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Involvement of General Transcriptional Coactivator PC4 in the Transcription of  
Medaka Fish Intestine-Specific Membrane Guanylyl Cyclase Gene (*OIGC6*)

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Running title: Transcriptional regulation of *OIGC6* by OIPC4

Note: Nucleotide sequence data reported are available in the DDBJ/EMBL/GenBank databases under the accession number AB188297.

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A recent study showed that the AGACCTTTGC nucleotides (between -90 and -79) contained in the *cis*-regulatory element in an intestine-specific membrane guanylyl cyclase gene, *OIGC6*, of the medaka fish, *Oryzias latipes*, are important for transcription of the gene in mammalian cultured cell line and in the medaka fish. Using sequence-specific DNA affinity chromatography, we purified a *cis*-regulatory element-binding protein from medaka fish intestinal nuclear extract and identified by mass spectrometry that it is a medaka fish homologue of general transcriptional coactivator PC4, which we designated as OIPC4. The expression of the *OIPC4* gene was detected in embryos, as well as in a large variety of tissues of the adult medaka fish. Using a 17-kDa recombinant OIPC4, we carried out an ultraviolet (UV) cross-linking experiment and an electrophoretic mobility shift assay (EMSA), demonstrating that the recombinant OIPC4 can be substituted for native OIPC4 in medaka fish intestinal nuclear extracts. In CACO-2 cells, cotransfection of the *OIGC6*-luciferase fusion genes with an OIPC4 expression vector resulted in 1.5-fold stimulation of the *OIGC6* 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 *OIGC6* 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 *OIGC6* gene are essential for transcription of the *OIGC6* 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 *OIGC6* 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 transcriptional regulation (10-12).

However, the consensus binding sequences for HNF-4 are not found in the 5'-flanking region of the *OIGC6* gene and those for Cdx2 are found although they do not seem to be involved (9), suggesting that the transcriptional regulatory mechanism of the *OIGC6* 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 a protein binding to that element play important roles in the transcription of the *OIGC6* gene (9). In this study, we purified that protein from medaka fish intestinal nuclear extracts, and we identified the protein as a medaka fish homologue (designated as OIPC4) of the mammalian positive cofactor 4 (PC4) by mass spectrometry. Here, we report the results of a UV cross-linking analysis and EMSA, which demonstrated that bacterially expressed recombinant OIPC4 can be substituted for native OIPC4 in medaka fish intestinal nuclear extracts. In addition, transient transfection of CACO-2 cells with an OIPC4 expression vector stimulates the promoter of the *OIGC6* gene through the *cis*-regulatory element. These results strongly support our hypothesis that OIPC4 is an important regulator of the *OIGC6* gene expression.

## MATERIALS AND METHODS

*Purification of a Cis-Regulatory Element-Binding Protein using the Oligonucleotide Trapping Method* — Single-stranded oligonucleotides (TG)<sub>5</sub> (5'-NH<sub>2</sub>-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)<sub>5</sub> Sepharose 4B. Single-stranded (CA)<sub>5</sub> protruding double-stranded oligonucleotides containing the *cis*-regulatory element were

synthesized using 5'-GCAGCGCACACACAGACCTTTGCACACCCA-3'-(CA)<sub>5</sub> and 5'-TGGGTGTGCAAAGGTCTGTGTGTGCGCTGC-3'-(CA)<sub>5</sub>, as described previously (9) and were used as a probe. A one-ml bed volume of (TG)<sub>5</sub> Sepharose 4B in a Polyprep empty column (BioRad, Hercules, CA, USA) was equilibrated with a binding buffer containing 10% glycerol, 100 mM NaCl, 2.5 mM MgCl<sub>2</sub>, 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 over a column five times under gravity flow. The column was washed three times with 10-ml aliquots of the binding buffer, and then the protein was eluted three times from the column by the addition of 2.5-ml portions of the 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), and then was purified by (TG)<sub>5</sub> Sepharose 4B column chromatography, as described above. The purified samples were precipitated with 75% acetone and were subjected to SDS-polyacrylamide gel electrophoresis (PAGE) with 15% gel and the samples were then silver-stained. 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 then was destained by treatment with 15 mM potassium hexacyanoferrate (III)/50 mM sodium thiosulfate for 10 min at room temperature. 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 they were 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 then were concentrated *in vacuo*. After desalting the peptide fragments with ZipTip<sub>C18</sub> (Millipore, Billerica, MA, USA), they 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 (OIPC4)* — Total RNA was prepared from the intestine of the 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<sup>TM</sup> First-Strand Synthesis System (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. The coding region of the *OIPC4* cDNA was amplified from the medaka fish

