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Involvement of General Transcriptional Coactivator PC4 in the Transcription of Medaka Fish Intestine–Specific Membrane Guanylyl Cyclase Gene (*OlGC6*)

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A recent study showed that the AGACCTTTGC nucleotides sequence (between –90 and –81) contained in the *cis*-regulatory element in an intestine-specific membrane guanylyl cyclase gene, *OlGC6*, of the medaka fish, *Oryzias latipes*, are important for the transcription of the gene in mammalian cultured cell line and in medaka fish. Using sequence-specific DNA affinity chromatography, we purified a *cis*-regulatory element–binding protein from a medaka fish intestinal nuclear extract and used mass spectrometry to identify it as a medaka fish homologue of general transcriptional coactivator PC4, which we designated as OlPC4. The expression of the *OlPC4* gene was detected in embryos, as well as in a large variety of tissues of adult medaka fish. Using a 17-kDa recombinant OlPC4, we carried out an ultraviolet (UV) cross-linking experiment and an electrophoretic mobility shift assay (EMSA), and demonstrated that the recombinant OlPC4 can be substituted for native OlPC4 in medaka fish intestinal nuclear extracts. In CACO-2 cells, cotransfection of the *OlGC6*-luciferase fusion genes with an OlPC4 expression vector resulted in 1.5-fold stimulation of the *OlGC6* promoter.

Key words: coactivator, intestine-specific membrane guanylyl cyclase, Medaka fish, positive cofactor 4, transcription.

The gastrointestinal tract of vertebrates expresses specific isoforms of membrane guanylyl cyclase (membrane GC). Mammalian GC-C has been reported to be expressed at high levels throughout the epithelium of the intestine (*1*), and has been shown to be the receptor for *Escherichia coli* heat-stable enterotoxin (STa) (*2*) and the endogenous peptides guanylin and uroguanylin (*3*, *4*). In response to the binding of these ligands to the extracellular domain of GC-C, the intracellular cyclase catalytic domain converts GTP to cGMP, and subsequent cGMP accumulation induces the secretion of fluid and electrolytes. Thus, GC-C regulates water and ion transport in the intestine (*5*). On the other hand, *GC-C*-deficient mice are viable, develop normally, and are fertile, but they are resistant to STa-induced diarrhea (*6*, *7*). However, considering that all vertebrates contain an intestine-specific membrane *GC* gene, this GC may serve some important yet undefined physiological roles.

The medaka fish *Oryzias latipes* is a small freshwater teleost with various traits that make it useful for molecular genetic studies. It has been reported that the *OlGC6* gene, a medaka fish homologue of the mammalian *GC-C* gene, is also expressed abundantly in the intestine (*8*). In a previous study, we demonstrated that the nucleotides between –98 and –89 in the 5′-flanking region of the *OlGC6* gene are essential for transcription of the *OlGC6* gene in CACO-2 cells (a human intestine–derived cell line), and the nucleotides between –98 and +50 are sufficient to induce expression of this gene in the medaka fish intestine (*9*). In that previous study, we also demonstrated that a nuclear protein from CACO-2 cells and from adult medaka fish intestinal cells binds specifically to the AGACCTTTGC nucleotides in the 5′-flanking region of the *OlGC6* gene (*9*).

It has been reported that hepatocyte nuclear factor 4 (HNF-4) and/or Cdx2 are involved in the regulation of mammalian *GC-C* transcription (*10–12*). However, the consensus binding sequences for HNF-4 are not found in the 5′-flanking region of the *OlGC6* gene, and those for Cdx2 are found, although they do not seems to be involved (*9*), suggesting that the transcriptional regulatory mechanism of the *OlGC6* gene differs from that of the mammalian *GC-C* gene. In our previous study, we demonstrated that a nuclear protein from both mammalian and medaka fish intestinal cells binds to the same nucleotides (AGACCTTTGC), suggesting that the *cis*-regulatory element and protein binding to that element play important roles in the transcription of the *OlGC6* gene (*9*). In this study, we purified this protein from medaka fish intestinal nuclear extracts, and, using mass spectrometry, identified it as a medaka fish homologue (designated as OlPC4) of the mammalian positive cofactor 4 (PC4). Here, we report the results of UV cross-linking analysis and EMSA, which demonstrated that bacterially expressed recombinant OlPC4 can be substituted for native OlPC4 in medaka fish intestinal nuclear extracts. In addition, transient transfection of CACO-2 cells with an OlPC4 expression vector stimulates the promoter of

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the *OlGC6* gene through the *cis*-regulatory element. These results strongly support our hypothesis that OlPC4 is an important regulator of *OlGC6* gene expression.

