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Modular Changes of Cis-Regulatory Elements From Two Functional Pit1 Genes in the Duplicated Genome of *Cyprinus carpio*

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Abstract The pituitary-specific transcription factor Pit1 is involved in its own regulation and in a network of transcriptional regulation of hypothalamo-hypophyseal factors including prolactin (PRL) and growth hormone (GH). In the ectotherm teleost *Cyprinus carpio,* Pit1 plays an important role in regulation of the adaptive response to seasonal environmental changes. Two Pit1 genes exist in carp, a tetraploid vertebrate and transcripts of both genes were detected by RT-PCR analysis. Powerful comparative analyses of the 5′-flanking regions revealed copy specific changes comprising modular functional units in the naturally evolved promoters. These include the precise replacement of four nucleotides around the transcription start site embedded in completely conserved regions extending upstream of the TATA-box, an additional transcription factor binding site in the 5′-UTR of gene-I and, instead, duplication of a 9 bp element in gene-II. Binding of nuclear factors was assessed by electro mobility shift assays using extracts from rat pituitary cells and carp pituitary. Binding was confirmed at one conserved Pit1, one conserved CREB and one consensus MTF1. Interestingly, two functional Pit1 sites and one putative MTF1 binding site are unique to the Pit1 gene-I. In situ hybridization experiments revealed that the expression of gene-I in winter carp was significantly stronger than that of gene-II. Our data suggest that the specific control elements identified in the proximal regulatory region are physiologically relevant for the function of the duplicated Pit1 genes in carp and highlight modular changes in the architecture of two Pit1 genes that evolved for at least 12 MYA in the same organism. J. Cell. Biochem. 99: 905–921, 2006.

Key words: Pit1; duplicate genes; promoter architecture; cis-regulatory elements; *Cyprinus carpio*

Powerful comparative genome analyses highlighted evolutionarily conserved non-coding sequences unexpected in extension and organization that might serve as critical cis-regulatory regions [Sandelin et al., 2004]. This is particularly true for promoter sequences from duplicated genes, which evolved in the same cell over millions of years.

The carp (*Cyprinus carpio*) experienced genome duplication some 12 million years ago in addition to the well-documented two rounds (2R) of genome duplication at the beginning of vertebrate radiation [David et al., 2003]. Consistent with the fish-specific genome duplication referred to as 3R-hypothesis, a complex set of gene copies is present in a single individual [Hoegg and Meyer, 2005]. Genome duplication may be followed by either the loss or inactivation of one of the duplicated gene copies, subfunctionalization whereby each gene copy acquires a specific expression pattern, and/or one copy acquires a novel function (neofunctionalization) [Ferris and Whitt, 1977; Hoegg and Meyer, 2005]. Many duplicated genes were shown to be maintained in carp [Arends et al., 1998; Futami et al., 2001; Hermesz et al., 2002; Molina et al., 2002; Figueroa et al., 2005], possibly due to the relatively recent occurrence of the duplication. Two divergent copies of the *Pit1* gene, both coding for a complete Pit1 protein and showing none of the features associated to pseudogenization, were identified in carp [Kausel et al., 1998, 1999]. Therefore,

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similarity analyses of the 5′-flanking regions were expected to reveal important features of the transcriptional regulation of these two recently duplicated Pit1 gene copies.

The transcription factor Pit1 is composed of two functional domains; the N-terminal serine/threonine-rich transactivating domain and the bipartite C-terminal DNA-binding domain of the POU-class homeo domain type. Pit1 is required for tissue-specific expression of pituitary hormones such as prolactin (PRL), growth hormone (GH), thyroid-stimulating hormone (TSH-β), and, in teleosts, somatolactin (SL) [Lopez et al., 2001]. It has also been shown to regulate its own expression during development and in the adult. Mutations in the Pit1 gene have been identified in patients affected by 'combined pituitary hormone deficiency' (CPHD) [Pfaffle et al., 1996; Pernasetti et al., 1998], in the Snell dwarf mouse [Lin et al., 1994] and in zebrafish [Nica et al., 2004].

Regulation of Pit1 gene expression has been shown during embryogenesis [Dasen and Rosenfeld, 2001; Nica et al., 2004] and during the entire life. In particular, Pit1 expression is strongly modulated during acclimatization of the common carp (*Cyprinus carpio*) between summer and winter season [Kausel et al., 1999]. This circannual regulation, high in summer and low in winter, correlates well with the seasonal modulation of Pit1 target genes, such as PRL [Figueroa et al., 1994], GH [Figueroa et al., 2005], SL [Lopez et al., 2001], and Pit1 itself [Kausel et al., 1999]. Except for the initial activation of Pit1 expression during development [Dasen and Rosenfeld, 2001], little is known concerning the mechanisms of regulation of this gene in the adult life. Pit1 gene promoters were analyzed in rat [Chen et al., 1990; McCormick et al., 1990], mouse [Rhodes et al., 1993], human [Ohta et al., 1992; Delhase et al., 1996], and activation by regulatory pathways acting on cAMP levels has been shown [Chen et al., 1990; McCormick et al., 1990; Elsholtz et al., 1991; Rhodes et al., 1993; Delhase et al., 1996; Sekkali et al., 1999; Ferry et al., 2005].

In this study, we show the presence and expression at the transcriptional level of two Pit1 genes in individual fish. As the upstream regulatory region of the Pit1 gene-II was already known, we further obtained the promoter region of the Pit1 gene-I and performed an extensive sequence comparison of these two regulatory regions. Moreover, we show that nuclear extracts from a Pit1 expressing cell line and from carp pituitary contain transcription factors binding to conserved identified consensus sites, but most importantly, also to sequences present only in one of the gene copies. Finally, we show that the presence of these additional binding sites correlates with a higher expression of Pit1 gene-I as compared to gene-II in winter carp.

MATERIALS AND METHODS

Animals and Tissue Preparation

Adult carp (*Cyprinus carpio*) were caught in the Calle-Calle river in Valdivia, Chile and maintained for 3 weeks at a natural photoperiod and temperature in a fixed 3×4 m cage submerged 2 m in an effluent of the same river. Pituitary glands from male carp were dissected and processed immediately for RNA or nuclear extract preparation.

Genomic DNA Southern Blot Analyses

Genomic DNA was prepared from blood, which in carp contains nucleated erythrocytes, using a modified method described by Garbutt et al. [1985]. Briefly, 1 ml of heparinized blood was diluted in one volume of lyses buffer (4% w/v natrium desoxycholate, 0.8% v/v Nonidet P40, 1 mM NaH_2PO_4, 1 mM $MgCl_2$), mixed carefully and brought to 10 ml with high salt buffer (10 mM Tris/HCl pH 8.3, 5 mM EDTA pH 8.0, 150 mM NaCl). After addition of 3.5 ml 5 M $NaClO_4$ pH 8.0 and 1.5 ml 10% SDS, the solution was carefully mixed for 15 min at room temperature. DNA was purified by phenol-chloroform extraction and precipitated according to standard procedures [Sambrook et al., 1989]. Residual proteins were proteinase-K digested (1 mg/ml, 37°C, 3 h) and phenol extraction was repeated. Finally, DNA was precipitated, dissolved in TE (10 mM Tris/HCl pH 7.4, 1 mM EDTA) and stored at 4°C.

Southern blot analyses were performed using genomic DNA from individual male carp cut to completion with *Eco*RI or *Hin*dIII. Twelve micrograms were fractionated on a 1% agarose gel and transferred to a nitrocellulose membrane (BioRad). The blots were probed using radiolabeled fragments corresponding to carp Pit1, gene-I cDNA (accession no. AF096863), or gene-II promoter (nucleotides 49–1036, accession no. AF132287) and washed under stringent

conditions. The probes were labeled using the random primer method (Random Primer DNA Labeling System, Invitrogen) with ^{32}P-α-dCTP (New England Nuclear, Boston, MA) and the blots were autoradiographed with X-Omat film (Kodak).

Isolation of Pit1, Gene-I Promoter Sequences

A λFixII carp genomic library (Stratagene) was screened according to standard procedures [Sambrook et al., 1989]. Of the 10^6 recombinant plaques screened, two clones hybridized strongly with the ^{32}P-labeled carp Pit1 gene-I cDNA probe (560 bp, accession number AF09686) [Kausel et al., 1999]. Positive clones were characterized by restriction enzyme mapping and Southern hybridization. The Pit1, gene-I promoter sequences were amplified from the gene-I genomic clone λGP4 using oligonucleotides oli5a (exon I specific antisense, Table I) and oliT7 (vector specific sense, Table I), subcloned into pGEM-T-Easy (Stratagene), yielding the recombinant $pGP4_{776}$ and sequenced.

Determination of the Transcription Start Site

The transcription initiation site for both Pit1 genes was determined by primer extension using carp pituitary RNA as template [Sambrook et al., 1989]. The oligonucleotide oli5a (Table I), which is complimentary to exon-I, was end-labeled with γ-^{32}P-ATP and T4 polynucleotide kinase. Fifteen micrograms of total RNA were incubated with 5 pmol of labeled primer at 70°C for 10 min and annealed on ice. The primer extension reaction was carried out with 100 units of SuperScript II RNase H$^-$ Reverse Transcriptase (Invitrogen) in a solution containing 50 mM Tris/HCl pH 8.3, 75 mM KCl, 3 mM $MgCl_2$, 10 mM DTT, and 1 mM of each dNTP at 42°C. The newly synthesized DNA strands were denatured and fractionated in a 8% polyacrylamide denaturing gel and revealed by autoradiography.