intestine cDNA by polymerase chain reaction (PCR) with paired primers (OIPC4-F: 5'-ATGCCTAAATCCAAGGAAGT-3' and OIPC4-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 sec at 96 °C, 30 sec at 53 °C, and 30 sec 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 OIPC4 was compared with those of known mammalian PC4s using the Clastal 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 the reverse transcription (RT)-PCR analysis, total RNA was prepared from medaka fish embryos (stages 35-39) and several adult medaka fish tissues using 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, the reactions without reverse transcriptase were also performed. PCR was then carried out with a specific primer set for the *OIPC4* gene (GenBank™ accession number AB188297), or for the *OICA1* gene (the cytoplasmic actin gene of the medaka fish, GenBank™ accession number D89627): OIPC4-F, 5'-ATGCCTAAATCCAAGGAAGT-3' and OIPC4-R, 5'-TCATGTTCTCTTGATGGCCT-3' (375-bp product); OICA1-F, 5'-GGGTCTTCATGACGGGC-3' and OICA1-R,

5'-CAAGTCGGAACACATGTGCA-3' (100-bp product), and 2  $\mu$ l of the reverse-transcribed cDNA solution. The following conditions for amplification were used: 30, 28, 25, and 22 cycles of 30 sec at 96 °C, 30 sec at 55 °C, and 30 sec at 72 °C. The PCR products were analyzed by electrophoresis on 1.5% agarose gel.

*Expression and Purification of Recombinant OIPC4* — A coding region of the *OIPC4* cDNA was amplified by PCR from the *OIPC4* cDNA subcloned into pBSII, described above, using paired primers, 5'-AGGAGAATTCTTATGCCTAAATCCAAGG-3' and 5'-AAGCGAATTCTCATGTTCTCTTGCTG-3'. The amplified fragment was digested with *EcoRI* and inserted into similarly digested pGEX-KG. *Escherichia coli* strain BL-21 was then transformed with a plasmid encoding a GST-OIPC4 fusion protein and expression was induced with 0.25 mM isopropyl  $\beta$ -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 OIPC4 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 OIPC4-containing supernatant was mixed with Benzamidine Sepharose 6B (Amersham Biosciences) to remove the thrombin, and then the mixture was centrifuged. The recombinant OIPC4 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 - 109 and - 79 in the 5'-flanking

region of the *OIGC6* gene, designated as E1) as a probe (9) and 50 ng of the recombinant OIPC4.

*Cell Culture and DNA transfections* — A coding region of the *OIPC4* cDNA subcloned into pGEX-KG (described above) was digested with *EcoRI* and inserted into similarly digested pCR<sup>®</sup>3.1 (Invitrogen). The *OIGC6*-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<sup>®</sup>, Logan, UT, USA) and 1x penicillin-streptomycin-glutamine (Invitrogen) under a humidified 5% CO<sub>2</sub> atmosphere. For the reporter-enzyme assay, 5x10<sup>5</sup> CACO-2 cells or 2x10<sup>5</sup> COS7 cells were plated per well in a six-well plate cultured for 24 h before transfection. One μg of the *OIGC6*-luciferase fusion gene, 2 μg of OIPC4- pCR<sup>®</sup>3.1 or 2 μg of pCR<sup>®</sup>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 a 48-h culture of 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 in order to normalize it in regard to 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 were 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) by 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 search 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.

The EST consensus sequence TC25781 contains an open reading frame (ORF) encoding a polypeptide consisting of 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 hypothetical *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 OIPC4.