MATERIALS AND METHODS

Purification of a Cis-Regulatory Element–Binding Protein Using the Oligonucleotide Trapping Method—Single-stranded oligonucleotides $(TG)_5$ (5′-NH_2-TGTGTGTGTG-3′) were coupled to CNBr-preactivated Sepharose 4B (Amersham Biosciences, UK) as described by Gadgil and Jarrett (*13*), and the resultant Sepharose was designated as $(TG)_5$ Sepharose 4B. Single-stranded $(CA)_5$ protruding double-stranded oligonucleotides containing the *cis*-regulatory element were synthesized using 5′-GCAGCGCACACACAGACCTTTGCACACCCA-3′-$(CA)_5$ and 5′-TGGGTGTGCAAAGGTCTGTGTGTGCGCTGC-3′-$(CA)_5$ as described previously (*9*), and used as probes. A 1-ml bed volume of $(TG)_5$ Sepharose 4B in a Polyprep empty column (BioRad, Hercules, CA, USA) was equilibrated with binding buffer containing 10% glycerol, 100 mM NaCl, 2.5 mM $MgCl_2$, 5 mM dithiothreitol (DTT), 1 mM EDTA, and 15 mM Tris-HCl (pH 7.5). The medaka intestinal nuclear extract (2.5 mg of protein) was prepared as described previously (*9*). The extracts were incubated with 500 pmol of the probe and 50 μg poly (dI-dC)-poly (dI-dC) (Amersham Biosciences) in the binding buffer for 1 h on ice, and were then passed through a column five times under gravity flow. The column was washed three times with 10-ml aliquots of binding buffer, and then the protein was eluted from the column by the addition of three 2.5-ml portions of binding buffer containing 2 M NaCl. After desalting the elute with a PD-10 column (Amersham Biosciences), the elute was incubated with 200 pmol of the probe and 20 μg poly (dI-dC)-poly (dI-dC), and then purified by $(TG)_5$ Sepharose 4B column chromatography, as described above. The purified samples were precipitated with 75% acetone and subjected to SDS–polyacrylamide gel electrophoresis (PAGE) in a 15% gel, and detected by silver staining. The protein concentration was determined according to the Bradford method (*14*).

Mass Spectrometry—The silver-stained band on the SDS-PAGE gel corresponding to a prominent 17-kDa protein was excised and destained by treatment with 15 mM potassium hexacynoferrate(III)/50 mM sodium thiosulfate for 10 min at room temperature. The destained proteins in the gel were reduced by incubating them with 10 mM EDTA/10 mM DTT/100 mM ammonium bicarbonate for 1 h at 50°C, and alkylated by treatment with 10 mM EDTA/40 mM iodoacetamide/100 mM ammonium bicarbonate for 30 min at room temperature. The proteins were digested in gel with lysyl endopeptidase (LEP) from *Achromobacter lyticus* (Wako Pure Chemical Industries, Japan) in 100 mM Tris-HCl (pH 8.9) for 15 h at 37°C. The resulting peptide fragments were extracted from the gel and concentrated *in vacuo*. After desalting with ZipTip_{C18} (Millipore, Billerica, MA, USA), the peptide fragments were subjected to mass spectrometric analysis. The mass spectra were acquired by direct infusion analysis on a Micromass Q-Tof2 hybrid quadrupole time-of-flight (Q-TOF) mass spectrometer equipped with a nano-electrospray ionization (ESI) source, in the positive mode. Tandem mass spectrometry (MS/MS) was performed by collision-induced dissociation using argon as the collision gas. Identification of Expressed Sequence Tags (EST) consensus sequence using the mass spectrometric data was performed by product ion mass fingerprinting run by the MS/MS Ions Search program in MASCOT (Matrix Science, UK) on our in-house server. An EST consensus sequence database, the Tentative Consensus (TC) sequence database of *O. latipes* (Ver. 4.0) in the Institute for Genomic Research (TIGR), *O. latipes* Gene Index (http://www.tigr.org/tdb/tgi/), was downloaded from the file transfer protocol server of TIGR to our in-house MASCOT server.

Cloning of Medaka PC4 cDNA (OlPC4)—Total RNA was prepared from the intestine of adult medaka fish (orange-red variety of *O. latipes*) by the acid guanidium thiocyanate/phenol/chloroform extraction method (*15*). The first-strand cDNA was synthesized from the total RNA (2 μg) using the SuperScript™ First-Strand Synthesis System (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. The coding region of the *OlPC4* cDNA was amplified from the medaka fish intestine cDNA by polymerase chain reaction (PCR) with paired primers (OlPC4-F: 5′-ATGCCTAAATCCAAGGAAGT-3′ and OlPC4-R: 5′-TCATGTTCTCTTGATGGCCT-3′), which were synthesized based on the TC data. The reaction was performed under the following conditions: 2 min at 96°C and 30 cycles of 30 s at 96°C, 30 s at 53°C, and 30 s at 72°C. The PCR product was purified using a MinElute Reaction Cleanup Kit (Qiagen, Germany), and was subcloned into pBluescript II (KS–) (Stratagene, La Jolla, CA, USA) and sequenced. The deduced amino acid sequence of OlPC4 was compared with those of known mammalian PC4s using the Clustal W program (*16*) and the sequence editor SeqPub (Gilbert, Indiana University, Bloomington, IN, USA). The DDBJ/EMBL/GenBank accession numbers for the sources were: human PC4 (P53999), mouse PC4 (P11031), and rat PC4 (Q63396). For reverse transcription (RT)–PCR analysis, total RNA was prepared from medaka fish embryos (stages 35–39) and several adult medaka fish tissues by the acid guanidium thiocyanate/phenol/chloroform extraction method (*15*). After digestion of the contaminating genomic DNA by DNase I (Invitrogen), the first-strand cDNA was synthesized from the total RNA (2 μg) using the SuperScript™ First-Strand Synthesis System (Invitrogen) according to the manufacturer's protocol. As a control, reactions without reverse transcriptase were also performed. PCR was then carried out with a specific primer set for the *OlPC4* gene (GenBank™ accession number AB188297), or for the *OlCA1* gene (the cytoplasmic actin gene of the medaka fish, GenBank™ accession number D89627): OlPC4-F, 5′-ATGCCTAAATCCAAGGAAGT-3′ and OlPC4-R, 5′-TCATGTTCTCTTGATGGCCT-3′ (375-bp product); OlCA1-F, 5′-GGGTCTTCATGACGGGC-3′ and OlCA1-R, 5′-CAAGTCGGAACACATGTGCA-3′ (100-bp product), and 2 μl of the reverse-transcribed cDNA solution. The following conditions for amplification were used: 30, 28, 25, and 22 cycles of 30 s at 96°C, 30 s at 55°C, and 30 s at 72°C. The PCR products were analyzed by electrophoresis in 1.5% agarose gels.

