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Transcription Factor Pit-1 Expression Is Modulated Upon Seasonal Acclimatization of Eurythermal Ectotherms: Identification of Two Pit-1 Genes in the Carp

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Abstract A second Pit-1 gene in carp (*Cyprinus carpio*), including the complete structural gene and 1.1 kb of promoter region, was identified and completely sequenced. The exon-intron structure was determined, and reverse transcription-polymerase chain reaction (RT-PCR) experiments suggest that only one Pit-1 splice variant is present in carp pituitary. The effect of seasonal acclimatization on the extent of Pit-1 gene expression was studied in summer- and winter-acclimatized carp. Quantitative RT-PCR analysis revealed a clear increase of Pit-1 mRNA in the pituitaries from summer-acclimatized carp compared with the winter-adapted fish. In situ hybridization of pituitary gland sections with riboprobes representing the complete 5′-transactivating region of carp Pit-1 depicted a significantly higher Pit-1 mRNA level in the rostral pars distalis of the summer-acclimatized fish where prolactin is expressed in a manner that resembles the seasonal increase observed in the proximal pars distalis and the pars intermedia. The cell- and temporal-specific transcription of Pit-1 supports its role in the molecular mechanisms that underly the acclimatization process undergone by eurythermal fish as a result of the physical effects of seasonal changes on their habitat. J. Cell. Biochem. 75:598–609, 1999.

Key words: Pit-1; genomic organization; Pit-1 gene expression; carp; fish; seasonal control; acclimatization

Eurythermal fish undergo a cyclical rearrangement of molecular and cellular functions to compensate for the circannual environmental seasonal changes of their habitat. This acclimatization process of eurythermal fish leads to a cyclical reprogramming of molecular processes, which in the carp (*Cyprinus carpio*) has been shown to involve modulation of transcriptional and translational events in various tissues [Saez et al., 1982; Vera et al., 1993, 1997; Figueroa et al., 1994]. Likewise, although acclimation is known to be insufficient in mimicking the complexity of acclimatization [Segner and Braunbeck, 1990], it has been observed that long-term adaptation to temperature changes also involves reprogramming of gene expression in different tissues, as has been reported for carp muscle [Goldspink, 1995] and pituitary glands [Figueroa et al., 1997; Arends et al., 1998].

In fish, the adaptive compensatory response to stressors or environmental habitat changes seems to involve the pituitary as a central node where the modulation of the expression of prolactin (PRL), the most versatile pituitary hormone, is clearly involved [Yada et al., 1992; Figueroa et al., 1997; Poncelet et al., 1997]. Accordingly, high expression of PRL mRNA occurs in the rostral pars distalis (RPD) of summer-acclimatized carp, which contrasts with the negligible level of transcription detected in winter-acclimatized fish [Figueroa et al., 1994]. Underlying the seasonal adaptive response, the photoperiod appears to be a particularly relevant modulator in the neuroendocrine cascade that activates PRL transcription in the carp [Figueroa et al., 1997].

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Cell- and temporal-specific expression of PRL (lactotrophs), growth hormone (somatotrophs in the proximal pars distalis, PPD), and the fish-specific somatolactin (SL) gene in the pars intermedia (PI) of the pituitary gland of teleosts appear to be under the control of Pit-1, a POU domain transcription factor restricted to the anterior pituitary gland [Andersen and Rosenfeld,. 1994; Poncelet et al., 1997; Ono et al.,1994]. The Pit-1 protein is composed of two functional domains, the N-terminal serine/threonine-rich transactivating STA domain and the C-terminal domain where the highly conserved DNA binding POU domain is located. This region binds to complex patterns of sites involving the promoters and enhancers of target genes, including its own gene [Jacobson et al., 1997; Chen et al., 1990]. The activity of Pit-1 may be modulated by other interacting factors [Andersen and Rosenfeld, 1994; Simmons et al.,1990; Palomino et al.,1998]. In mammals, several Pit-1 isoforms, generated by differential splicing, were shown to display distinct transactivation properties depending on the precise target promoter context [Majumdar et al., 1996].

To assess whether the remarkable rise in PRL mRNA and protein, which occurs in summer-acclimatized carp, was associated with a concomitant increase of Pit-1 expression in lactotrophs, we performed in situ hybridization assays using a probe derived from a carp Pit-1 genomic clone containing the 3′ region of the third exon [Kausel et al., 1998]. Although Pit-1 mRNA levels were significantly higher in the PPD and PI from summer-acclimatized carp compared with winter-adapted fish, singularly, in both seasons only a basal Pit-1 mRNA content was observed in the RPD. Because of the nature of the probe, i.e., a short oligonucleotide could be missing a variant of a Pit-1 mRNA, we constructed a Pit-1 5′ cDNA clone to derive longer and putatively more specific riboprobes to assess the behavior of Pit-1 transcription in the RPD of carp undergoing seasonal acclimatization. In addition, we isolated a new genomic clone and characterized the first entire Pit-1 gene of a teleost.