Comparison of the deduced amino acid sequence of OIPC4 with the known sequence of mammalian PC4 demonstrated similarities between OIPC4 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, which 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) most significantly contributes to the activity of 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 OIPC4 (positions 1-21) showed 77% similarity to the SEAC motif of human PC4, and contained six putative recognition sites for CKII, suggesting that OIPC4 exhibits cofactor activity (Fig. 2A). RT-PCR analysis demonstrated that the *OIPC4* gene is expressed ubiquitously, in embryos (Fig. 2B) and in almost all adult medaka fish tissues (Fig. 2C). The PCR at different cycles demonstrated that *OIPC4* was expressed in the ovary slightly more abundant than in other tissues (Fig. 2C).

To determine whether or not the bacterially expressed recombinant OIPC4 possesses the ability to bind the *cis*-regulatory element, we carried out both a UV cross-linking analysis and EMSA using medaka fish intestinal nuclear extracts and the

recombinant OIPC4. 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 OIPC4, 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 OIPC4 (Fig. 4A). However, no complex was formed when the E1 probe was incubated with the recombinant GST-OIPC4 (Fig. 4A), suggesting that GST, which was attached to the amino terminus of the recombinant OIPC4, 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 OIPC4 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 the competition of unlabeled mutated competitors for labeled wild-type E1 binding to the recombinant OIPC4. 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 almost 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). OIPC4 contained both of these positively charged regions (Fig. 2A),

suggesting that OIPC4 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 OIPC4 exhibits certain preferences with respect to binding sequences, a finding which suggests that PC4 may exert still unknown functions in the recognition of the binding sequence.

To assess the role of OIPC4 in the transcription of *OIGC6* gene, the *OIGC6*-luciferase fusion genes were cotransfected with OIPC4 expression vector (OIPC4-pCR<sup>®</sup>3.1) or empty expression vector (pCR<sup>®</sup>3.1) in 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 activated about 1.5 fold by OIPC4, whereas the luciferase activity of an empty luciferase construct was not, indicating a specific effect of OIPC4 on *OIGC6* promoter activity (Fig. 5).

Incorporation of the 3-bp mutation in the binding site of OIPC4 (the region between -91 and -89) into the construct (-1757mutCAG/+50) abolished the effect of OIPC4 (Fig. 5). Although fold activation effect of the OIPC4 on the *OIGC6* gene transcription seems to be low, it was clearly demonstrated that OIPC4 activate the transcription of the *OIGC6* gene through the *cis*-regulatory element and essential for the expression of the *OIGC6* gene. On the other hands, in COS7 cells, OIPC4 failed to activate all examined constructs (data not shown), suggesting that OIPC4 could activate the promoter of the *OIGC6* gene only in the intestine-derived CACO-2 cells.

As shown in Fig. 2C, RT-PCR analysis demonstrated that the *OIPC4* 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 the 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 the OIPC4 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 OIPC4 (Fig. 2C and Fig. 4A). Although E1 weakly binds to other protein(s) in the brain, testis, or liver nuclear extracts, the binding of OIPC4 to E1 may require additional factor(s) which are present only in the intestinal nuclear extracts. Furthermore, the results of the coexpression experiments that OIPC4 could activate the transcription of the *OIGC6* gene only in the intestine-derived cell line also suggest that the activity of OIPC4 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 coactivators, 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 like 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 OIPC4 involves in the transcription of the intestine-specific membrane GC gene, *OIGC6*, suggesting that

OIPC4 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 the 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 that mammalian PC4 requires upstream activators and interacts with many factors (19, 23-25), and the results that the activity of the OIPC4 required the additional factor(s) expressed in the intestine, OIPC4 may also interact with some unknown factor(s) and/or activator(s) binding to the upstream region of the *OIGC6* gene; although this region has yet to be identified. The identification of the partner factor(s) of OIPC4 will reveal more details about the mechanisms of the transcriptional regulation of intestine-specific *GC* genes.

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## Figure legends

Fig. 1, Biochemical purification of a *cis*-regulatory element binding protein. Protein samples were subjected to electrophoresis on 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.