Expression and Purification of Recombinant OlPC4—A coding region of the *OlPC4* cDNA was amplified by PCR from the *OlPC4* cDNA subcloned into pBSII, described above, using paired primers, 5′-AGGAGAATTCTTATGCCTAAATCCAAGG-3′ and 5′-AAGCGAATTCTCATGTTCTCTTGCTG-3′. The amplified fragment was digested with *Eco*RI and inserted into similarly digested pGEX-KG. *Escherichia coli* strain BL-21 was then transformed with a plasmid encoding a GST-OlPC4 fusion protein, and expression was induced with 0.25 mM isopropyl β-D(–)-thiogalactopyranoside (IPTG). The expressed fusion protein was purified by Glutathione Sepharose 4B chromatography of the bacterial lysate according to the manufacturer's protocol (Amersham Biosciences). To purify OlPC4 lacking a tag, GST fusion protein–bound beads in phosphate-buffered saline (PBS) were incubated with thrombin for 20 h at 4°C. After recovery by centrifugation, the resultant OlPC4-containing supernatant was mixed with Benzamidine Sepharose 6B (Amersham Biosciences) to remove the thrombin, and then the mixture was centrifuged. The recombinant OlPC4 was recovered from the supernatant. The protein concentration was determined by the Lowry method (*17*, *18*).

Electrophoretic Mobility Shift Assay (EMSA) and UV Cross-Linking Analysis—EMSA and UV cross-linking analysis were performed as described previously using the labeled *cis*-regulatory element (the region between –108 and –79 in the 5′-flanking region of the *OlGC6* gene, designated as E1) as a probe (*9*) and 50 ng of the recombinant OlPC4.

Cell Culture and DNA Transfections—A coding region of the *OlPC4* cDNA subcloned into pGEX-KG (described above) was digested with *Eco*RI and inserted into similarly digested pCR®3.1 (Invitrogen). The *OlGC6*-luciferase fusion genes were constructed as described previously (*9*). CACO-2 cells and COS7 cells were cultured in DMEM supplemented with 10% heat-inactivated FBS (HyClone®, Logan, UT, USA) and 1× penicillin-streptomycin-glutamine (Invitrogen) under a humidified 5% CO_2 atmosphere. For the reporter-enzyme assay, 5×10^5 CACO-2 cells or 2×10^5 COS7 cells were plated per well in a six-well plate cultured for 24 h before transfection. One microgram of the *OlGC6*-luciferase fusion gene, 2 μg of OlPC4-pCR®3.1 or 2 μg of pCR®3.1 were co-transfected with 1 μg of pSV-β-galactosidase (Promega) into CACO-2 cells or COS7 cells using LIPOFECTAMIN 2000 (LF2000) Reagent according to the manufacturer's protocol (Invitrogen). After 48 h of culture, the CACO-2 cells or COS7 cells were lysed in Reporter Lysis Buffer (Promega), and the luciferase activity was assayed using a Luciferase Assay System (Promega) according to the manufacturer's protocol. β-Galactosidase activity was also assayed to normalize for variations in transfection efficiency.

RESULTS AND DISCUSSION

From 2.5 mg of medaka fish intestinal nuclear extracts, 700 ng of protein were obtained in two steps of purification by DNA affinity chromatography. The proteins were further separated by SDS-PAGE, and subsequently silver-stained. One prominent 17-kDa protein, which was most enriched in the final fraction of the DNA affinity chromatography (Fig. 1), was consistent with the putative molecular mass for a binding protein estimated by UV cross-linking analysis, as described previously (*9*). We carried out three identical purifications and obtained almost identical results with each purification. In order to identify the protein, an in-gel digest was subjected to nano-ESI-Q-TOF mass spectrometry. An intense peak of a double-charge ion at m/z 639.79 was observed in the spectrum. The product ion mass list obtained by MS/MS of the ion at m/z 639.79 was searched against the nr database of the National Center for Biotechnology Information (USA) using MASCOT. In the product ion mass fingerprinting (MS/MS Ions Search) by MASCOT, none of the proteins matched the list. Therefore, the product ion mass list from the ion at m/z 639.79 was then searched against an EST consensus sequence database, Tentative Consensus (TC) sequence database of *O. latipes*. It matched an amino acid sequence, DQMSEIDEAIK, in an EST consensus sequence, TC25781. The product ion mass fingerprinting against the EST consensus sequence database demonstrated that the preparation included a protein that was an *in silico* protein coded by a virtual transcript in TC25781.