The molecular size standard was prepared by performing a sequencing reaction with the fmol DNA Cycle Sequencing System (Promega) on $pGP4_{776}$ DNA using the ^{32}P-labeled oli5a as primer according to the supplier's instructions.

Detection of Gene-Copy Specific Transcripts

Total RNA was isolated from carp pituitary according to Chomzynski and Sacchi [1987]. RNA was treated with RNase-free DNase I (Invitrogen) and reverse transcribed using SuperScript II (Invitrogen) and oligo dT_{15} (Invitrogen). PCR was carried out with 2.5 units of Taq DNA Polymerase (Invitrogen) on 0.5 μg cDNA in a solution containing 20 mM Tris/HCl, pH 8.4, 50 mM KCl, 1.5 mM $MgCl_2$, 0.5 mM dNTPs, and 0.5 mM forward and reverse primer. A pilot reaction was performed to determine the number of cycles required to reach the exponential amplification phase for each primer pair. Amplification was performed for 30 cycles of denaturation for 30 s at 94°C, annealing for 30 s at 55°C, and elongation for 1 min at 72°C.

TABLE I. Oligonucleotides Used in This Study

		Gene-I (AY273789)	Gene-II (AF132287)
Primer extension			
oli5a	5′-GTAGTGAAGCAATCAGTGGTG-3′	756–776	1129–1149
RT-PCR			
oli6s	5′-CGATGCCTCGTGTGCAGGAC-3′	688–707	—
oli7s	5′-ACAGGTGGGACCCTGAGTAG-3′	—	1061–1080
oli3a	5′-GCGGCGGTAAGCTGTGGGTC-3′	305–324 (U92542)	6684–6665
EMSA (only coding strand primers are shown)			
ccP1	5′-GTTTGAAC**ATGATTTTT**AAAGC-3′	—	126–148
ccP2	5′-GCTTTTTTGTAG**TAAATCAT**ATTTTTG-3′	—	286–312
ccP3	5′-GAGCTC**ATTAACAT**GGATGAG-3′	605–625	989–1009
ccP4	5′CTCCAT**ACTTATGCAAC**TGGAC-3′	374–395	—
ccP5	5′-GGTTTAAT**ACTCATGCA**TTTTT-3′	323–345	(655–665 partial)
ccP6	5′-TTTAT**AAATATTCATG**TGCTCCTC-3′	277–300	—
ccA1	5′-GTAAT**CAGACTCA**TTCCCTC-3′	—	765–784
ccA2	5′-GAGCTT**TGCCTCAGC**GGGCCG-3′	564–584	947–967
ccC1	5′-CAGTGGGC**CGTCA**TGGTTTGC-3′	575–595	959–979
ccM1	5′-CGATGCCTC**GTGTGCA**GGAC-3′	688–707	—
ccM2	5′-ATAC**CTGCAC**ACGCCCTGCTC-3′	242–262	1033–1053

Numbers refer to nucleotide position in sequence according to GenBank accession number in brackets. Primer extension: oli5a, antisense oligonucleotide in the first exon; RT-PCR: oli6, oli7, gene specific primers For EMSA analysis only coding strand primers are shown; predicted transcription factor binding sites is highlighted in bold; Pit1 sites ccP1, ccP2, ccP3, ccP4, ccP5, ccP6, and P2; AP-1 site, ccA1, ccA2; CREB site, ccC1; MTF1 site, ccM1 (~~o~~li6), ccM2, control site oli7.

Amplification products were digested with *Pst*I and fractionated on 1% agarose gel.

Protein–DNA Interaction Analyses

Binding of nuclear factors to predicted transcription factor binding sites was analyzed by electrophoretic mobility-shift assays (EMSA) using nuclear extracts (NE) from GH3B6 cells, a Pit1 expressing rat pituitary cell line, and from carp hypophysis. Extract from cells grown in plastic Petri dishes of 137 mm diameter to 60% confluence were prepared as follows. Medium was eliminated, cells washed once with 1× PBS, then 1 ml 1× PBS was added, cells scraped off with a rubber policeman and transferred to a sterile microcentrifuge tube; cells were collected by centrifugation at 1000 rpm for 5 min at 4°C, supernatant discarded; cells were resuspended in 400 μl hypotonic buffer (10 mM HEPES pH 7.9, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM DTT, 0.5 mM PMSF) and incubated 15 min on ice, then 20 μl 10% NP40 were added (final conc. 0.2%–0.5% NP40), mixed by vortexing for 30 s and nuclei collected by centrifugation at 2000 rpm for 5 min at 4°C; supernatant was discarded and the pellet resuspended in 100 μl 4× binding buffer (4× binding buffer: 20 mM HEPES pH 7.9, 400 mM KCl, 20% glycerol, 2 mM DTT, 0.5 mM PMSF), the solution was centrifuged at full speed for 30 min at 4°C to eliminate genomic DNA, the supernatant (=NE) was stored in three aliquots at −80°C.

Carp pituitary NE were prepared as follows. One pituitary (ca. 30 mg) was homogenized by three strokes in a dounce homogenizer (tight) in 1 ml icecold hypotonic buffer (10 mM HEPES pH 7.9, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM DTT, 0.5 mM PMSF), incubated 15 min on ice, dounced three more times and briefly centrifuged (30 s, 12,000*g*, 4°C). The sediment was resuspended in 100 μl 4× binding buffer (20 mM HEPES pH 7.9, 400 mM KCl, 20% glycerol, 2 mM DTT, 0.5 mM PMSF). The lysate was frozen in liquid nitrogen, thawed on ice, centrifuged (12,000*g*, 4°C, 30 min) and the pellet discarded. Aliquots were stored at −80°C. Protein concentration was determined by using a Bradford assay with bovine serum albumin as standard.

DNA templates were prepared from synthetic oligonucleotides annealed in buffer (70 mM Tris/HCl pH 7.6, 0.1 M KCl, 10 mM $MgCl_2$, 1 mM 2-mercaptoethanol) by heating for 10 min to 95°C and cooling gradually to 4°C. The annealed oligonucleotides were end-labeled with γ-^{32}P-ATP and T4 polynucleotide kinase (Promega). For the binding assays with cell extract only sense oligonucleotide was labeled prior to annealing.

The binding reactions were performed with 2 μg of nuclear protein extract, 1 μg of poly (dI:dC), and 8,0000 cpm of ^{32}P-labeled DNA fragment in 5 mM HEPES pH 7.8, 100 mM KCl, 0.5 mM DTT, 5% (v/v) glycerol. For competition experiments, a 200-fold molar excess of unlabeled binding site was added. Reactions were incubated for 15 min at room temperature and loaded on a prerun 4% (w/v) polyacrylamid gel (acrylamide:bisacrylamid 40:1). Gels were run at 4°C in buffer 0.5× TBE (44.5 mM Tris, 44.5 mM boric acid, 1 mM EDTA) at 150 V, dried, exposed to an X-ray-sensitive Imaging screen-K (BioRad, Hercules, CA) and the image was analyzed on a Molecular Imager FX System (BioRad).

In Situ Hybridization

Gene copy specific antisense oligonucleotides oli6a (gene-I) and oli7a (gene-II) were used as probe in serial cuts of sagittal sections of pituitary from male winter-acclimatized carp as described in Kausel et al. [1999]. Control hybridizations were performed with the corresponding sense oligonucleotides (not shown). Specific signal was quantified as integrated optical density (IOD) by Image-Pro-Plus in three to five sections from each individual, the ratio gene-I to gene-II was determined resulting in mean and standard deviation.