Fig. 2, A. Alignment of the amino acid sequences of OIPC4 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. *OIPC4* expression in embryos at stages 35-39. C. *OIPC4* expression in several adult medaka fish tissues. PCR cycles numbers are indicated at the left. RT+ and RT- represent amplification with and without reverse transcriptase, respectively. *OICAI* was amplified as an internal control.

Fig. 3, UV cross-linking analysis using medaka fish intestinal nuclear extracts or bacterially expressed recombinant OIPC4. Competitive complex formation experiments were conducted by the addition of a 40-fold excess of unlabeled E1.

Fig. 4, A. Electrophoretic mobility shift assay (EMSA) with an E1 probe using medaka fish intestinal nuclear extracts (lane 1), bacterially expressed recombinant OIPC4 (lane 2), and GST-OIPC4 (lane 3). B. Competitive EMSA of labeled E1 and

unlabeled wild-type or mutated competitors using the recombinant OIPC4. Complex competition was achieved by the addition of unlabeled E1 (in 40-fold excess or 100-fold excess) (left panel). Recombinant OIPC4 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.

Fig. 5, Potentiation of the *OIGC6* promoter activity by OIPC4 in CACO-2 cells. The *OIGC6*-luciferase fusion gene constructs are indicated at the left of figure. White boxes show the 5'-leader sequence of the *OIGC6* gene, black boxes denote the luciferase open reading frame. An untranscribed 5'-flanking region of the *OIGC6* gene is indicated by horizontal line. Each constructs was cotransfected with the OIPC4 expression vector (solid bars) or empty expression vector (open bars). Data represented the luciferae-to- $\beta$ -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.

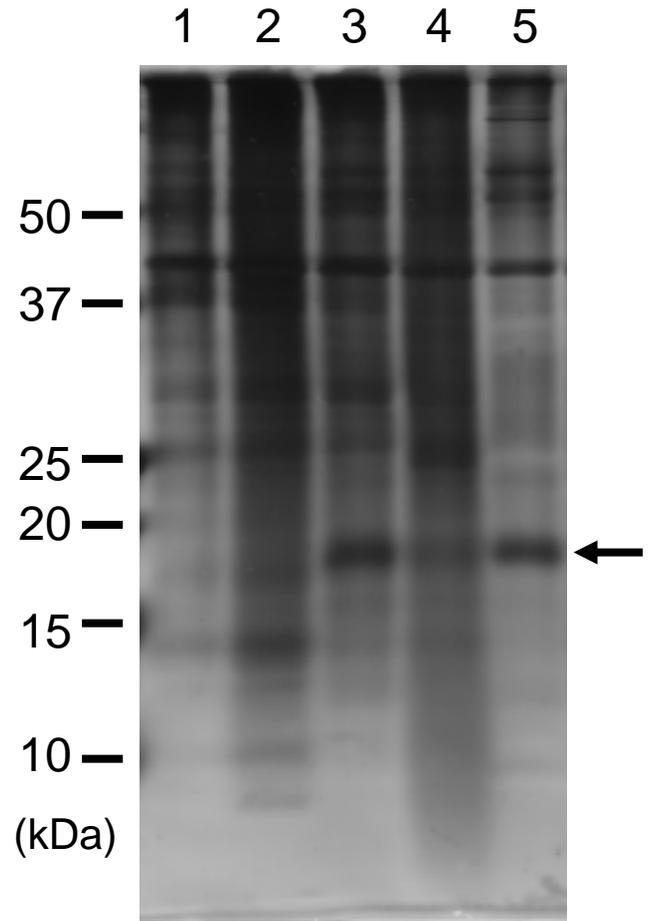
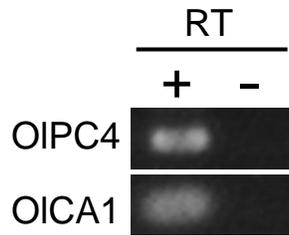