In this article, we show that Pit-1 mRNA levels, in the RPD where PRL is expressed, as in the PPD and the PI, also depict differences in summer- and winter-acclimatized carp, and that in this fish this transcription factor is encoded for at least two genes with only one splice variant.

MATERIALS AND METHODS

Animals and Tissue Preparation

Male carp (*Cyprinus carpio*) weighing about 1,000–1,500g were caught during winter and summer and maintained in a fixed 3 × 4-m cage submerged 2 m in an affluent of the same river. The water temperatures in winter and in summer were 8–10°C and 18–20°C, respectively.

Pituitary glands from winter- and summer-acclimatized carp were dissected and either fixed immediately for in situ hybridization or frozen in liquid nitrogen and stored at −80°C for RNA extraction, as reported elsewhere [Vera et al., 1997].

Isolation of Pit-1 Clones

A probe containing a carp Pit-1 exon III sequence was obtained by PCR amplification using as primers the oligonucleotides 5′-CTTTGACCCACAGCTTAC-3′ and 5′-TAGAAACTAATCAGCTAAGC-3′ derived from the primary structure of $pGP5_{1700}$, a clone comprising about two-thirds of the estimated carp Pit-1 coding sequence [Kausel et al., 1998]. During the PCR reaction (30 cycles at 93°C for 30 s, 55°C for 30 s, and 72°C for 10 s), high specific labeling with ^{32}P of the 264-bp fragment was achieved as described by Mertz and Rashtchian [1994]. A λFixII carp genomic library (Stratagene) was screened according to standard procedures [Sambrook et al., 1989]. The positive clones were characterized by restriction enzyme mapping and Southern hybridization, using the insert of the recombinants $pGP5_{1700}$ [Kausel et al., 1998] and pCP_{560a} (accession number AF096863) as probes. The positive clone (λGP7) was subcloned in pBluescript SK^+ vector (Stratagene) and sequenced as indicated bellow.

A Pit-1 5′ cDNA was obtained using as template carp pituitary gland RNA and the 5′-AmpliFINDER™ RACE-Kit (CLONTECH). A total of 5 µg of total RNA was reverse transcribed with Superscript II RT (Moloney Murine Leukemia Virus RT, Gibco-BRL) and an antisense oligonucleotide derived from the POUs region in exon IV (5′-CTCTGCTTCGTCCAGCCACTT-3′) [Kausel et al., 1998]. Pit-1 5′ cDNA was amplified using nested antisense primers derived from exon III (Oli3: 5′-GCGGCGGTAAGCTGTGGGTC-3′; Oli4: 5′-GCACAG-

GAGCCCCCACCCAG-3′) [Kausel et al., 1998]. Amplification conditions were as follows: after a denaturation step of 95°C for 3 min, 2.5 U of *Taq* DNA Polymerase were added and 30 cycles were performed (94°C for 30 s, 55°C for 30 s, 72°C for 1 min), followed by a final extension at 72°C for 10 min. The unique amplification product was cloned in the TA-cloning vector pCR2.1 (Invitrogen), yielding the recombinant pCP_{560a}. Sequencing was performed using the T7 Sequencing Kit (Pharmacia) or an automated DNA sequencer (Perkin-Elmer).

RT-PCR Analysis of Pit-1 Expression

A primer set, cMIP1 (5′-CTTTGACCCACAGCTTAC-3′) and cMIP2 (5′-GGATGGATTTGAGCTTGC-3′), for the reverse transcription-polymerase chain reaction (RT-PCR) analyses was derived from exon III and exon IV contained in $pGP5_{1700}$ [Kausel et al., 1998]. When cDNA was used as a template, the amplification, resulted in a 315-bp fragment while genomic DNA yielded one of 820 bp. To obtain a competitive Pit-1 DNA for the quantitative RT-PCR reactions [Siebert and Larrick,1993], a 412-bp *Hin*dIII-*Eco*RI-fragment of a carp ribosomal RNA clone [Vera et al., 1997] was ligated to the oligonucleotides cMIP1 and cMIP2 and the phosphorylated complementary oligonucleotides cCOMIP1 (5′-AGCTGTAAGCTGTGGGTCAAAG-3′) and cCOMIP2 (5′-AATTGCAAGCTCAAATCCATCC-3′) harboring *Hin*dIII- or *Eco*RI- compatible ends, respectively. A PCR reaction was performed on the resulting ligated product using cMIP1 and cMIP2 as primers. Different dilutions of the competitive Pit-1 DNA solution were prepared in 50 µg/ml glycogen.

Total RNA (5 µg) was isolated from winter- and summer-acclimatized carp pituitaries and digested with 2 U of DNase I free of RNase. Aliquots containing 0.5 µg of this RNA were reverse transcribed with Superscript II and oligo dT_{15} (Gibco-BRL). The competitive PCR reactions were performed with the cMIP1 and cMIP2 primers using one-twentieth of the cDNA as templates and a serial dilution of the competitive Pit-1 DNA. The products were fractionated on agarose gels and quantified with an analytical Imager System (UN-SCAN-IT).