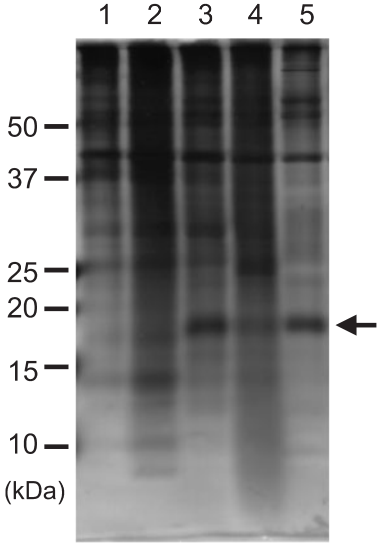


Fig. 1. **Biochemical purification of a *cis*-regulatory element binding protein.** Protein samples were subjected to electrophoresis in a 15% polyacrylamide gel, which was then silver-stained. The arrow indicates a major 17-kDa protein. Lane 1, crude medaka fish intestinal nuclear extract; lane 2, flow-through fraction of the first affinity chromatography; lane 3, fraction eluted from the first affinity chromatography; lane 4, flow-through fraction of the second affinity chromatography; lane 5, fraction eluted from the second affinity chromatography.

The EST consensus sequence TC25781 contains an open reading frame (ORF) encoding a polypeptide comprising 125 amino acid residues. The theoretical molecular weight of the polypeptide is 13,865, which is smaller than the molecular weight of the 17-kDa protein estimated by SDS-PAGE, assuming that the protein is modified post-translationally, as discussed below. According to the nucleotide sequence, PCR was performed in order to obtain a full-length cDNA clone and to confirm the hypo-

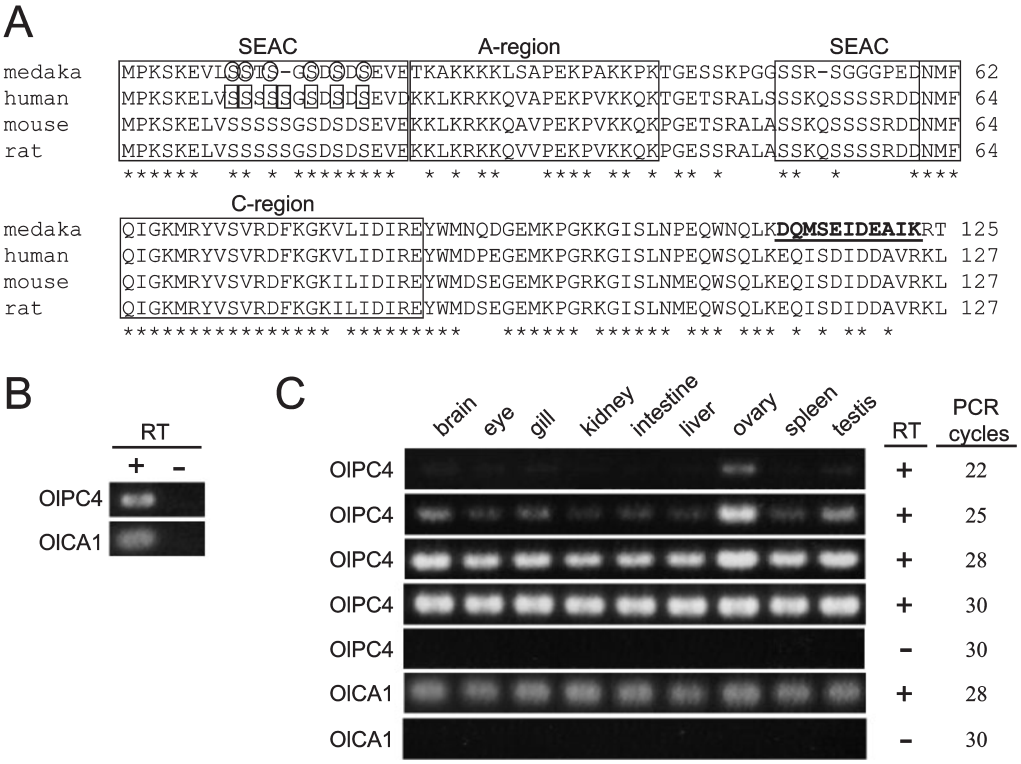


Fig. 2. **A: Alignment of the amino acid sequences of OlPC4 and mammalian PC4s.** The amino acid sequence determined by mass spectrometry is indicated in underlined boldface letters. Open circles and open squares indicate putative recognition sites for CKII in the medaka fish and human PC4, respectively. **B: *OlPC4* expression in embryos at stages 35–39. C: *OlPC4* expression in several adult medaka fish tissues.** PCR cycles numbers are indicated at the right. RT+ and RT− represent amplification with and without reverse transcriptase, respectively. *OlCA1* was amplified as an internal control.

thetical *in silico* nucleotide sequence. No nucleotide gaps were found between the ORF regions of the nucleotide sequences of the PCR product and those of TC25781. The deduced amino acid sequence was found to be homologous to a mammalian protein, RNA polymerase II transcription cofactor p15 (PC4). Thus, we concluded that "p17" is an *O. latipes* homologue of PC4, and we referred to it as OlPC4.