Fig. 1

**A**

	SEAC	A-region	SEAC	
medaka	MPKSKEVLSSTG-GSDSDSEVE	TKAKKKKLSAPEKPAKKPKT	GESSKPGGSSR-SGGPEDNMF	62
human	MPKSKELVSSSSSGSDSDSEVD	KKLKRKKQVAPEKPVKKQKT	GETSRALSSSKQSSSRDDNMF	64
mouse	MPKSKELVSSSSSGSDSDSEVE	KKLKRKKQAVPEKPVKKQKP	GETSRALASSKQSSSRDDNMF	64
rat	MPKSKELVSSSSSGSDSDSEVE	KKLKRKKQVVPEKPVKKQKP	GESSRALASSKQSSSRDDNMF	64
	***** ** * *****	* * * * * * * * * * * * *	** * ****	
	<b>C-region</b>			
medaka	QIGKMRYVSVRDFKGVLIDIRE	YWMNQDGEMKPGKKGISLNPEQWNQLK	<u>DQMSEIDEA</u> IKRT	125
human	QIGKMRYVSVRDFKGVLIDIRE	YWMDPEGEMKPGRKGISLNPEQWSQLKEQ	ISDIDDAVRKL	127
mouse	QIGKMRYVSVRDFKGVLIDIRE	YWMDSEGEMKPGRKGISLNMEQWSQLKEQ	ISDIDDAVRKL	127
rat	QIGKMRYVSVRDFKGVLIDIRE	YWMDSEGEMKPGRKGISLNMEQWSQLKEQ	ISDIDDAVRKL	127
	*****	*****	***** * * * * *	