In Situ Hybridization

Carp pituitaries were removed, and in situ hybridization was performed on sections of different specimens (summer- and winter-acclimatized carp) as previously described [Figueroa et al. 1994], except that the washing step was carried out at 42°C. To obtain the Pit-1 riboprobe, the 560-bp fragment of 5′cDNA was excised from pCP_{560a} with *Eco*RI and cloned in pBluescript SK^+ (Stratagene) yielding pCP_{560b}. The Digoxigenin-UTP RNA Probe Labeling System was employed to generate labeled RNA according to the manufacturer's instructions (Boehringer-Mannheim). Antisense cRNA probes were synthesized with T7 RNA polymerase using pCP_{560b} linearized with *Hin*dIII as the template and sense cRNA probes with T3 RNA polymerase and the same plasmid linearized with *Bam*HI. Quantification of the label in the tissue sections was attained using an automated image digitizing system as described earlier [Kausel et al., 1998]. Differences were assessed using the Student's *t*-test. $P < 0.03$ was considered significant.

RESULTS

Characterization of a Complete Carp Pit-1 Genomic Sequence

We previously described a partial carp Pit-1 gene sequence [Kausel et al., 1998] covering 4 exons coding for the entire POU domain but lacking the region coding for the N-terminal end of the protein. To obtain 5′ end coding and genomic sequences and to identify the upstream promoter region, we isolated new genomic recombinants and constructed another clone containing 5′ cDNA, based on the information contained in $pGP5_{1700}$ [Kausel et al., 1998].

When we screened a carp genomic library using a probe derived from $pGP5_{1700}$, two clones were obtained. Upon Southern blot analysis and restriction mapping, one was shown to correspond to λGP5 [Kausel et al., 1998]. The other clone, λGP7, was clearly different, representing an additional carp Pit-1 gene. Its insert was therefore subcloned and completely sequenced.

As shown in Figure 1, the λGP7 insert contains the entire carp Pit-1 gene entailing about 1.1 kb of the 5′ flanking sequence containing promoters, the entire structural region of about 7.8 kb and 4.3 kb of 3′ sequences. In addition to the consensus TATA-box, consensus sites for Pit-1/GHF-1 binding sequences (positions 134, 297, and 994) [Ohkubo et al., 1996], binding to target genes such as PRL [Poncelet et al., 1997] and GH [Ohkubo et al., 1996], CREB (position 966) and AP-1 binding site (position 769) were

identified upstream of the coding region. We also found three putative polyadenylation signals downstream of the stop codon (positions 9343, 9475, and 9930).

The exon-intron structure of the gene was first analyzed by searching the open reading frames in the sequence and comparing the translated peptides with known Pit-1 amino acid sequences from other vertebrates. Seven exons coding for the entire amino acid sequence (exon I: amino acid 1–47; exon II: 48–97; exon γ: 98–130; exon III: 131–213; exon IV: 214–267; exon V: 268–288; exon VI: 289–357) are separated by six introns of varying sizes (intron 1: 3711 bp; intron 2: 147 bp; intron 3: 1249 bp; intron 4: 1401 bp; intron 5: 132 bp; intron 6: 96 bp). Exons III–VI could be clearly localized in the genomic sequence using this approach (Fig. 1), whereas exons I and II could not be unequivocally assigned. The intron 4 sequence displayed an 82% homology with the internal inverted repeats found in the transposon Tol2 of the medaka fish *Oryzae latipes* [Koga et al., 1996].

In an attempt to identify coding sequences comprising exons I–II of carp Pit-1, and thereby complete our knowledge about the sequence that codes for this transcription factor in *C. carpio*, we used a 5′-RACE strategy and, by reverse transcription, isolated the Pit-1 cDNA sequence from total carp pituitary RNA with exon III specific oligonucleotides derived from $pGP5_{1700}$ [Kausel et al., 1998]. Only one fragment was obtained, cloned (pCP_{560a}) and sequenced (Fig. 2).

Alignment of the amino acid sequences deduced from the genomic clones λGP7 and λGP5 shows some minor differences in the highly conserved POU domain. Similarly, comparison of the N-terminal region deduced from λGP7 and from pCP_{560a} (5′ cDNA) shows some minor differences in the amino acid sequences (Fig. 3). As compared with mammals, the derived carp Pit-1 amino acid sequence comprises a 26-amino acid β-insert and a 33 amino-acid γ-insert, which are both also present in other teleost and avian species (Fig. 3). The β-insert represents a 5′ extension of exon II, similar to the turkey and mammalian β-variants resulting from alternative splicing [Kurima et al., 1998; Theill et al., 1992; Majumdar et al., 1996], whereas the γ-insert is coded by a separate, additional exon γ [Majumdar et al., 1996]. A methionine is present at position 26 that could be used as an alternative translation start site as was shown in mammals [Voss et al., 1991].

