MFRP interacts with the C1q domain in CTRP5, suggesting that these two may be related functionally, similar to other dicistronic genes.[29] We previously reported the identification of a human cDNA clone containing the ORFs of both these genes, further suggesting that that *CTRP5* and *MFRP* are at least structurally dicistronic in mammals.[38]

Earlier studies indicated the expression of CTRP5 independent of the MFRP. Northern blot analysis with probes specific to human or mouse *CTRP5* identified two CTRP5 transcripts of 4.4 kb and 1.4 kb in the eye and brain tissue.[20,38] The larger band detected by Northern blot analysis corresponds to the dicistronic transcript, whereas the smaller band may represent a transcript containing the CTRP5 sequence alone, indicating the expression of CTRP5 independent of MFRP. The expression profile of CTRP5 and MFRP in tissues is not similar. The expression of *CTRP5* was detected in most tissues, whereas the expression of *MFRP* was restricted to the RPE and ciliary body, suggesting independent regulation of the expression of these two genes.[16,38] It has recently been reported that several CTRPs, including *CTRP5*, *CTRP1*, *CTRP2*, *CTRP3*, and *CTRP7* transcripts, are expressed predominantly by adipose tissue.[17] In addition, all these CTRPs, including CTRP5, are secreted glycoproteins, and most of them are found in the plasma, indicating that they can function as potential endocrine hormones.[17] None of the other CTRPs is on a dicistronic transcript.[17] These observations suggest that the expression of CTRP5 may be regulated independently of MFRP.

The presence of an insulator region between *MFRP* and *CTRP5* has not been reported to date. In vertebrates, the versatile transcription regulator CCCTC-binding factor (CTCF) is the only identified transacting factor that confers enhancer-blocking insulator activity. CTCF binding sites are commonly distributed along the vertebrate genome and nearly 20,262 human CTCF-binding sites, identified by Zhao et al. using massive direct sequencing of ChIP DNA, are denoted by iden-