RESULTS

The Two Pit1 Genes Are Present in Each Individual Carp Fish

In order to ensure that the previously identified carp Pit1 genes [Kausel et al., 1998, 1999] truly represent a duplicated gene, genomic DNA from four individual carp were analyzed by Southern blot using a probe corresponding to the Pit1, gene-II promoter, and to the Pit1, gene-I cDNA. In both *Eco*RI and *Hin*dIII digested DNA, the cDNA probe (Fig. 1B,C) clearly revealed a fragment corresponding to the Pit1, gene-II (see Fig. 1A), and an additional fragment revealing the presence of gene-I in each individual. Occasional duplicate bands illustrate the presence of two alleles of the

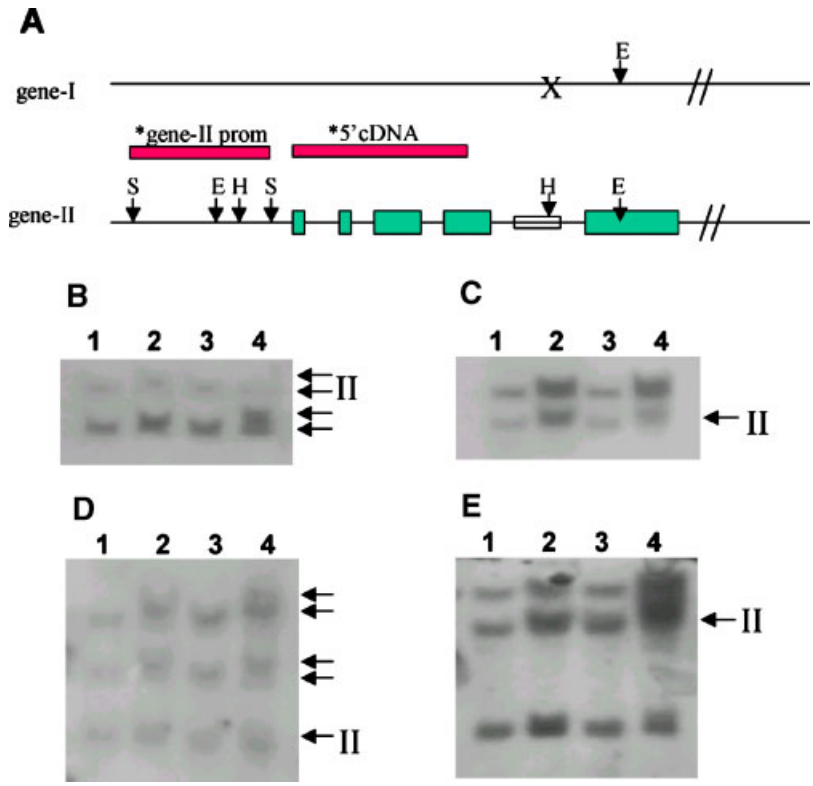

Fig. 1. Two Pit1 genes in carp genomic DNA. **A**: Schematic representation of gene-I and gene-II genomic sequences. Only relevant sites are indicated. **B**–**E**: Southern blot analyses of 10 μg genomic DNA (1–4: carp#1, #2, #3, #4) digested with *Eco*RI (B, D) and *Hin*dIII (C, E), and probed with ^{32}P labeled 560 bp 5′cDNA (B, C) and 992 bp gene-II promoter fragment (D, E). Arrows indicate fragments, II those derived from gene-II. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

corresponding gene in this particular fish. The promoter probe revealed in each case the same band corresponding to the Pit1, gene-II and two additional bands, again supporting the presence of two Pit1 genes in each individual fish.

Both Pit1 Genes Are Expressed in Individual Carp

We next determined whether each of the two carp Pit1 genes is expressed.

Comparison of the cDNA sequence of Pit1, gene-I with the cDNA deduced from the gene-II genomic sequence [Kausel et al., 1999] revealed the presence of divergent sequences in the 5′-untranslated region (Fig. 2A). Gene-specific primers were designed against this divergent region and used, in combination with a reverse primer directed against exon-III, to amplify cDNA fragments corresponding to the two Pit1 genes from carp pituitary mRNA. Two fragments of identical size were obtained (Fig. 2C, lanes 2,3). To confirm the identity of the two cDNA fragments, we took advantage of the presence of an additional *Pst*I site in the Pit1, gene-I cDNA. Digestion of the amplified fragments clearly revealed the patterns expected for each of the two cDNAs (Fig. 2B,C lanes 5,6). These results show that the two carp Pit1 genes are indeed expressed in the pituitary of an individual carp.

The Two Pit1 Gene Promoters Have Significantly Diverged Since Duplication

In order to gain insight into the mechanisms of transcriptional control of the duplicated Pit1 genes, we obtained the sequence of the Pit1, gene-I promoter. A positive phage (λGP4) was isolated from a *Cyprinus carpio* genomic library screened with the gene-I cDNA probe (accession number AF09686). Restriction mapping revealed that this phage contained the complete gene-I sequence (not shown). The 5′-flanking region was amplified by PCR, using a forward primer directed against the vector T7 sequence and a reverse primer against exon I, and subcloned to generate $pGP4_{776}$. Sequence analysis revealed the presence of 41 bp of coding sequence, as expected, and 735 bp upstream from the translation start codon ATG. A TATA-box is

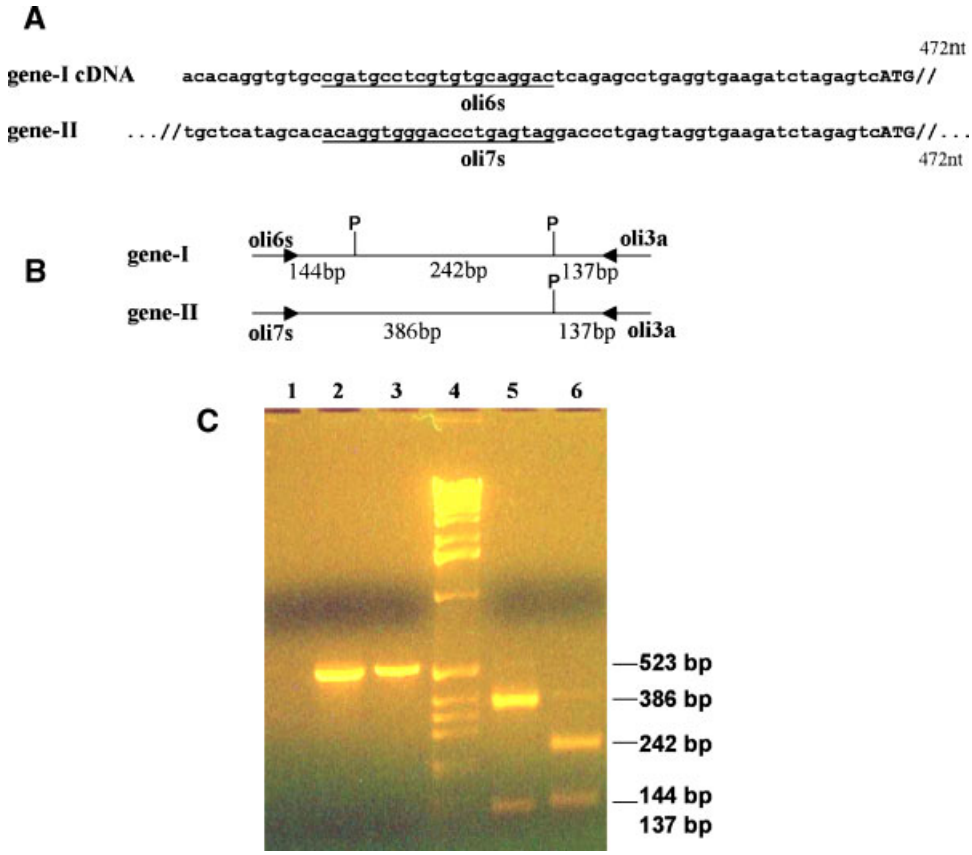

Fig. 2. Both Pit1 genes are expressed in carp pituitary gland. **A**: Alignment of the region upstream from the ATG of carp Pit1, gene-I 5′-cDNA (accession No. AF09686), and gene-II (accession No. AF132287). The location of gene-copy specific oligonucleotides is underlined. **B**: Schematic representation of the restriction site polymorphism in gene-specific RT-PCR products. **C**: RT-PCR amplification products of carp pituitary cDNA with gene-copy specific sense primer and exon-III specific antisense primer (oli3a) fractionated in a 1% agarose gel stained with ethidium bromide. (1) Negative control without template DNA; (2) oli7s/oli3a; (3) oli6s/oli3a; (4) DNA size standard; (5) PCR amplification product from (2)/*Pst*I; (6) PCR product from (3)/*Pst*I. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

observed 91 bp upstream from the ATG (see Fig. 3B). Furthermore, the analysis confirmed that this gene (gene-I) indeed encodes the previously identified cDNA [Kausel et al., 1999].

When the gene-I promoter (776 bp) was plotted against the corresponding gene-II sequence (1150 bp), the diagonals in the dot-matrix plot revealed highly conserved regions as well as completely divergent domains (Fig. 3A). An overall conservation of 49% identical nucleotides concentrated in the partial exonI and 5′ adjacent 300 bp in addition to three shorter stretches further upstream interspersed by completely divergent sequences (Fig. 3B). The distance between TATA-box and ATG in gene-I (91 bp) is 10 bp longer than the respective distance in gene-II (81 bp); the length difference results from changes in a defined region. Interestingly, for this region of 26 nucleotides starting at position −51 relative to the translation initiation site in gene-I, no matches could be found in gene-II (Fig. 3B). This divergence was also apparent in the interruption to the end of the longest diagonal in the right corner of the dot-matrix plot (Fig. 3A). The rest of this 353 bp (position 424–776 in gene-I, 806–1150 in gene-II) proximal promoter region is highly conserved (88.1% identity, 311/353). Further upstream, two additional highly conserved domains are apparent, from position 11–67 in gene-I to position 12–71 in gene-II (identity 91.7%, 55/60), and 303–344 in gene-I to 634–665 in gene-II (96.8%, 30/31). The region separating the two most distal homology regions contains a large, 563 bp insertion in gene-II relative to gene-I.