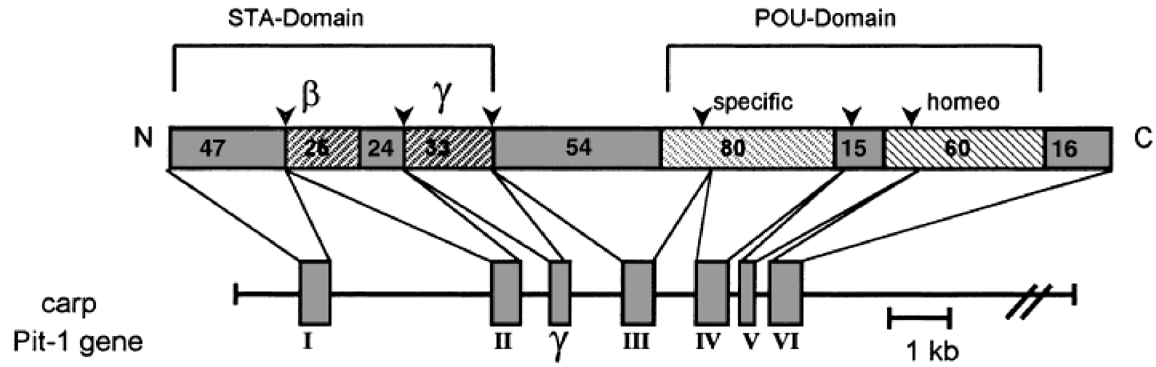
Pit-1 Expression During Acclimatization

Modulation of Pit-1 expression during seasonal adaptation was first adressed using a quantitative RT-PCR approach. Specific primers spanning exons III–IV were designed to amplify a fragment of the cDNA. As shown in Figure 4, a clear increase of Pit-1 mRNA content is observed in summer-acclimatized carp, as compared with winter-adapted fish.

To map the cellular distribution of Pit-1 mRNA expression, in situ hybridization was performed. Using antisense riboprobes corresponding to the 5′-region of carp Pit-1 cDNA, specific transcripts were detected in the PPD, PI and RPD of carp pituitary sections (Fig. 5). No signal was obtained after incubation of the samples with digoxigenin-labeled Pit-1 sense probe (Fig. 5). As expected, the extension and intensity of the in situ hybridization signals observed in the PPD and PI from summer fish were significantly higher as compared with winter-acclimatized carp. This becames evident with the semiquantitative analyses obtained upon digitalization of the hybridization signals (Fig. 5). In the lactotroph cells of the RPD, the hybridization signal was also low in winter, but a clear increase could be observed using the cDNA-derived riboprobe probe.

DISCUSSION

We have cloned and characterized λGP7, a second additional gene coding for the transcription factor Pit-1 in carp. This clone represents the first complete gene for Pit-1 from a teleost described to date. In addition, we constructed and analyzed a cDNA clone corresponding to the 5′ region of the carp Pit-1 mRNA. The gene structure deduced from these data is similar to the recently described Pit-1 gene from turkeys [Kurima et al., 1998]. As in mammals, the exons coding for the DNA binding module, the POU domain, (exons III, IV, V, and VI) are close together (introns of about 100 bp), whereas the exons constituting the transactivating domain (exons I, II, and γ in carp; exons I and II in mice) are farther apart. The intron between exon III and IV is longer in the genomic clone λGP7 compared with the genomic clone λGP5 [Kausel et al., 1998], possibly originating from transposon sequences identified in that intron (Fig. 1). By comparing mammalian, avian, and ichthyic Pit-1 genes Majumdar et al. [1996]

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           10         20         30         40         50         60         70         80         90        100
gagctctaatacgactcactatagggcgtcgactcgatcacacattgaccttttgtgacaacatgccccattagcgcastcactcatgtttattacagtt
tttaaatatatttttaaacgtattgtttgaacatgattttaaagcagtttgacaatctacaattcatcagcagtttgaaatgcttagttaatctaaa
attaatgcttcagctccttctatattaatctgcatacactttttggacctcacattaattgagaaaacacataaaatatttgtgctttttgtagtaaa
tcatatttttgtagtttcttttagaattagtgaaggtgaaacttaaaataataataattccaccattcagtgcagagaaaatataattaaaatatta
attgatcacactttgaccttttttttttgaaaacatgccccttagcacaactatattacagaccttcatcaaaatttcaactgtaaaacagttatgaa   500
acacaataaaatgtaattaaatatgtaaaaacaaatcatgcaatgaaacaacaaatcattgttttggctaaataaatgtcacgtttttttttttcatt
taaatataattaataaaggaatgtgcttctcagaattctttattactataccatggtttaatactttaaaattagtaataccacaaagctttttttatac
tcatgcacttataataaatcctaaattttccatcatatattcaatgataacttcattatttatgtaatcagactcattccctcattgcatctaaaatgta
                                                                         AP-1
tctaaatgtaaacacatcatgaaatggctaattaatcttaagtcccataagccgtggtttgtcagacgtgtcgctggtgtttgttgtacgccccacacag
tggaagtggacagtatcagattgcacctctttgcccctaattaccagagctttgcctcagtgggccgtcatggtttgctaacagcaagagctcattaaca   1000
                                                                   CREB                           Pit-1
tggatgaggtggtctgaggggctcagtataaatacctgcacacgccctgctcatagcacacaggtgggaccctgagtaggaccctgagtaggtgaagat
ctagagtcATGACCTGCCAGGCCTTCAGCACCACTGATTGCTTCACTACTCTGAGCGGAGACTCGGCTGCTCTGCCGCTGCTCATGCATCACACCGGAGC
        M  T  C  Q  A  F  S  T  T  D  C  F  T  T  L  S  G  D  S  A  A  L  P  L  L  M  H  H  T  G  A   ExI
CGCAGACTGTCTGCCCGTCTCCAGCCACACCACCAACATGATGCCTTCAGgtaaatctcacgctcttggacgatcacacagtggttctctactgagagag   1300
 A  D  C  L  P  V  S  S  H  T  T  N  M  M  P  S
[3718 nt Intron 1]tgtctaggatttatcacagatataagtggctggttagTCCCCTCAGGTCTGCCACTGGTTCAGTCATCCAAACGCTCACATAT   5000
                                                     V  P  S  G  L  P  L  V  Q  S  S  K  R  S  H  M
                                                     ------------------------------ β-insert -------
GCACCTGTCCACTTCTGCTTTGGGCAATGCGTCAGCCAGCCTGCACTATCCAATGCCCCCGTGTCACTATAGCAACCAGCAGACCACCTACGGCATGATG
 H  L  S  T  S  A  L  G  N  A  S  A  S  L  H  Y  P  M  P  P  C  H  Y  S  N  Q  Q  T  T  Y  G  M  M   ExII
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GCAGgtatgtcaagttacgcacaagctacgcaagtgattcaagtattcttcttctgtgcatatgcattaactgtcataaacacacacagaaaaacaggat
A  g  m  s  s  y  a  q  a  t  q  v  i  q  v  f  f  f  c  a  y  a  l  t  v  i  n  t  h  r  k  t  g  f   In2