Comparison of the deduced amino acid sequence of OlPC4 with the known sequence of mammalian PC4 demonstrated similarities between OlPC4 and human, mouse, and rat PC4 of 71%, 68%, and 66%, respectively (Fig. 2A). It has been shown that human PC4 contains two short stretches, referred to as SEAC motifs, that are rich in serine and acidic residues within the amino-terminal half, and that the function of human PC4 is entirely dependent on the 61 amino-terminal amino acids (containing two SEAC motifs) (*19*, *20*). The amino-terminal SEAC motif (positions 1–22 in human PC4) contributes most significantly to the activity of the cofactor, and is the target of casein kinase II (CKII), which negatively regulates the activity of PC4. There are seven putative recognition sites for CKII in the amino-terminal SEAC motif of human PC4. Phosphorylation of human PC4 by CKII inhibits the acetylation of PC4, and, in turn, induces conformational changes in PC4, which then lead to a loss of transcriptional and DNA-binding activity (*19*–*22*). The amino-terminal portion of OlPC4 (positions 1–21) shows 77% similarity to the SEAC motif of human PC4, and contains six putative recognition sites for CKII, suggesting that OlPC4 exhibits cofactor activity (Fig. 2A). RT-PCR analysis demonstrated that the *OlPC4* gene

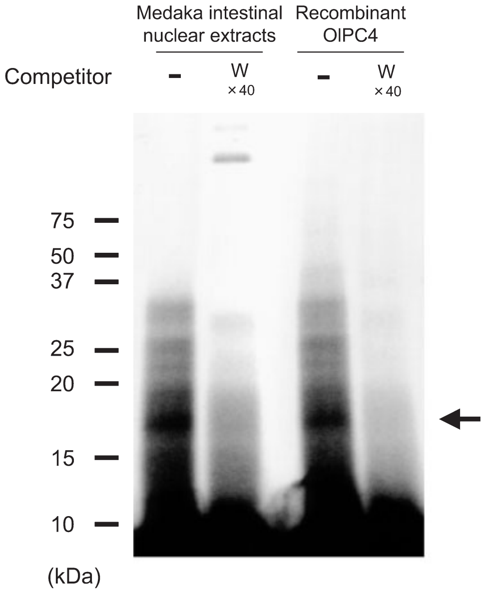
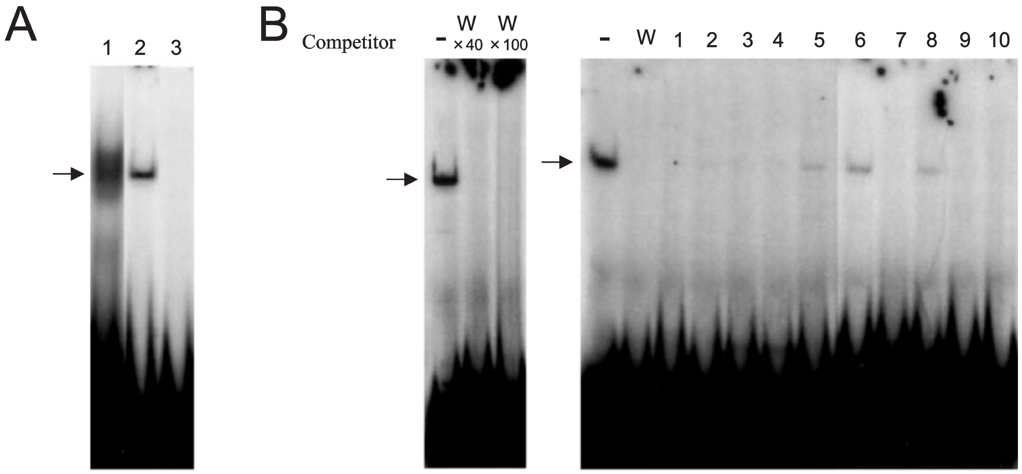


Fig. 3. **UV cross-linking analysis using medaka fish intestinal nuclear extracts or bacterially expressed recombinant OlPC4.** Competitive complex formation experiments were conducted by adding a 40-fold excess of unlabeled E1.



W: 5′-G AGCCTGCAGC GCACACACAG ACCTTTGCAC C-3′
1: 5′-G AGCCTGCAGC **TA**ACACACAG ACCTTTGCAC C-3′
2: 5′-G AGCCTGCAGC GC**CA**ACACAG ACCTTTGCAC C-3′
3: 5′-G AGCCTGCAGC GCAC**CA**ACAG ACCTTTGCAC C-3′
4: 5′-G AGCCTGCAGC GCACAC**CA**AG ACCTTTGCAC C-3′
5: 5′-G AGCCTGCAGC GCACACAC**CT** ACCTTTGCAC C-3′
6: 5′-G AGCCTGCAGC GCACACACAG **CA**CTTTGCAC C-3′
7: 5′-G AGCCTGCAGC GCACACACAG AC**AG**TTGCAC C-3′
8: 5′-G AGCCTGCAGC GCACACACAG ACCT**GG**GCAC C-3′
9: 5′-G AGCCTGCAGC GCACACACAG ACCTTT**TA**AC C-3′
10: 5′-G AGCCTGCAGC GCACACACAG ACCTTTGC**CA** C-3′