Further comparative aspects were derived by manual inspection of the region between the TATA-box and the translation initiation codon ATG of the two carp Pit1 genes (Fig. 4B). The

coding sequences and 18 bp upstream from the ATG are identical. On the 5′ end, the TATA-box and the following 40 bp are identical, except for four nucleotides located between 26 and 30 bp downstream of the TATA-box (Fig. 4B). Indeed, the gtgt in gene-I is exchanged for atag in gene-II, embedded in invariant surrounding sequence (Fig. 4B). In addition, a gaccctgag element was detected once in gene-I at −25 to −17 relative to the ATG, while this sequence occurred twice in gene-II in the divergent region, positions −40/−32 and −28/−20 relative to the translation initiation codon, the latter flanked by a direct 3 bp tag repeat (−31/−29 and −19/−17); these triplets are absent from the corresponding unique copy in gene-I (Fig. 4B). The precise sequence changes clearly detected by comparative analyses suggest physiologically relevant modular cis-element evolution.

The transcription start sites (tss) were determined by primer extension analysis. Due to the highly conserved sequences between the two Pit1 cDNAs, a single reaction was performed using a labeled reverse primer hybridizing to both mRNAs. A major product of 104 nucleotide was observed suggesting that Pit1 transcripts of the corresponding 5′-UTR size predominated in carp pituitary RNA (Fig. 4A). In addition, a less intense band corresponding to a 85 nucleotide fragment was observed suggesting that shorter Pit1 mRNAs were also present. We propose that the major product corresponds to gene-I tran-

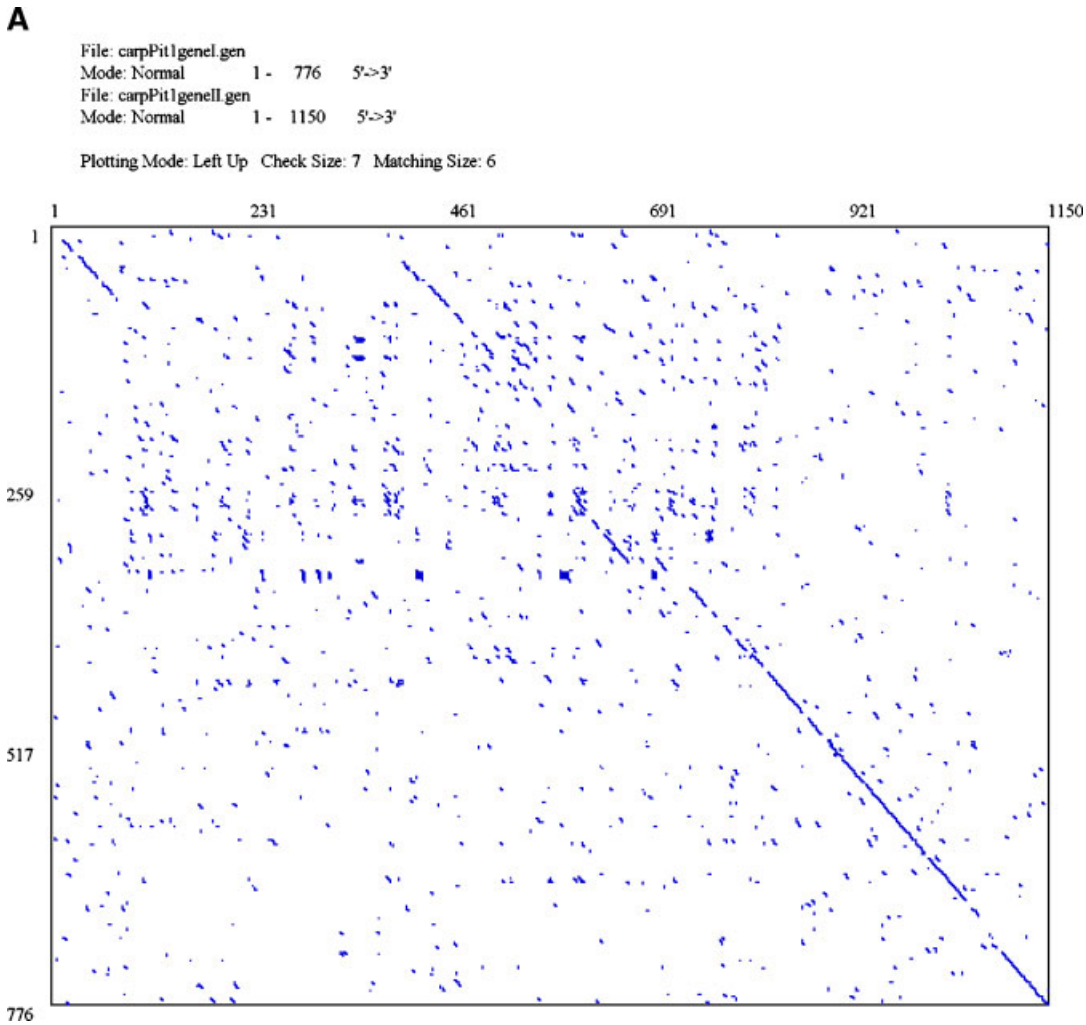

Fig. 3. Conserved regions in the promoters of the duplicated carp Pit1 genes. **A**: Dot matrix plot of the two promoter regions. The conserved domains are clearly illustrated by the diagonal lines. **B**: Alignment of Pit1 gene-I and gene-II promoter sequences. Stars between the lines indicate identical nucleotides, the putative transcription factor binding sites are in bold and underlined. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

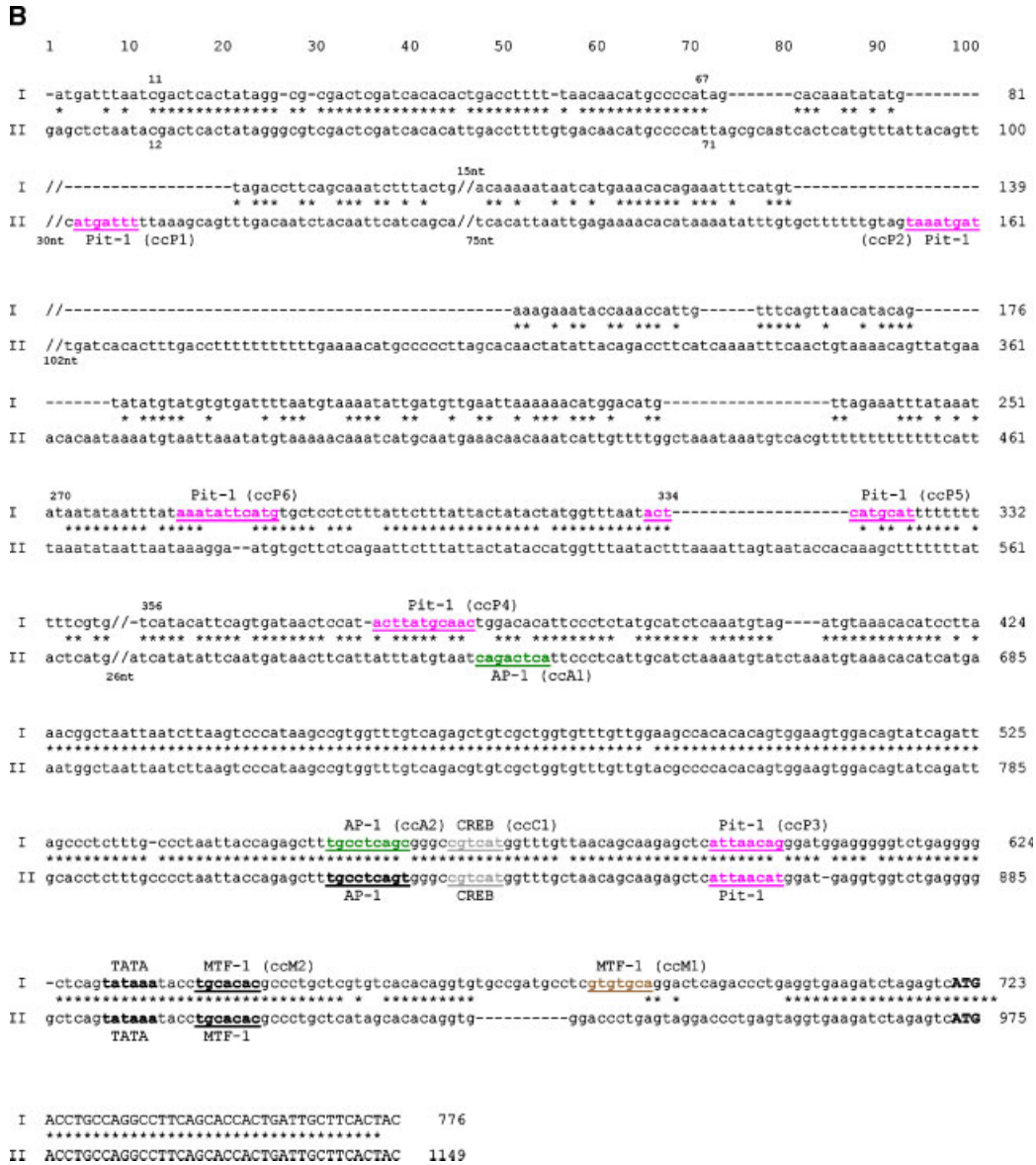

Fig. 3. (*Continued*)

scripts, based on three independent arguments. First, projection of the 104 nucleotide fragment on the Pit1 gene-I sequence places the transcription start site at a G 29 nucleotides downstream from the TATA-box, a distance expected for TATA-box containing eukaryotic promoters (Fig. 4B). Second, the previously reported gene-I 5′-cDNA [Kausel et al., 1999] starts 32 nucleotides downstream from the TATA-box, consistent with the tss located 3 bp upstream. This latter observation also excludes that the tss be located 20 bp downstream, as would be suggested by the 85 nucleotide signal in the primer extension. The Pit1 cDNA from grass carp (deposited under GenBank accession number AY694156) is more related to carp Pit1, gene-I, and starts at a similar position (Fig. 4C). On the other hand, projection of the 104 nucleotide fragment on the Pit1, gene-II would place the gene-II tss at a C 19 nucleotides downstream of the TATA-box, a distance unlikely for initiation of transcription (Fig. 4B). Third, the predicted tss of zebrafish Pit1 coincides in sequence and position with the gene-I arrangement (Fig. 4C). Furthermore, the gene-I inr-site ctcgtgt displays 71% (5/7) similarity to the mammalian consensus inr site py,py,A,N,T/A,T/C,T/C [Fukue et al., 2005] compared to the