**B**



**C**

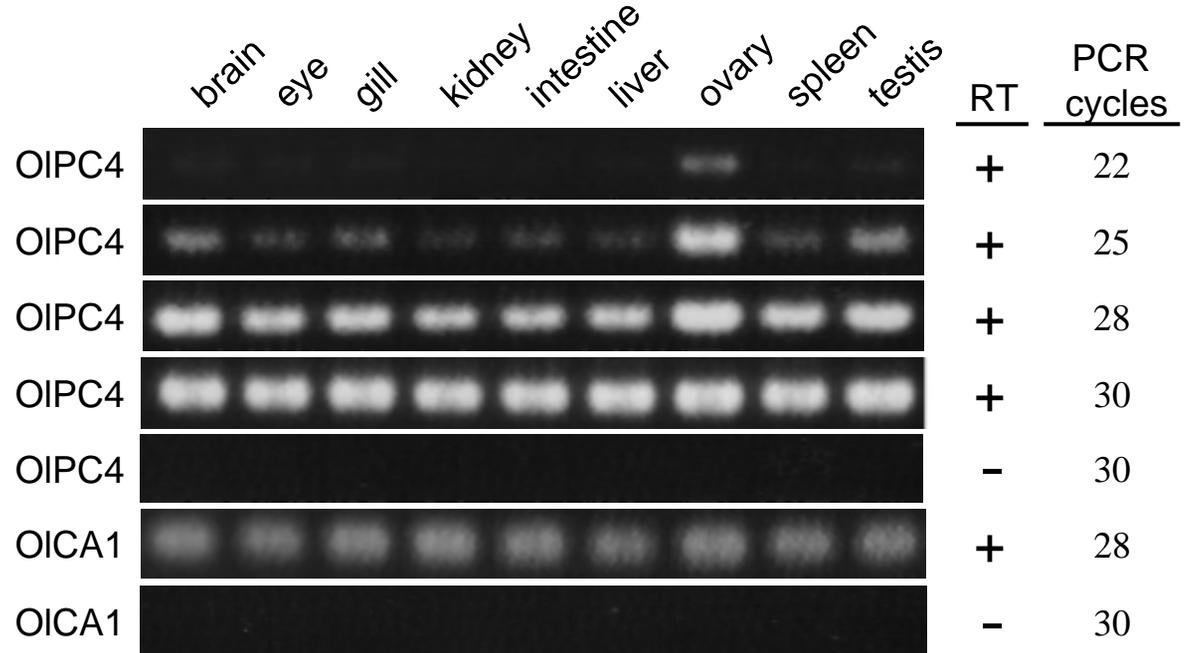


Fig. 2

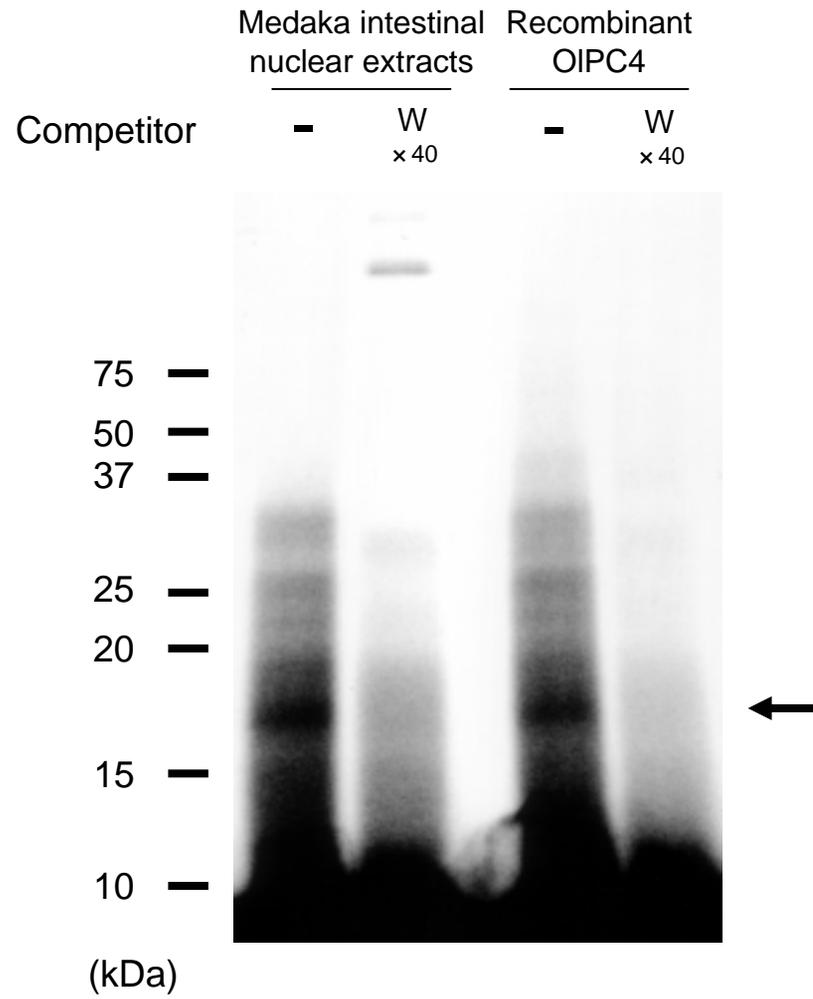
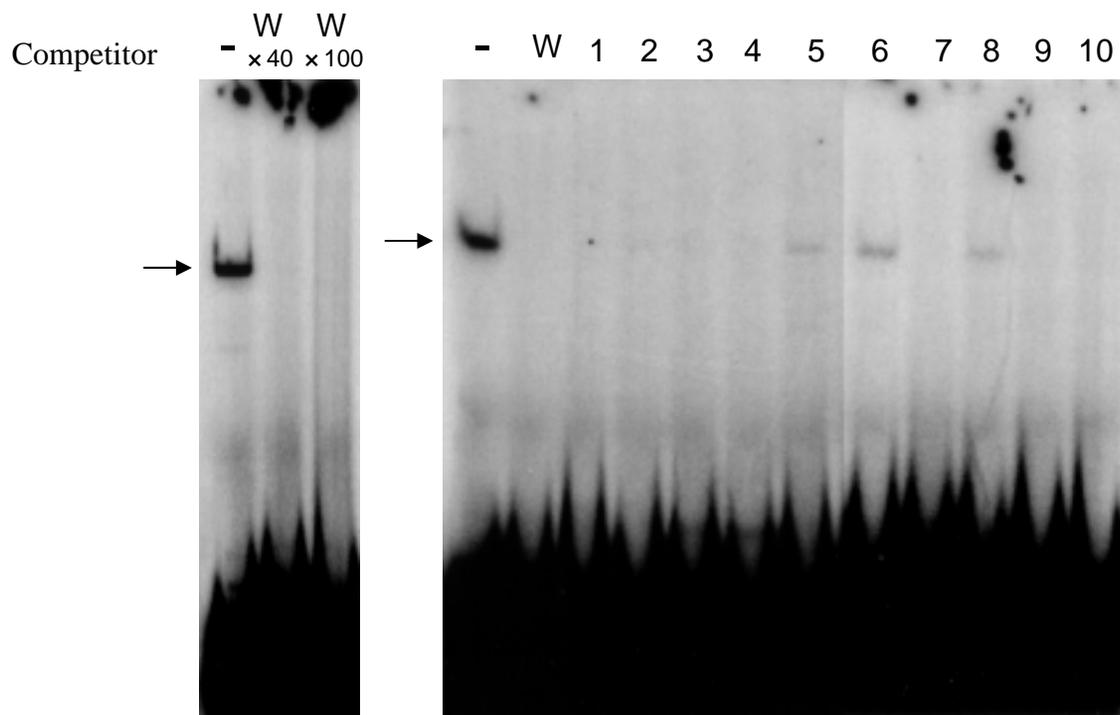