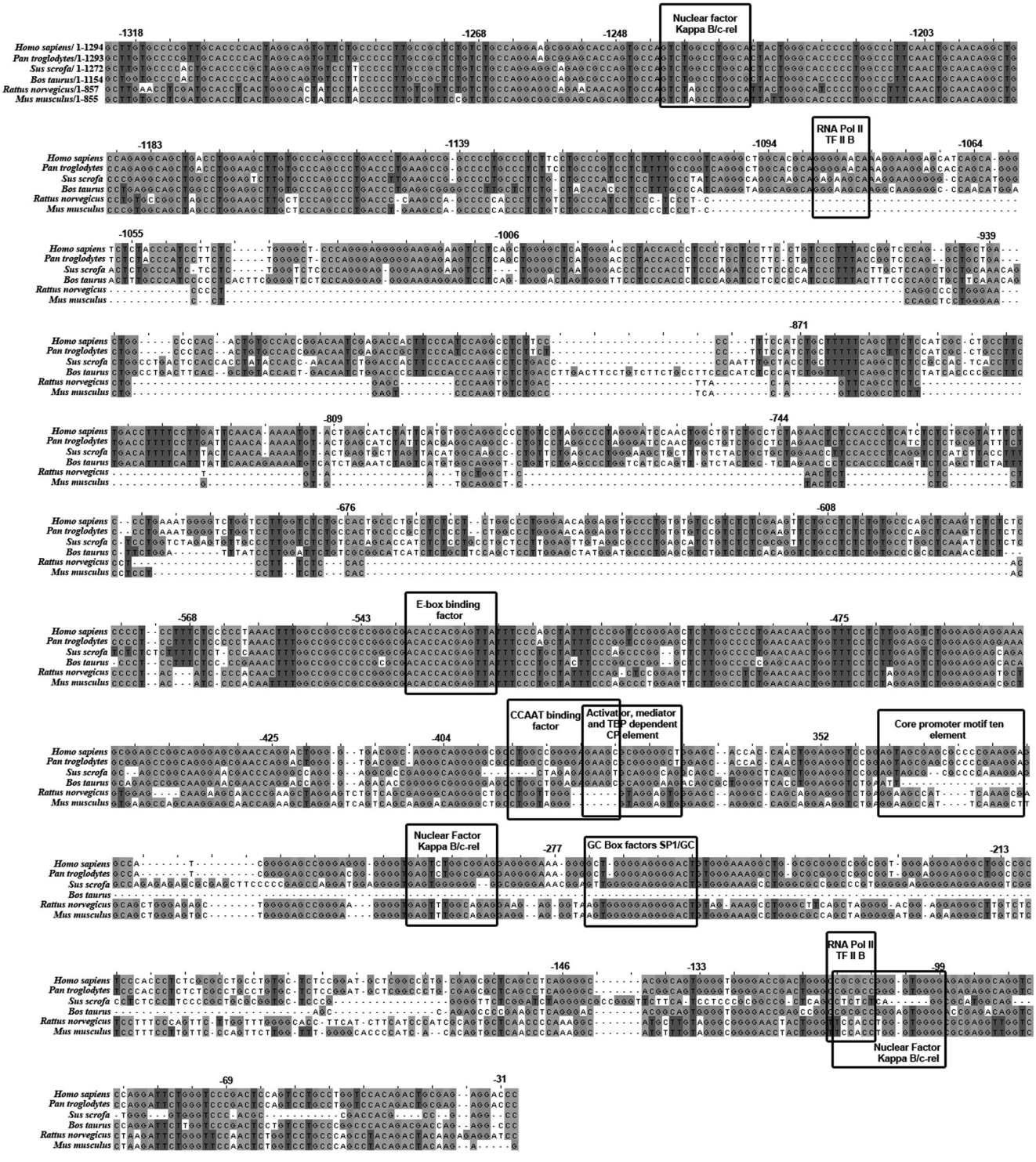

FIGURE 5. Evolutionary conservation of the putative *CTRP5* promoter region. The evolutionary conservation of the putative *CTRP5* promoter is shown by aligning the genomic sequences from *Pan troglodytes*, *Sus scrofa*, *Bos taurus*, *Rattus norvegicus*, and *Mus musculus* with the *Homo sapiens* putative promoter region. *Open boxes*: predicted transcription factor binding sites, encompassing the binding sequences. *Shaded boxes*: conserved residues in the upstream promoter regions of different species.

tifiers starting with INSUL_ZHAO.[39] We report the presence of two CTCF binding sites in the region between *MFRP* and *CTRP5* that may confer insulator activity and independent expression of CTRP5 and MFRP. Given that the CTCF binding sites are present between the two genes, they might also act as the blocking elements to protect against the activating influence of any distal enhancers associated with MFRP.

CpG islands are present in the promoters and exonic regions of approximately 40% of mammalian genes.[40] A CpG island is a region with at least 200 bp, a GC percentage greater than 50%, and an observed/expected CpG ratio greater than 60%.[41] Approximately 70% of human promoters have high CpG content. The methylation of CpG sites in the promoter of a gene may inhibit the expression of a gene. The CpG island