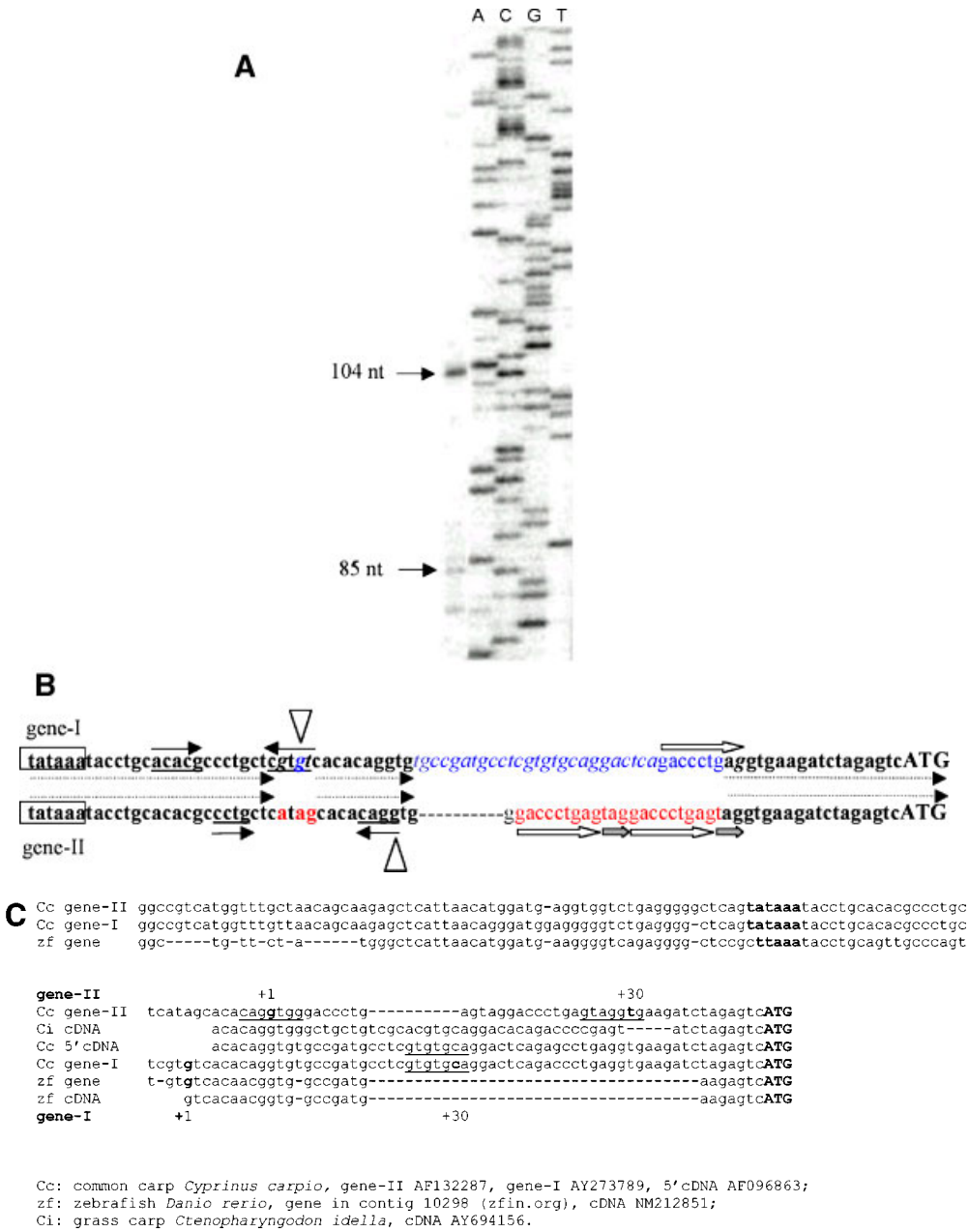

Fig. 4. Promoter architecture of duplicated carp Pit1 genes. **A**: Transcription site mapping by primer extension. Primer extension cDNA fragments were obtained by reverse transcription of carp hypophyseal mRNA with the exon I specific antisense oli5a labeled with ^{32}P, fractionated in a 4% denaturing polyacrylamid gel, and autoradiographed. As molecular size marker, a sequencing reaction (A, C, G, T) with the same oli5a of gene-I ($pGP4_{776}$) was run in parallel. **B**: Alignment of promoter sequences of carp Pit1 gene-I and gene-II. In italics: gene-I specific regions; dotted line arrow: identical stretches in both promoters; open arrow: sequence repeat; shaded arrow: tandem flanking triplet repeat; underlined and full line arrow: inverted repeat; open triangle: transcription start site. **C**: Alignment of Pit1 gene and cDNA sequences from common carp (*Cyprinus carpio*, Cc), grass carp (*Ctenopharyngodon idella*, Ci), and zebrafish (*Danio rerio*, zf). The TATA-box and the aligned ATGs are in bold, the Inr sequence and the downstream promoter elements (DPE) are underlined. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

57% (4/7) similarity of the aligned gene-II sequence ctcatag. Similarly, the sequence located 30 bp downstream of the gene-I tss (cctcgtg) displays 71% (5/7) similarity with the downstream promoter element DPE consensus sequence G/A,G,T/A,C,G,T,G [Fukue et al., 2005]. Consequently, the shorter primer extension product (85 nucleotides) is proposed as gene-II product based on the following observations: first, expression of both genes is clearly

demonstrated (Fig. 2); second, the deduced tss is located at a G 38 nucleotides downstream from the TATA-box, a reasonable distance for transcription initiation in gene-II; third, three of four nucleotides composing the +1 site in gene-I (gt**g**t) are altered in the corresponding site in gene-II (atag) that might relate to changes of functionality (Fig. 4). Only in combination with the proposed tss at cag**g**tgg in gene-II, a DPE occurs at the respective +30 position (gtaggtg, consensus 5/7).

Consensus Sites Bind-Specific Nuclear Factors From Pit1 Expressing Cells

To investigate the transcriptional regulation of the two duplicated carp Pit1 genes, we analyzed the isolated promoter sequences for the presence of putative transcription factor binding sites. Taking into account the highest scores for consensus sequences obtained with two independent programs (Tess: http://www. cbil.upenn.edu/tess/ and MatInspector: http://www.genomatix.de/cgi-bin/matinspector/matinspector.pl), binding sites were predicted for Pit1, AP1, CREB, and the metal transcription factor MTF1 (Fig. 3B and Table I). First, the binding potential of predicted Pit1 and MTF1 sites was assessed utilizing nuclear extracts from the Pit1-expressing cell line, GH3B6, derived from a rat pituitary tumor. To evaluate the complexes obtained in mobility shift assays with carp Pit1 sites, we used in addition a labeled oligonucleotide corresponding to the known Pit1 binding site P2 in the human PRL promoter [Caccavelli et al., 1998; Fig. 5A lane 8–11]. In the Pit1 sites tested, the formation of a complex of similar mobility increased in a dose-dependent manner with increasing amounts of nuclear extract (Fig. 5A,B) and disappeared in the presence of specific cold competitor, but not of unrelated oligonucleotides. Out of six potential Pit1 sites tested, specific complex formation was observed with ccP3 (Fig. 5B lanes 1–4), ccP5 (Fig. 5A lanes 1–4 and Fig. 5B lanes 15–18), and ccP6 (Fig. 5A lanes 12–15), but not with ccP1 and ccP2 (data not shown). Only a faint signal occurred with the ccP4 site (Fig. 5B lanes 8–11). Using ccP3 and, much stronger, ccP6 resulted in the formation of a second complex of lower mobility, probably corresponding to a dimeric Pit1 complex (Fig. 5A lanes 13–15). To further assess the specificity and the relative affinities of the different sites, the ccP6 probe was incubated in the presence of different cold competitor sequences. Indeed, the specific complexes formed at ccP6 disappeared upon addition of the canonical P2 (Fig. 5C lane 7) and ccP3 (Fig. 5C lane 6), whereas ccP4 did not compete (Fig. 5C lane 5). Non-related sequences such as the putative MTF1 (ccM2) or AP-1 (ccA2) sites did not compete (Fig. 5C lane 8–9). These results strongly suggest that the most proximal ccP3, present in both promoters, and the distal ccP5 and ccP6, unique to the gene-I promoter, are indeed specific Pit1 binding sites.