Fig. 3

A



B



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 ACCTTTG**CA** C-3'

Fig. 4

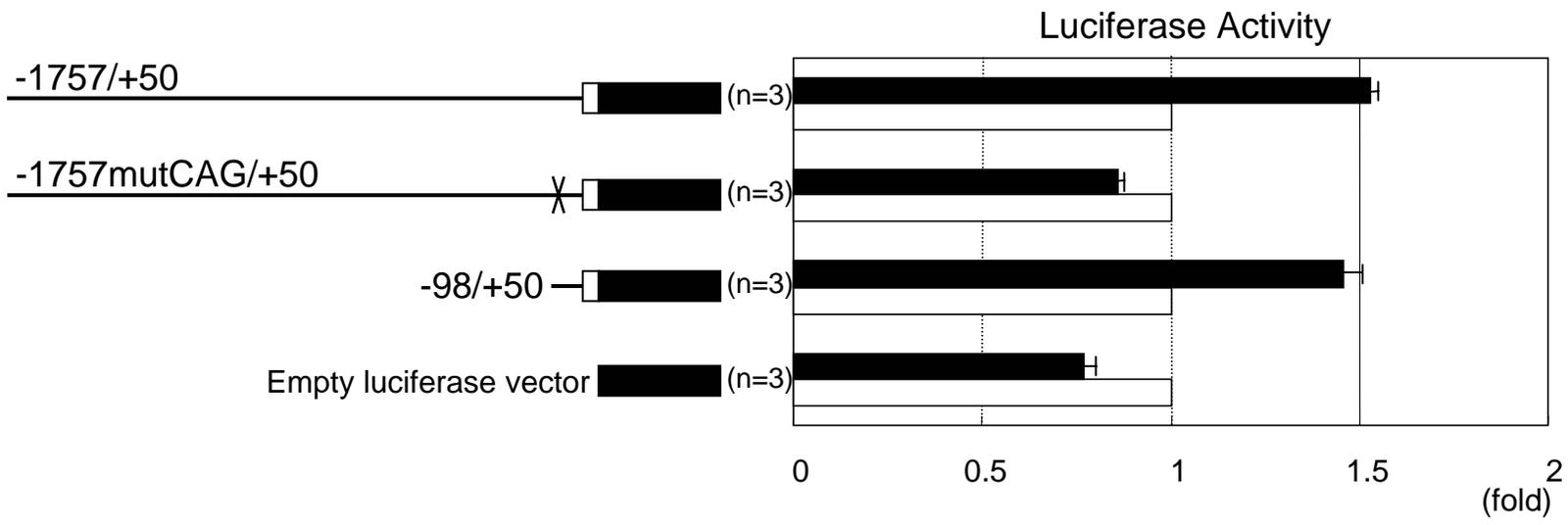


Fig. 5