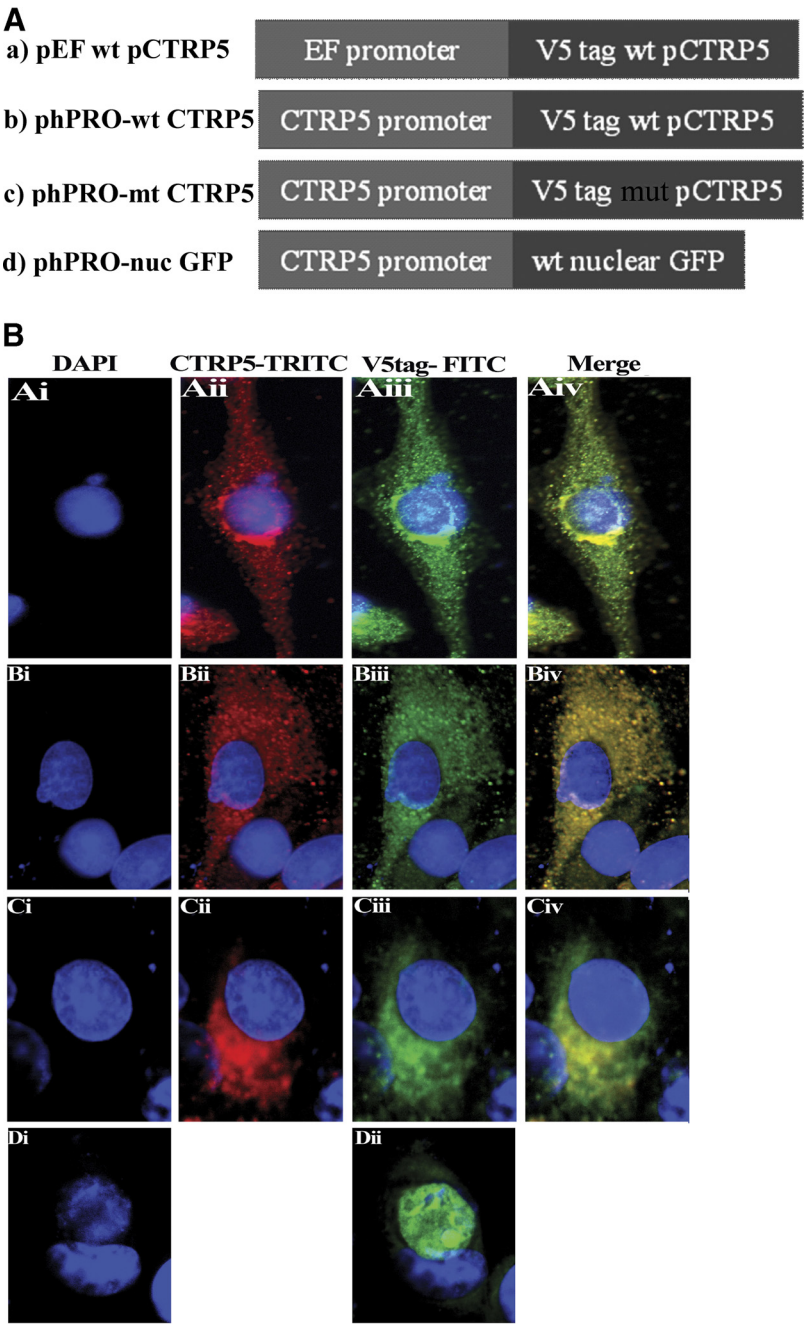

FIGURE 6. (**A**) Expression constructs designed for testing *CTRP5* promoter activity. Four constructs were made to investigate the activity of the putative *CTRP5* promoter: (**a**) pEF V5 tag-wt *pCTRP5*; (**b**) phPRO-V5 tag-wt *pCTRP5*; (**c**) phPRO-V5tag mutCTRP5; (**d**) phPRO-nuclear GFP. wt, wild-type Ser163 allele; mt, S163R mutation. (**B**) Expression of CTRP5 in ARPE-19 cells under the control of the human *CTRP5* promoter. ARPE-19 cells transfected with pEF *pCTRP5*wt V5 tag (**Ai–Aiv**), phPRO-CTRP5wt V5 tag (**Bi–Biv**), and phPRO-mutCTRP5 V5 tag (**Ci–Civ**) were labeled with DAPI, V5 antibody, and CTRP5 polyclonal antibody, whereas phPRO+nGFP (**Di**, **Dii**) was labeled with DAPI.

search software predicted the presence of a CpG island in the 5′ region of the human *CTRP5* gene sequence. No CpG island was detected in the 5′ region of the mouse *Ctrp5* gene. Despite these differences between human and mouse sequences, it is reported that a gene promoter is usually present between the 5′ boundary of the CpG island and the transcription initiation site.[42] The presence of a CpG island in the *CTRP5* putative promoter region suggests an alternative means of regulation for this protein.

The putative promoter of the *CTRP5* gene is possibly a TATA-less promoter. Although the TATA sequence is absent, one potential CCAAT transcription factor binding site is present in the promoter in reverse orientation (Table 1). The CCAAT box is frequently found in promoters of many species.[43] CCAAT boxes may function in either the forward or the reverse orientation, and they may be found in multiple copies in a promoter.[44,45] A number of transcription factors have been found to bind to or regulate gene expression positively or negatively at CCAAT boxes.[44] Two sites for the transcription factor IIB are present in the *CTRP5* promoter, with the highest matrix similarity of 1. Transcription factor IIB serves as a bridge between transcription factor IID, the factor that initially recognizes the promoter sequence, and RNA polymerase II.[46] A transcription factor binding site commonly present in the TATA-less promoters (the activator-, mediator-, and TBP-dependent core promoter element for RNA polymerase II) was also detected in the putative promoter sequence of the *CTRP5* promoter. The presence of this site further supports the nature of the *CTRP5* promoter as a potential TATA-less promoter.

Although the data presented here indicate the presence of a promoter that can regulate the expression of *CTRP5*, the role of this promoter on the expression of *MFRP* is unknown. In addition, the current studies do not exclude the possibility of a common promoter located upstream of the *MFRP* transcrip-

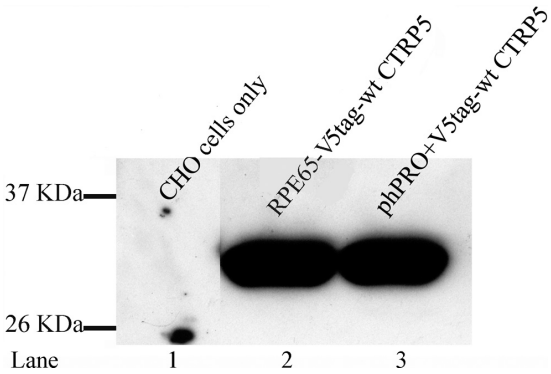

FIGURE 7. Expression of genes cloned downstream of the putative *CTRP5* promoter. CHO-K1 cells were transfected with plasmids into which the *pig CTRP5* gene was cloned downstream of either RPE65 promoter (*Lane 2*) or the human *CTRP5* predicted promoter (*Lane 3*) and the expression of fusion protein was tested by Western analysis with V5 antibody.

tion start site regulating the expression of both *MFRP* and *CTRP5*. Additional studies are needed to determine whether the expression of CTRP5 is solely controlled by the promoter sequence located in its 5′ region or whether other sequences play a role in regulating its expression either in concert with or independently of *MFRP* gene expression.

In summary, we identified the promoter sequence of the human *CTRP5* gene, which is expressed in the RPE and ciliary epithelium. Two well-characterized promoters of RPE65 and vitelliform macular dystrophy-2 genes expressed in RPE are being used for the expression of proteins in the RPE of mouse.[47,48] The *CTRP5* promoter may provide an additional choice for selective expression of proteins in the RPE. Further characterization of the *CTRP5* promoter will help identify regulatory elements (enhancers, repressors) and control elements necessary for its specific expression in selected tissues. Understanding the regulation of *CTRP5* gene transcription may also provide insight into the possible role of CTRP5 in the normal retina and in causing late-onset retinal degeneration in patients. In addition, these studies will help determine whether CTRP5 and MFRP are functionally dicistronic.

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