Fig. 4. **A: Electrophoretic mobility shift assay (EMSA) with an E1 probe using medaka fish intestinal nuclear extracts (lane 1), bacterially expressed recombinant OlPC4 (lane 2), and GST-OlPC4 (lane 3). B: Competitive EMSA of labeled E1 and unlabeled wild-type or mutated competitors using the recombinant OlPC4.** Complex competition was achieved by the addition of unlabeled E1 (in 40-fold excess or 100-fold excess) (left panel). Recombinant OlPC4 was combined with labeled E1 in the presence or absence of a 10-fold excess of the indicated unlabeled mutated competitor (right panel). Competitors used in EMSA are indicated at the bottom of the right panel, and the mutated nucleotides are indicated in underlined boldface letters.

is expressed ubiquitously in embryos (Fig. 2B) and in almost all adult medaka fish tissues (Fig. 2C). The PCR at different cycles demonstrated that *OlPC4* is expressed in the ovary slightly more abundantly than in other tissues (Fig. 2C).

To determine whether or not the bacterially expressed recombinant OlPC4 possesses the ability to bind the *cis*-regulatory element, we carried out both UV cross-linking analysis and EMSA using medaka fish intestinal nuclear extracts and the recombinant OlPC4. Cross-linking and subsequent SDS-PAGE experiments indicated that both the apparent molecular mass of the complex formed with E1 and the binding protein obtained from the medaka fish intestinal nuclear extracts and the recombinant OlPC4 were similar (Fig. 3). Furthermore, a complex showing the same mobility was formed when the E1 probe was incubated with either the medaka fish intestinal nuclear extract or with the recombinant OlPC4 (Fig. 4A). However, no complex was formed when the E1 probe was incubated with the recombinant GST-OlPC4 (Fig. 4A), suggesting that GST, which was attached to the amino terminus of the recombinant OlPC4, may interrupt DNA binding, because the region involved in DNA binding is located in the amino-terminal portion, as noted above. The ability of the E1 probe to form a complex with the recombinant OlPC4 was abolished by the addition of increasing amounts of unlabeled E1 (Fig. 4B), which suggested that the DNA-protein complex is probe sequence-specific. The nucleotides in E1 responsible for protein binding were identified by EMSA, which was carried out to examine competition of unlabeled mutated competitors for labeled wild-type E1 binding to the recombinant OlPC4. Competitors 5, 6, and 8 did not compete with labeled wild-type E1, suggesting that the nucleotides AGAC and TT in the *cis*-regulatory region are important for binding (Fig. 4B). This finding was mostly consistent with our previous results using medaka fish intestinal nuclear extracts (*9*). On the other hand, it has been suggested that the amino acids (positions 22–87) of human PC4 containing two positively charged regions (referred to as the A- and C-regions) contribute to non-specific binding to double-stranded DNA; therefore, the A-region, which consists of an extremely lysine-rich motif, may play a role in the recognition of double-stranded DNA (*19*, *20*). OlPC4 contains both of these positively charged regions (Fig. 2A), suggesting that OlPC4 may bind to double-stranded DNA at these regions. Although there is still no evidence for the sequence-specific binding of human PC4 to any human genes, it has been demonstrated that the binding of human PC4 to a random sequence is much less efficient than the binding of human PC4 to a promoter-containing sequence (*19*, *20*). In this regard, we demonstrated that OlPC4 exhibits certain preferences with respect to binding sequences, a finding that suggests that PC4 may exert still unknown functions in the recognition of the binding sequence.

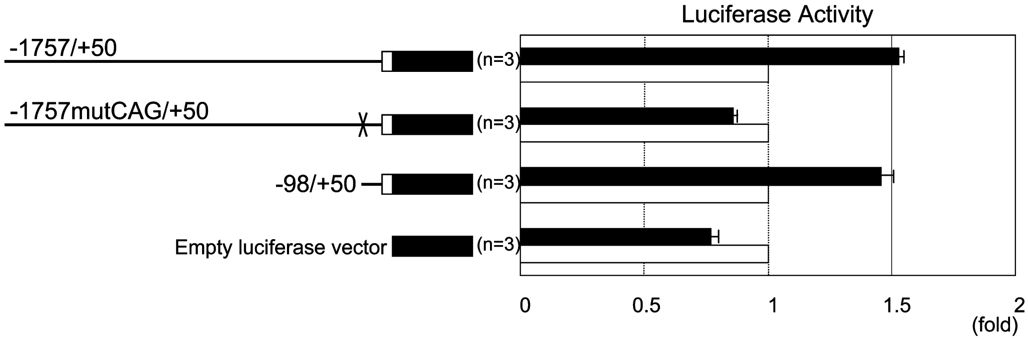


Fig. 5. **Potentiation of *OlGC6* promoter activity by OlPC4 in CACO-2 cells.** The *OlGC6*-luciferase fusion gene constructs are indicated at the left of the figure. White boxes show the 5′-leader sequence of the *OlGC6* gene, black boxes denote the luciferase open reading frame. An untranscribed 5′-flanking region of the *OlGC6* gene is indicated by the horizontal line. Each constructs was cotransfected with the *OlPC4* expression vector (solid bars) or empty expression vector (open bars). Data represent the luciferae-to-β-galactosidase ratio and are expressed as fold activation for each construct (relative to the empty expression vector as 1). "*n*" denotes the number of independent transfections for each construct.