Immediately downstream of the TATA-box, a highly conserved MTF1 consensus sequence (Fig. 3B) was detected in both promoters (ccM2) and an additional one was present only in the gene-I sequence downstream of the tss in reverse orientation (ccM1). EMSA analyses of the ccM2 and ccM1 site incubated with GH3B6 cell nuclear extract lead to specific complex formation on each probe, yet the completely different pattern suggests that different factors or different complexes were bound (Fig. 6). Furthermore, cold ccM1 competed only slightly with ccM2 or vice versa (Fig. 6 lane 4, lane 11). Similarly, an excess of cold oli7 and ccAP2 reduced the specific signal apparent with ccM2 probe (Fig. 6 lanes 5, 7), but not ccP6 (Fig. 6 lane 6).

Selected Consensus Sites Bind-Specific Nuclear Factors From Carp Pituitary

We further examined several of the potential binding sites for their capacity to recognize nuclear factors extracted from carp pituitary. To test the quality of these nuclear extracts, we first incubated them with a labeled oligonucleotide corresponding to the Pit1 binding site P2 and analyzed the obtained complexes in an electrophoretic mobility shift assay. A large complex was observed (Fig. 7A lane 1), which disappeared in the presence of specific antibodies against human Pit1 or an excess of cold P2 probe, but not in the presence of ccA1 or ccC1 competitor. This complex presumably consisted of carp Pit1 present in the pituitary extract. When the putative Pit1 site ccP3, present in both carp Pit1 genes, was tested, a complex of similar size was obtained (Fig. 7B), which was competed by an excess of cold ccP3 or ccM1 probe but not by cold ccA1 or ccC1 probes. This specific complex was unaffected by the addition of antibodies against Pit1, suggesting that it consists of either a different isoform of Pit1 or a completely different factor. When the putative

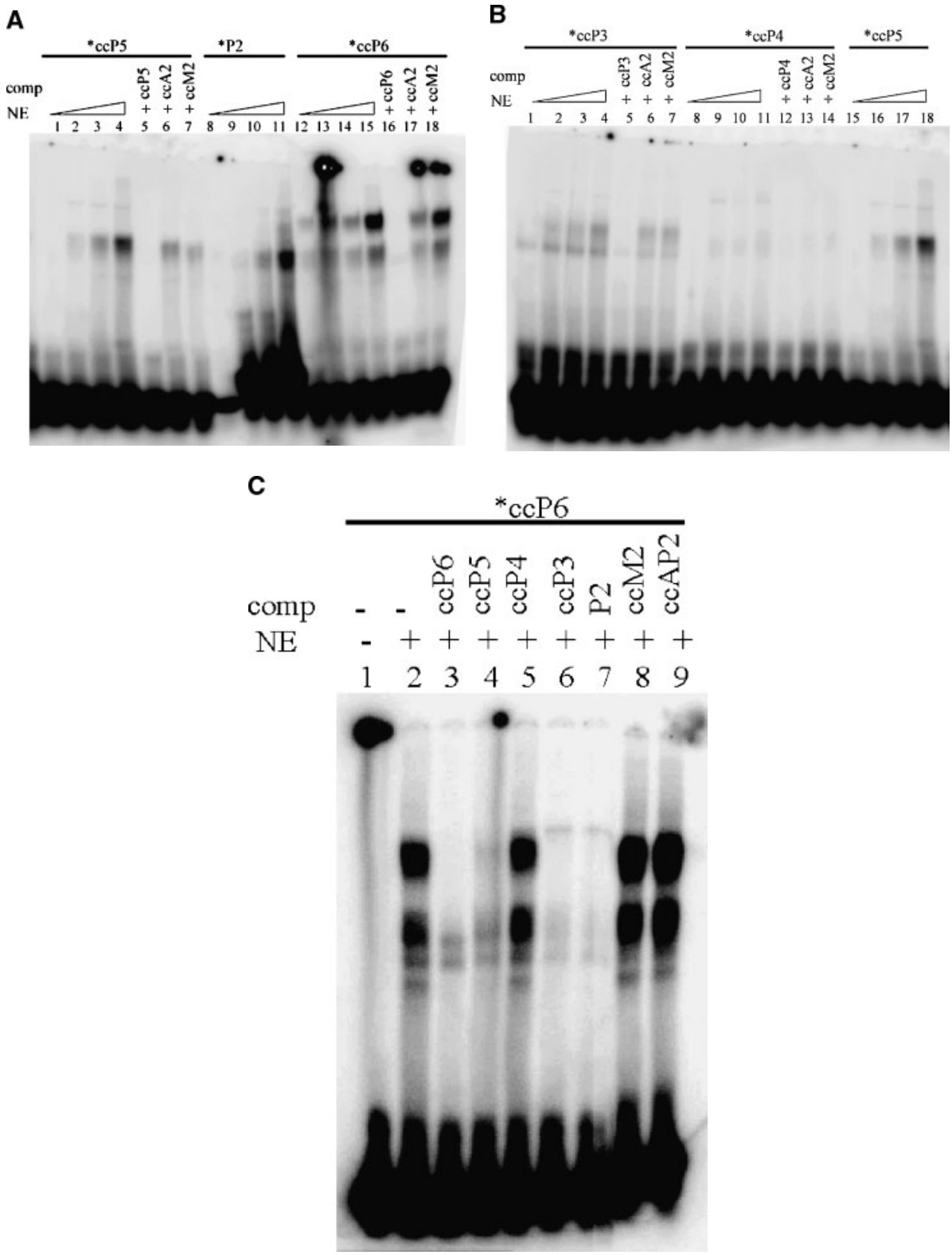

Fig. 5. Binding specificity of factors to predicted Pit1 binding sites. EMSA analyses with increasing amounts of nuclear extract from the Pit1 expressing rat cell line GH3B6 binding to (**A**) ccP5, P2, ccP6, in (**B**) binding to ccP3, ccP4, ccP5, in (**C**) to ccP6 and competition with the indicated predicted Pit1 binding sites. The strong slow migrating bands observed occasionally in EMSA experiments, for example lanes 13, 17, 18 in (A) correspond to material retained in the slots.

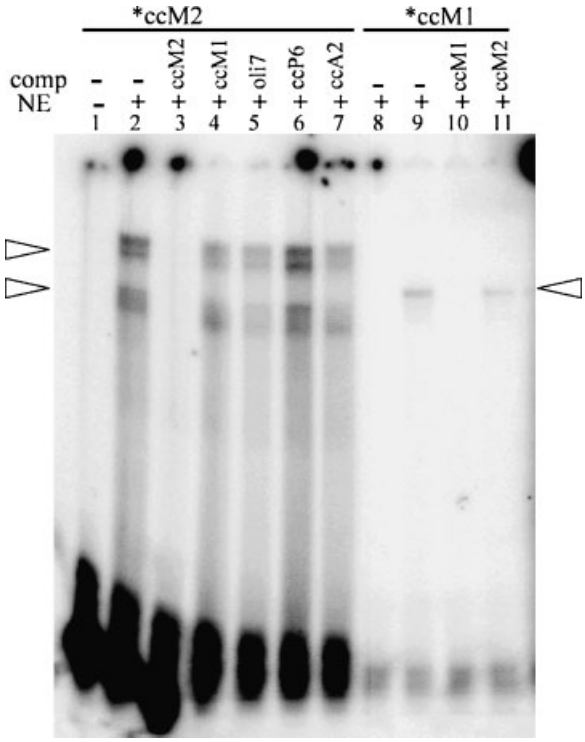

Fig. 6. Regulatory module unique in gene-I. EMSA analyses of GH3B6 nuclear extract with ccM2 and ccM1 sites.

CREB binding site ccC1 was used, a complex was observed that disappeared upon addition of an excess of cold ccC1 probe, but not in the presence of cold ccA1 or ccP1. A specific antibody against human CREB/ATF-1 also slightly competed for this complex (Fig. 7C).

In order to address a possible differential regulation of the duplicated carp Pit1 genes, we concentrated our attention on the divergent region around the transcription initiation site. When a probe corresponding to ccM1, present only in the gene-I promoter, was tested in the presence of pituitary nuclear extract, a strong specific complex was observed, which was competed by an excess of cold ccM1 probe, but not of cold ccC1, ccA1, ccP1, ccP2 (Fig. 8). A weak competition was observed with cold ccP3.

Our results indicate that putative binding sites common to both carp Pit1 genes, but most interestingly also specific to gene-I, are able to recognize specific transcription factors present in carp pituitary.