ttaggatatgtgtgtgtgtgtgtggtaatgatgtaagatctgtgtctccagCACAGGAAATGCTCTCTGCCAGCATCTCCCAGACCCGCATCCTGCAGAC
 r  i  c  v  c  v  c  g  n  d  v  r  s  v  s  p  A  Q  E  M  L  S  A  S  I  S  Q  T  R  I  L  Q  T
                                                 ''''''''''''''''''''''' γ-Insert ''''''''''''''''    Exγ
CTGCAGCATGCCTCATGCCAACATGGTCAACGGAGCCAACTCACTGCAAGgtaggcctacaacctgtgctcgaatccaaaccttcagccccatgacattc   5400
 C  S  M  P  H  A  N  M  V  N  G  A  N  S  L  Q
''''''''''''''''''''''''''''''''''''''''''''''''''

[1118 nt intron 3]aaaataaatgatggttagatattttaagatttgatctccttgaacagttattattcgctctaacccctgtgtcctgtccgcag   6600
                                                                                                  G
GAGCTCTGGCTTCACGCCTCTATAAGTTCCCAGAGCACAGTTTGGGTGGGGGCTCCTGTGCTTTGACCCACAGTTTACCGCCGCTGCCCCAGGCCCTGCT
  A  L  A  S  R  L  Y  K  F  P  E  H  S  L  G  G  G  S  C  A  L  T  H  S  L  P  P  L  P  Q  A  L  L
CATGGACGAGCCCACCCTAGGAGACATCAAGCAGGAGCTGCGCAGGAAAAGCCGGCCCCTGGAAGAGCCGCCCGACATGGACTCACCTCAGATCCGTGAA
 M  D  E  P  T  L  G  D  I  K  Q  E  L  R  R  K  S  R  P  L  E  E  P  P  D  M  D  S  P  Q  I  R  E   ExIII
                                                                ========================================

CTGGAGAAGTTTGCTAACGATTTCAAACTGAGGAGAATCAAACTAGgtttgtcttctgagctggaccatatttttataaaattatgtaaaacactgccac   6900
L  E  K  F  A  N  D  F  K  L  R  R  I  K  L  G
==============================================

[617 nt intron 4]gataacttttgaatgagctttaaatttttatttgaagggattggtttcaccgaaaaaaaagaaggaaaattctgtcattaata   7600
                                                         -.-.-.-.-.-.-.-.-.-.-.-.-.-.-.-.-.-.-.-.-.-.-.-.-.-.-.-.-.-.
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Figure 1.

have suggested that exon γ, which is present only in fish and birds, may have been lost in mammals during evolution.

Comparison of the deduced amino acid sequences shows the close relation of carp Pit-1 to other fish Pit-1 transcription factors (Fig. 3). The difference in size of the carp Pit-1 to mammalian Pit-1 is mainly due to insertions of 26-amino acid (β-insert) and 33-amino acids (γ-insert) in the N-terminal half as reported in other fish and avian Pit-1-derived amino acid sequences [Vila et al., 1995].