To assess the role of OlPC4 in the transcription of *OlGC6* gene, the *OlGC6*-luciferase fusion genes were cotransfected with OlPC4 expression vector (OlPC4-pCR®3.1) or empty expression vector (pCR®3.1) into CACO-2 cells (intestine-derived cell line) or COS7 cells (kidney-derived cell line). In CACO-2 cells, the luciferase activity of (–1757/+50) and (–98/+50) constructs were stimulated about 1.5 fold by OlPC4, whereas the luciferase activity of an empty luciferase construct was not, indicating a specific effect of OlPC4 on *OlGC6* the promoter activity (Fig. 5). Incorporation of the 3-bp mutation in the binding site of OlPC4 (the region between –91 and –89) into the construct (–1757mutCAG/+50) abolished the ability of OlPC4 to stimulate the *OlGC6* promoter (Fig. 5). Although the fold stimulation effect of OlPC4 on *OlGC6* gene transcription seems to be low, it was clearly demonstrated that OlPC4 stimulates the transcription of the *OlGC6* gene through the *cis*-regulatory element, and thus we presume that it is essential for the expression of the *OlGC6* gene. On the other hands, in COS7 cells, OlPC4 failed to stimulate the luciferase activity of all examined constructs (data not shown), suggesting that OlPC4 could stimulate the *OlGC6* promoter only in the intestine-derived CACO-2 cells.

As shown in Fig. 2C, RT-PCR analysis demonstrated that the *OlPC4* gene is ubiquitously expressed in almost all adult medaka fish tissues, and it was demonstrated that several complexes were formed when the labeled E1 probe was incubated with brain, testis, or liver nuclear extracts, although all of these complexes exhibited different mobility patterns from that of the intestine-specific complex (*9*). The different mobility-complex formation of E1 with the nuclear extract from OlPC4 expressing-tissues appears to be inconsistent with the result that only an intestinal complex exhibits the same mobility as that of the complex with the recombinant OlPC4 (Figs. 2C and 4A). Although E1 binds weakly to other protein(s) in the brain, testis, and liver nuclear extracts, the binding of OlPC4 to E1 may require additional factor(s) that are present only in the intestinal nuclear extracts. Furthermore, the results of the coexpression experiments that OlPC4 stimulates the transcription of the *OlGC6* gene only in the intestine-derived cell line also suggest that the activity of OlPC4 may require some unknown factor(s) expressed in the intestine.

It has been demonstrated that human PC4 activates the transcription of some genes by interacting with several types of activators and general transcriptional factors (TFIID, TFIIA, and TFIIH), and also with TATA-binding protein (TBP)–associated factors (TAFs), as well as with a specific type of coactivator, thereby acting as an adaptor that links upstream activators with the basal transcriptional machinery (*23–25*). These results suggest that PC4 plays an important role in the regulation of the genes transcribed by RNA polymerase II, although its physiological roles are still largely unknown. Recently, it was shown that PC4 exhibits a growth-suppressive effect on human teratocarcinoma cells; in other words, PC4 can function in the manner of tumor suppressors that interact with AP-2 (*26*) and activate p53, resulting in the stimulation of tumor suppressor activity (*27*). In this study, we demonstrated that OlPC4 is involved in the transcription of the intestine-specific membrane GC gene, *OlGC6*, suggesting that OlPC4 play an important role in the some physiological processes such as intestinal ion transport.

To date, there have been several papers that demonstrate the presence of the *cis*-regulatory element in the 5′-flanking region of medaka fish *GC* genes (*28*). The results presented here provide the first demonstration of the transcriptional factor acting through the *cis*-regulatory element of the medaka fish *GC* genes. Considering the results that mammalian PC4 requires upstream activators and interacts with many factors (*19*, *23–25*), and our results presented here that the activity of OlPC4 seems to require additional factor(s) expressed in the intestine, OlPC4 may also interact with some unknown factor(s) and/or activator(s) binding to the upstream region of the *OlGC6* gene, although this region has yet to be identified. The identification of the partner factor(s) of OlPC4 will reveal more details about the mechanisms of the transcriptional regulation of intestine-specific GC genes.

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Culture of Japan (no. 11236202) and from the National Project on Protein Structural and Functional Analyses. Nucleotide sequence data reported are available in the DDBJ/EMBL/GenBank databases under the accession number AB188297. We would like to thank the staff members of the Center for Advanced Science and Technology, Hokkaido University, for the use of their laboratory facilities.