Differential Expression of Pit1 Genes in Pituitary of Winter-Acclimatized Carp

To directly compare the expression levels of the two Pit1 genes, in situ hybridization was performed using pituitaries from winter-acclimatized carp. Labeled oligonucleotides specific for gene-I or gene-II were used on serial sagittal sections. A significantly higher expression was observed for Pit1 gene-I, as compared to Pit1 gene-II (Fig. 9). Quantification of the specific signals was performed on serial sections from four different individuals and revealed an expression ratio for Pit1 gene-I/gene-II of 4.6 ($n = 4$). In winter condition a clear difference of gene copy specific signal was observed, revealing 4.6 times more transcripts of gene-I with respect to gene-II (standard deviation 1.1; $n = 4$) (Fig. 9).

DISCUSSION

The Pit1 gene codes for a transcription factor that is of paramount importance for the setup and maintenance of the hypothalamo-pituitary axis, which places Pit1 in the center of a regulatory network that is of determinant importance for the development and homeostasis in vertebrates [Dasen and Rosenfeld, 2001]. The transcriptional control of Pit1 expression has been studied in several mammalian species, however, little is known concerning this regulation in lower vertebrates such as teleosts [Dasen and Rosenfeld, 2001]. Comparison of regulatory pathways between closely related and distant species appears as a powerful means to gain more profound understanding of the underlying principles [Cossins and Crawford, 2005].

We recently described the isolation of a second Pit1 gene from carp [Pit1, gene-II, Kausel et al., 1999], in addition to the previously described Pit1, gene-I [Kausel et al., 1998]. The two genes code for divergent copies of the Pit1 transcription factor. In the context of the known tetraploidization of the carp genome, we first determined that no further Pit1 gene amplification had taken place in the duplicated carp genome such as frequently observed in plant genomes [Haberer et al., 2004]. We revealed gene copy specific transcripts and confirmed that the mRNAs derived from each gene copy are present in an individual fish. A similar situation was observed for the duplicated GH genes in carp, which are both expressed and the ratio of gene-I to gene-II transcripts is not affected by seasonal acclimatization [Figueroa et al., 2005].

Gene duplication plays a fundamental role in evolution providing the genetic material from which new functions can arise [Ohno, 1970]. In particular, gene copies may acquire differential expression patterns under determined spatial or temporal circumstances, as was reported in common carp [Arends et al., 1998; Futami et al., 2001; Polley et al., 2002]. In order to gain insight into the transcriptional regulation of the two carp Pit1 genes, we isolated a genomic clone corresponding to the gene-I promoter, the 5′-untranslated region, and part of the coding sequence. Sequence comparison of the two genes revealed an interesting region located at the 3′-end of the regulatory sequence, between the TATA-box and the translation initiation codon ATG. Indeed, the region surrounding the TATA-box is highly conserved until position +40. Only three nucleotides differ in this homologous block, which are grouped precisely around the position of the transcription start site for gene-I, as determined by primer extension (Fig. 4). The position of these divergent nucleotides, in the middle of an otherwise highly conserved domain, lends strong support to their functional relevance [Fairley et al., 2002]. Our observations suggest that these mutations are responsible for the observed shift downstream of the transcription initiation site observed in gene-II. Interestingly, these bases are also part of an inverted 5 bp repeat in gene-I that is disrupted in gene-II. The tss determined for Pit1, gene-II is located 38 bp downstream from its TATA-box, a distance that is unusual but not impossible. The existence of such an mRNA is supported by the fact that clearly the two genes are expressed and by the additional signal observed in the primer extension experiment. Possibly, the choice of this unusual position for transcription initiation results from the destruction of the more canonical tss more upstream, as previously discussed. Surprisingly,

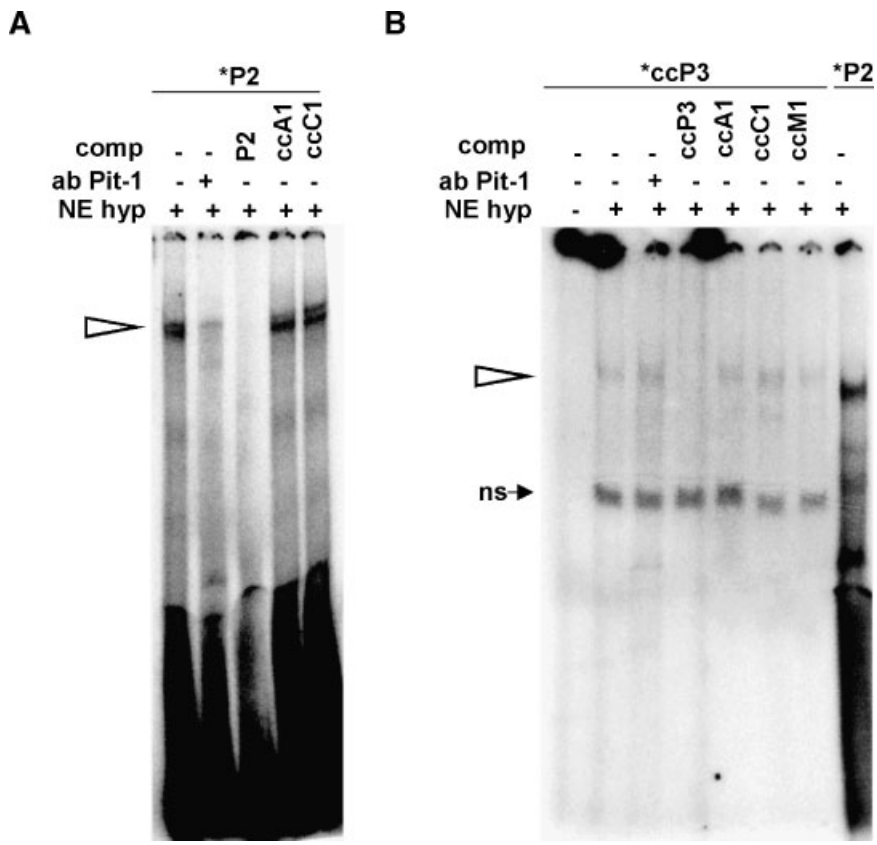

Fig. 7. Specificity of binding of hypophyseal factors to binding sites present in the carp Pit1 genes. EMSA analyses of nuclear extracts from carp pituitary (**A**) with the canonical Pit1 binding site P2, (**B**) with the proximal Pit1 site ccP3, (**C**) with the CRE element ccC1. The effect on complex formation upon addition of specific antibodies or an excess of unlabelled specific or unspecific competitor is shown. Open triangle indicates specific binding; ns = non-specific.

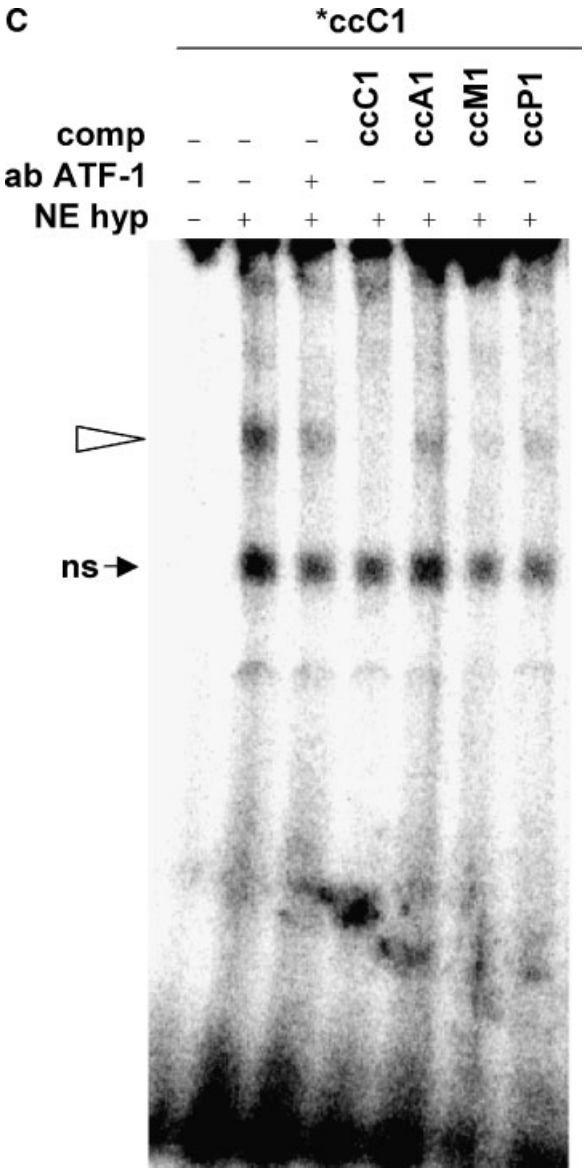

Fig. 7. (*Continued*)