The presence of several splice variants of Pit-1 in the N-terminal transactivation domain

Fig. 1. (Continued.) Nucleotide and deduced amino acid sequences of a carp transcription factor Pit-1 gene (accession no. AF 132287) The schematical representation depicts the STA and POU functional domains in the organization of the complete Pit-1 gene. ▽, exon-intron boundaries. In the primary structure, predicted coding sequences are given in uppercase letters and intronic sequences in lower case letters. The complete intron sequences are available under the accession no. AF 132287; the number of the omitted nucleotides is indicated in brackets. TATA-Box, ATG start codon, TGA stop codon, gt/ag-splice sites and polyadenylation signals are in boldface. Predicted Pit-1, AP-1, and CREB-binding sites toward the 5′ of ATG are underlined, and Pit-1 sites are boldface and underlined; ---, the β-insert; ′′′ the γ-insert; = = =, the POU-specific and POU-homeo region. The sequence indicated with -.-.-.-.-. in intron 4 reveals 82% identical nucleotides with the inverted terminal repeats of the fish transposon Tol2 (accession no. D84375). The deduced amino acid sequence for carp Pit-1 is represented in uppercase. As intron 2 continues in an open reading frame, the putative amino acids are depicted in lowercase.

has been described in mammals and turkeys [Vila, et al., 1995; Kurima et al., 1998]. In mammals, differential effects on gene expression have been suggested for these Pit-1 isoforms [Theill et al., 1992; Diamond et al., 1996; Kurima et al., 1998]. One of these variants, Pit-2, results from alternative splicing between exons I and II of the Pit-1 gene and contains a 26-amino acid insertion similar to the β-insert observed in fish Pit-1 proteins. Pit-2 retains the binding activity of Pit-1 and can activate the GH promoter but has lost the ability to activate the PRL and Pit-1 promoter [Theill et al., 1992]. In turkeys, three Pit-1 variants are described, all harboring a 38-amino acid insertion in the N-terminal region corresponding to the fish γ-insert and additional sequences arising from varying splicing events in the intron between exons I and II [Kurima et al., 1998]. The presence of the β- and γ-insert in fish Pit-1 proteins may be required to carry out novel tasks compared with mammals, such as the differentiation of

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ggcttcctgggtcggctcacctctgaaggttccagaatcgatagttaactcgtgacacag    60

gtgtgccgatgcctcgtgtgcaggactcagagcctgaggtgaagatctagagtcATGACC   120
                                                        M  T      2

TGCCAGGCCTTCAGCACCACTGATTGCTTCACTACTCTTAGCGGAGACTCGGCTGCTCTG   180
C  Q  A  F  S  T  T  D  C  F  T  T  L  S  G  D  S  A  A  L      22

CCGCTGCTCATGCATCACACCGGAGCTGCAGACTGTCTGCCCGTCACCAGCCACGCCACC   240
P  L  L  M  H  H  T  G  A  A  D  C  L  P  V  T  S  H  A  T      42

AACATGGTGCCTCCAGTCCCCTCAGATCTCCCTCTGGTCCAGTCGTCCAAACGCTCTCAT   300
N  M  V  P  P  V  P  S  D  L  P  L  V  Q  S  S  K  R  S  H      62
               ------------------------------------------------------

ATGCACCTGTCCGCTTCTGCTTTGGGCAATGCGTCAGCCAGCCTGCACTACCCATTGCCC   360
M  H  L  S  A  S  A  L  G  N  A  S  A  S  L  H  Y  P  L  P      82
-----------------------------------------------

CCATGTCACTATAGCAACCAGCAGACCACTTACGGCATGATGGCAGCACAGGAAATGCTC   420
P  C  H  Y  S  N  Q  Q  T  T  Y  G  M  M  A  A  Q  E  M  L     102
                                              ''''''''''''''''

TCTGCCAGCATCTCTCAGACCCGCATCCTGCAGACCTGCAGCATGCCTCATGCCAACATG   480
S  A  S  I  S  Q  T  R  I  L  Q  T  C  S  M  P  H  A  N  M     122
''''''''''''''''''''''''''''''''''''''''''''''''''''''''''''''

GTCAACAGTGCCAACTCACTGCAAGGAGCTCTGGCTCCACGCCTCTATAAGTTCCCAGAG   540
V  N  S  A  N  S  L  Q  G  A  L  A  P  R  L  Y  K  F  P  E     142
''''''''''''''''''''''''

CACAGTCTGGGTGGGGGCTCCTGTGCAAGCC                                571
H  S  L  G  G  G  S  C                                         150
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Fig. 2. Nucleotide and deduced amino acid sequence of a carp Pit-1 5′ cDNA (accession no. AF096863). ---, the β-insert; ′′′ the γ-insert.

somatolactin (SL)-producing cells and/or cell-specific expression of the SL gene. As shown by Majumdar et al. [1996], at least one salmon Pit-1 insert is needed to control salmon PRL promoter, whereas none is required for the activation of the growth hormone promoter and both for somatolactin promoter stimulation.

In carp, RT-PCR reactions covering the mRNA region encoding the β- and γ-insert resulted in the production of only one band, suggesting that no alternatively spliced form is present. Moreover, in the λGP7 gene, the continuing frame present in intron 2 (Fig. 1) could lead to an even larger protein if the intron is not spliced. Analyses of the putative amino acid composition and the codon usage make this possibility very unlikely. Thus, our data suggest that, as in salmon, only one single Pit-1 splice variant is present in carp.