REFERENCES

1. Giannella, R.A. and Mann, E.A. (2003) *E. coli* heat-stable enterotoxin and guanylyl cyclase: new functions and unsuspected actions. *Trans. Am. Clin. Climatol. Assoc.* **114**, 67–86
2. Schulz, S., Green, C.K., Yuen, P.S., and Garbers, D.L. (1990) Guanylyl cyclase is a heat-stable enterotoxin receptor. *Cell* **63**, 941–948
3. Currie, M.G., Fok, K.F., Kato, J., Moore, R.J., Hamra, F.K., Duffin, K.L., and Smith, C.E. (1992) Guanylin: an endogenous activator of intestinal guanylate cyclase. *Proc. Natl Acad. Sci. USA* **89**, 947–951
4. Hamra, F.K., Forte, L.R., Eber, S.L., Pidhorodeckyj, N.V., Krause, W.J., Freeman, R.H., Chin, D.T., Tompkins, J.A., Fok, K.F., Smith, C.E, Duffin, K.L., Seigel, N.R., and Currie, M.G. (1993) Uroguanylin: structure and activity of a second endogenous peptide that stimulates intestinal guanylate cyclase. *Proc. Natl Acad. Sci. USA* **90**, 10464–10468
5. Vaandrager, A.B. (2002) Structure and function of the heat-stable enterotoxin receptor/guanylyl cyclase C. *Mol. Cell. Biochem.* **230**, 73–83
6. Schulz, S., Lopez, M.J., Kuhn, M., and Garbers, D.L. (1997) Disruption of the guanylyl cyclase-C gene leads to a paradoxical phenotype of viable but heat-stable enterotoxin-resistant mice. *J. Clin. Invest.* **100**, 1590–1595
7. Mann, E.A., Jump, M.L., Wu, J., Yee, E., and Giannella, R.A. (1997) Mice lacking the guanylyl cyclase C receptor are resistant to STa-induced intestinal secretion. *Biochem. Biophys. Res. Commun.* **239**, 463–466
8. Mantoku, T., Muramatsu, R., Nakauchi, M., Yamagami, S., Kusakabe, T., and Suzuki, N. (1999) Sequence analysis of cDNA and genomic DNA, and mRNA expression of the medaka fish homolog of mammalian guanylyl cyclase C. *J. Biochem.* **125**, 476–486
9. Nakauchi, M. and Suzuki, N. (2003) Promoter analysis of a medaka fish intestinal guanylyl cyclase gene. *FEBS Lett.* **536**, 12–18
10. Swenson, E.S., Mann, E.A., Jump, M.L., and Giannella, R.A. (1999) Hepatocyte nuclear factor-4 regulates intestinal expression of the guanylin/heat-stable toxin receptor. *Amer. J. Physiol.* **276**, G728–G736
11. Park, J., Schulz, S., and Waldman, S.A. (2000) Intestine-specific activity of the human guanylyl cyclase C promoter is regulated by Cdx2. *Gastroenterol.* **119**, 89–96
12. Di Guglielmo, M.D., Park, J., Schulz, S., and Waldman, S.A. (2001) Nucleotide requirements for CDX2 binding to the cis promoter element mediating intestine-specific expression of guanylyl cyclase C. *FEBS Lett.* **507**, 128–132
13. Gadgil, H. and Jarrett, H.W. (2002) Oligonucleotide trapping method for purification of transcription factors. *J. Chromatogr. A* **966**, 99–110
14. Bradford, M.M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72**, 248–254
15. Chomczynski, P. and Sacchi, N. (1987) Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal. Biochem.* **162**, 156–159
16. Thompson, J.D., Higgins, D.G., and Gibson, T.J. (1994) CLUSTAL W: Improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Res.* **22**, 4673–4680
17. Lowry, O.H., Rosebrough, N.J., Farr, A.L., and Randall, R.J. (1951) Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193**, 265–275
18. Schacterle, G.R. and Pollack, R.L. (1973) A simplified method for the quantitative assay of small amounts of protein in biologic material. *Anal. Biochem.* **51**, 654–655
19. Kretzschmar, M., Kaiser, K., Lottspeich, F., and Meisterernst, M. (1994) A novel mediator of class II gene transcription with homology to viral immediate-early transcriptional regulators. *Cell* **78**, 525–534
20. Kaiser, K., Stelzer, G., and Meisterernst, M. (1995) The coactivator p15 (PC4) initiates transcriptional activation during TFIIA-TFIID-promoter complex formation. *EMBO J.* **14**, 3520–3527
21. Ge, H., Zhao, Y., Chait, B.T., and Roeder, R.G. (1994) Phosphorylation negatively regulates the function of coactivator PC4. Phosphorylation negatively regulates the function of coactivator PC4. *Proc. Natl Acad. Sci. USA* **91**, 12691–12695
22. Kumar, B.R., Swaminathan, V., Banerjee, S., and Kundu, T.K. (2001) p300-mediated acetylation of human transcriptional coactivator PC4 is inhibited by phosphorylation. *J. Biol. Chem.* **276**, 16804–16809
23. Ge, H. and Roeder R.G. (1994) Purification, cloning, and characterization of a human coactivator, PC4, that mediates transcriptional activation of class II genes. *Cell* **78**, 513–523
24. Malik, S., Guermah, M., and Roeder, R.G. (1998) A dynamic model for PC4 coactivator function in RNA polymerase II transcription. *Proc. Natl Acad. Sci. USA* **95**, 2192–2197
25. Wu, S.Y. and Chiang, C.M. (1998) Properties of PC4 and an RNA polymerase II complex in directing activated and basal transcription *in vitro*. *J. Biol. Chem.* **273**, 12492–12498
26. Kannan, P. and Tainsky, M.A. (1999) Coactivator PC4 mediates AP-2 transcriptional activity and suppresses ras-induced transformation dependent on AP-2 transcriptional interference. *Mol. Cell. Biol.* **19**, 899–908
27. Banerjee, S., Kumar, B.R., and Kundu, T.K. (2004) General transcriptional coactivator PC4 activates p53 function. *Mol. Cell. Biol.* **24**, 2052–2062
28. Kusakabe, T. and Suzuki, N. (2000) The guanylyl cyclase family in medaka fish *Oryzias latipes*. *Zool. Sci.* **17**, 131–140