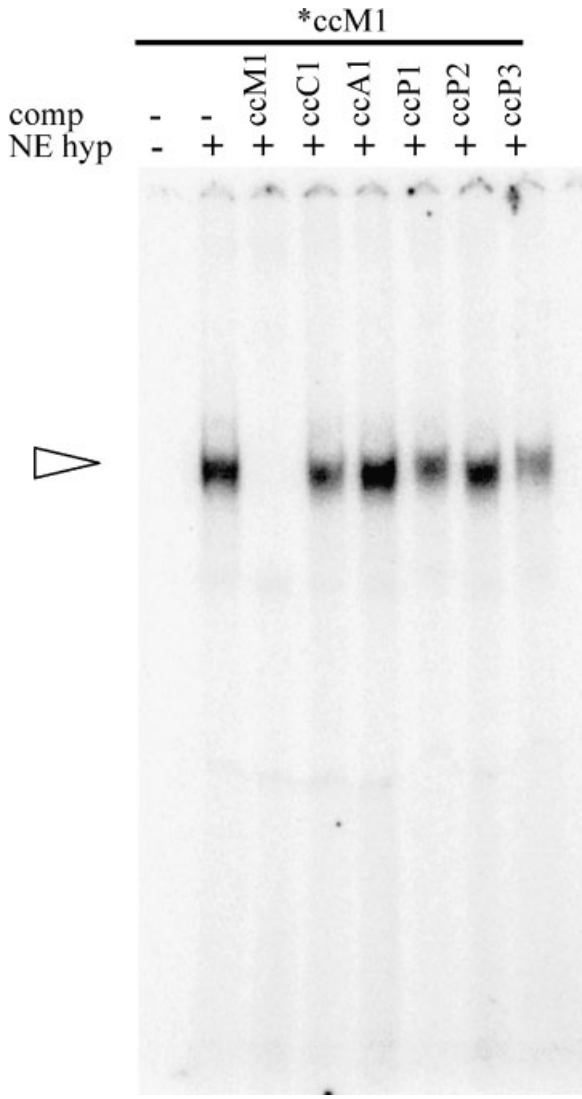

Fig. 8. Binding of hypophyseal factors to the ccM1 site unique to Pit1 gene-I. EMSA analyses of the ccM1 site with nuclear extract from carp pituitary. Open triangles indicate specific binding.

the tss determined for gene-II is again part of a 4 bp inverted repeat only present in gene-II. Whether the particular sequence of the tss or this particular feature is operative in defining the tss remains at present unknown.

When we compared the sequences of the two Pit1 promoters, large stretches of high conservation were apparent separated by regions presenting a high divergence. A 379 bp insertion was also identified in gene-II (positions 71–450). Two putative binding sites for Pit1 were revealed in this inserted region, however, preliminary DNA binding experiments failed to detect a clear binding activity in GH3B6 cell and pituitary extracts (not shown). In contrast, we found two Pit1 sites specific to gene-I (ccP5, ccP6) and one (ccP3) site present in both genes that were able to bind Pit1 from GH3B6 extracts. These observations are consistent with the autoregulatory loop described for Pit1 transcriptional control [Rhodes et al., 1993]. Surprisingly, the complex formed on ccP3 using pituitary extracts was not recognized by antibodies against human Pit1, in contrast to the one formed on the control fragment P2. It is unclear at present whether this reflects the binding of a different Pit1 isoform or of a different factor. In contrast, a clear binding

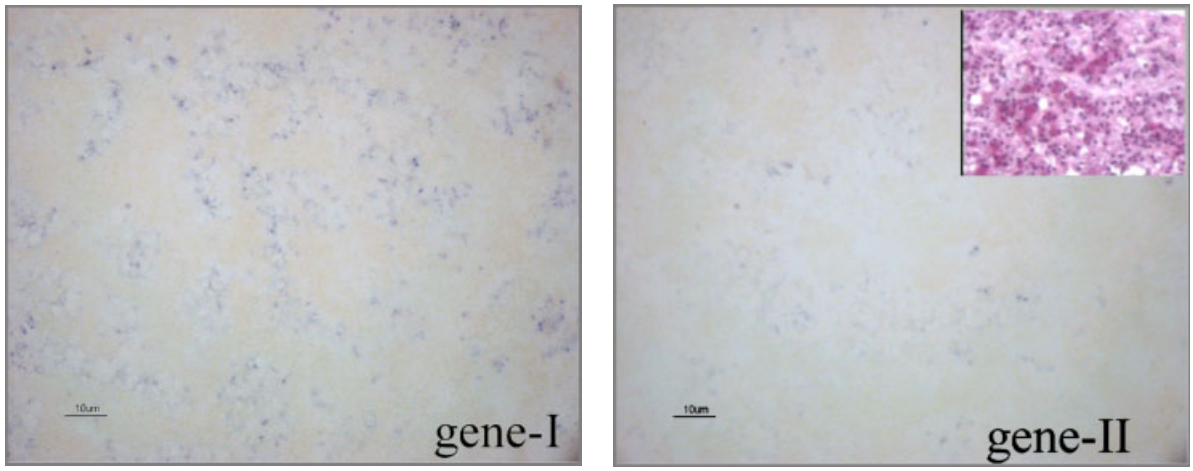

Fig. 9. In situ hybridization comparing the expression levels of Pit1 gene-I (**left**) and Pit1 gene-II (**right**). Quantification revealed a 4.6-fold higher expression for Pit1 gene-I. Inset shows hematoxilin-eosin stained section of carp pituitary. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

activity was detected to three other identified putative sites. The conserved CRE sequence appears to bind a factor identified as CREB/ATF-1 by using a specific antibody in the EMSA assays (Fig. 7C).

The region downstream of the TATA-box is of particular interest. The highly conserved region immediately downstream presents a putative binding site for MTF1 (ccM2), while the highly divergent region further downstream harbors an MTF1 site specifically in gene-I (ccM1). The complexes formed on these two sites are clearly different (Fig. 6). Interestingly, the ccM1 site present only in the Pit1, gene-I at position 697–703 was also clearly able to bind a similar, specific factor from carp pituitary extracts (Fig. 8). In addition, this region harbors a direct repeat sequence present only in gene-II, gene-I containing only a single copy of this repeat at this location. All these observations point at this region surrounding the tss as a possible candidate for differential regulation of the two expressed Pit1 genes in carp.

The conservation of extended conserved regions present in the 5′ region of Pit1, gene-I and gene-II could be attributable on the one hand to the rather recent event of genome duplication and therefore short divergence time [David et al., 2003]. Alternatively, the conservation of non-coding sequences could reflect the functional conservation of the Pit1 gene regulation. In vertebrate genomes, genes that work as master regulators, for example, the transcription factors encoded in the hox clusters, were shown to be flanked by arrays of ultraconserved non-coding regions [Sandelin et al., 2004]. The cyprinid lineage experienced genome duplication relatively recently [12 MYA, David et al., 2003] in its evolutionary history leading, among others, to the common carp (*Cyprinus carpio*). The precise sequence of the two gene copies at the time of duplication is unknown, however known mechanisms of genome duplication argue against a high divergence [Conlon and Larhammar, 2005]. Therefore, in the context of the tetraploid carp fish, the presence of highly divergent regions in the regulatory sequences of the two copies of the crucial transcription factor Pit1 embedded into otherwise well-conserved regions, is of particular interest. Among the most closely related species, the grass carp and zebrafish Pit1 cDNAs are more closely related to carp Pit1, gene-I, while more distant species such as fugu, *Salmo salar*, *Oncorhynchus mykiss*, or *Oncorhynchus keta* differed extensively from the sequences (not shown). No evidence for a second Pit1 gene, the ortholog of carp Pit1, gene-II was described to date in any of these species. From our data, the Pit1, gene-I promoter has gained two Pit1 sites and an additional MTF1 site relative to gene-II. In addition, extensive reorganization has occurred in the region surrounding the tss. As far as we know, the ccM1 site in gene-I is a completely new feature never before connected to regulation of Pit1 gene expression. In grass carp Pit1 cDNA, the ccM1 sequence unique to gene-I is not conserved, while in zebrafish cDNA, the whole block of 36 nucleotides specific for gene-I is absent (Fig. 4C). The modular changes of

cis-regulatory elements between the two carp Pit1 genes described here suggest an additional molecular switch in gene-I that might imply an adaptive response to changes in environmental conditions, such as winter adaptation. Therefore, gene-I might constitute an example for the specialization of a recently duplicated gene [Prince and Pickett, 2002]. The functional importance of these additional binding sites in the Pit1 gene-I promoter is supported by our observation that this gene is expressed at 4.6-fold higher levels in winter-acclimatized carp, while the overall Pit1 expression is dramatically reduced as compared to summer (Kausel et al., 1999; data not shown). Whether the additional Pit1 sites or the ccM1 site is responsible for this higher expression of gene-I remains to be elucidated.

The carp fish provides a model system to study the dynamics of duplicated genes after genome duplication [Cossins and Crawford, 2005]. Well-illustrated examples exist for gene loss, pseudogenization and sub- or neo-functionalization [Postlethwait et al., 2004]. Sequence divergence of the two gene copies might affect the protein coding region, leading to a protein presenting slightly different biological properties, or the regulatory regions of the gene, leading to a modification of its spatial or temporal expression. An important example in carp was described with the POMC gene, another gene specifically expressed in the pituitary [Arends et al., 1998]. Here, we concentrated on the characterization of the regulatory regions of the duplicated Pit1 genes in carp. We show that the two genes are expressed, and we characterize the conserved and divergent regions in the transcriptional control regions of the two genes. In addition, we show a 4.6-fold higher expression of Pit1 gene-I with respect to gene-II in winter-acclimatized carp. Further work will be required to determine whether, and under which circumstances, the two Pit1 genes are differentially regulated and to correlate this divergent regulation to the particular features that we described here.

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