Restriction mapping and sequence comparison clearly show that the two genomic clones λGP5 and λGP7 represent two different carp Pit-1 genes. Comparison of the nucleotide sequence in the small overlapping part between λGP5 and the 5′ cDNA reveals a 1-bp difference.

The presence of duplicate genes is a common feature in teleosts, particularly in tetraploids

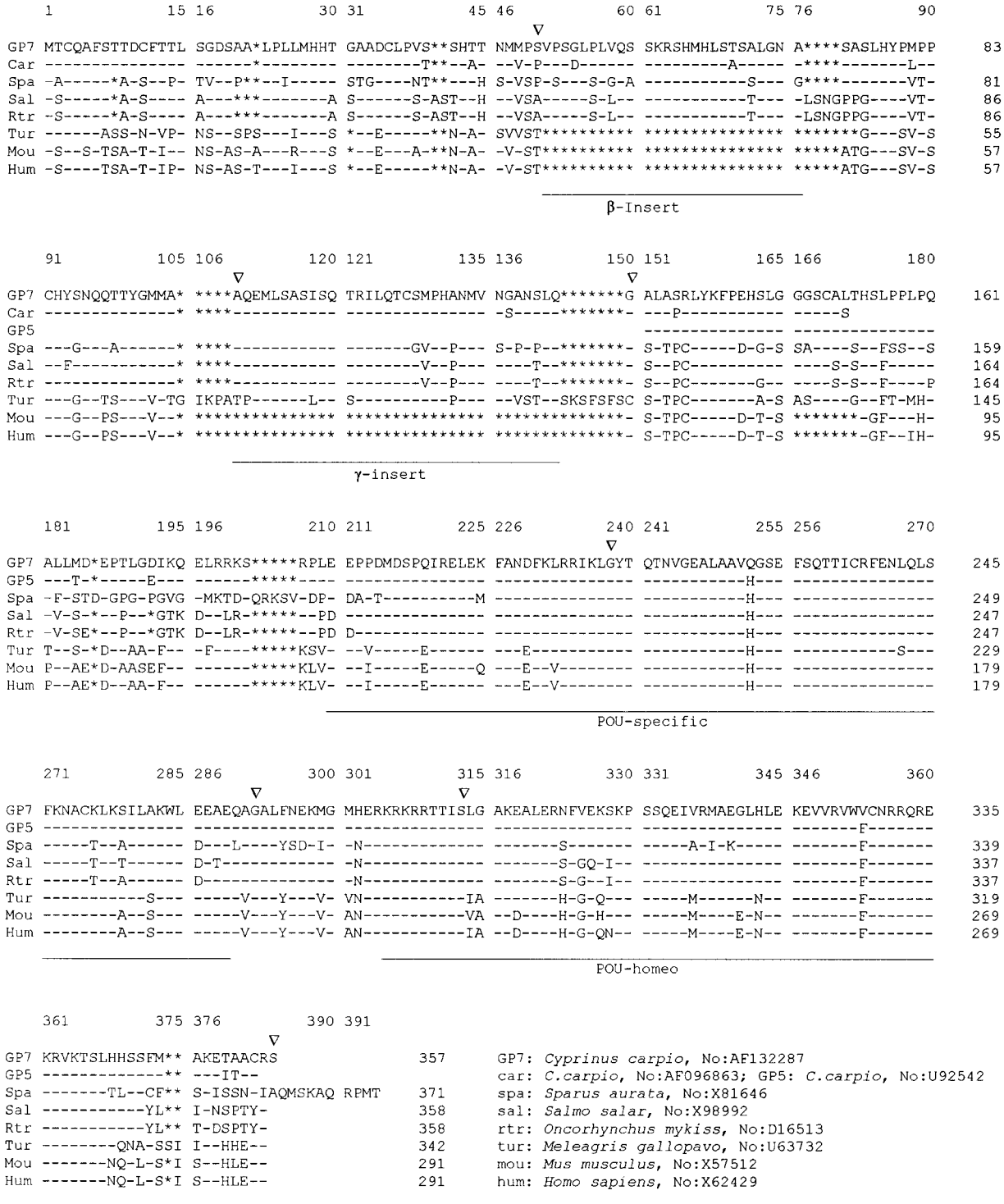

Fig. 3. Alignment of Pit-1-deduced amino acid sequences from ichthyic, avian, and mammalian species. The amino acids derived from *Cyprinus carpio* genomic clone GP7 serve as a reference; identical amino acids are represented by hyphens, whereas stars apply to gaps. The β- and γ-insert, POU-specific and POU-homeo region are underlined. ▽, exon/intron boundaries.

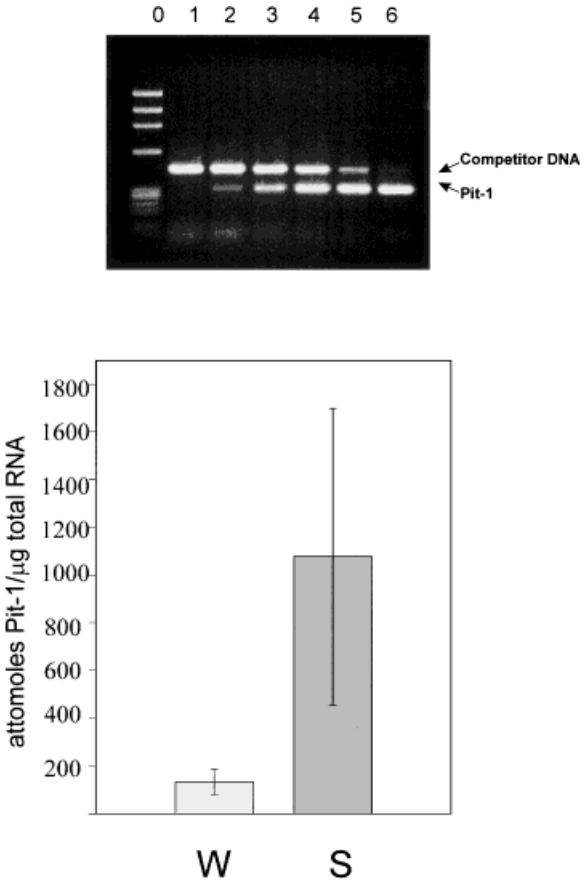

Fig. 4. Quantification of Pit-1 transcripts with reverse transcription-polymerase chain reaction (RT-PCR) from carp pituitaries. Amplification products of competitive PCR obtained with the Pit-1-specific primers cMIP1 and cMIP2 with a constant amount of cDNA (315-bp fragment) complemented with dilutions of competitor DNA (450-bp fragment). Fractionation on a 1.4% agarose gel: (**0**) ϕX174/*Hae*III-DNA, (**1**)–(**6**): 10^{-14}, 10^{-15}, 10^{-16}, 10^{-17}, 10^{-18}, 10^{-19} moles competitor DNA. The ethidium bromide-stained amplification products were quantified using an automated image digitizing system. The histogram depicts Pit-1 transcripts in individually analyzed summer (S) and winter (W)-acclimatized carp pituitary glands (n = 5 for each season; Student's *t*-test $P < 0.01$).

like the carp [Ferris and Whitt, 1977]. In some of these duplicate loci only one copy is effectively transcribed. In others, both gene copies are expressed [Lorens et al., 1996]. In Atlantic salmon, two Pit-1 cDNAs were described, differing in 4 codons in the coding region and the presence of two Pit-1 genes was thus suggested [Lorens et al., 1996]. In carp, we clearly identified at least two different Pit-1 genes, possibly giving rise to mRNAs differing in the nucleotide sequence, but not in their splicing pattern.

Analyses of the nucleotide sequences revealed the presence of several transcription factor binding sites in the promoter region of the λGP7 Pit-1 gene. Consensus sequences for binding of Pit-1 and for specific factors like AP-1 or CREB could be involved in the cell-specific activation of the Pit-1 gene during development and/or the modulation of Pit-1 expression by external stimuli [Xu et al., 1998]. Interestingly, a Pit-1 binding site located downstream of the transcription start site, shown to mediate negative regulation by Pit-1 in mammalian Pit-1 genes, was not detected in the carp gene [Chen et al., 1990]. Further studies will be required to define the function of these different putative elements in transcriptional regulation.

Pit-1 is the main regulatory factor of genes expressed in the anterior pituitary, i.e., PRL [Curlewis, 1992; Poncelet et al., 1997], GH [Farchi-Pisanty et al., 1995; Tuggle and Trenkle, 1996], and exclusively in fish, SL [Ono et al., 1994]. In teleosts, the expression of PRL and GH is modulated upon seasonal adaptation of the fish [Figueroa et al., 1994, 1995]. As observed by Rand-Weaver et al. [1995], pronounced seasonal changes occur in the rainbow trout plasma somatolactin level, which reaches a maximum during summer.

Concerning the expression of PRL, we have assessed the remarkable increase in PRL mRNA levels in summer-acclimatized carp as compared with the pituitary from winter-acclimatized fish [Figueroa et al., 1994]. During the seasonal acclimatization of the carp, photoperiod appears to act as a relevant modulator of PRL expression [Figueroa et al., 1997]. We therefore investigated whether Pit-1 mRNA levels are modulated during the seasonal acclimatization of the carp, and particularly whether the changes follow the same pattern depicted by PRL mRNA. In an earlier study, we undertook the study of this question, performing in situ hybridization using an oligonucleotide probe specific for exon III of the carp Pit-1 gene [Kausel et al., 1998]. Semiquantitative analyses of the hybridization signals showed that Pit-1 mRNA was clearly higher in the PI (SL expression) and in the PPD (GH expression) of the pituitary glands from the warm-season-aclimatized carp compared with the winter-acclimatized fish [Kausel et al., 1998]. Strikingly, in both summer and winter, only a basal Pit-1 mRNA level was detected in the rostral pars distalis vis-à-vis the signals detected in the pars intermedia and proximal pars distalis [Kausel et al., 1998]. To ensure that this was

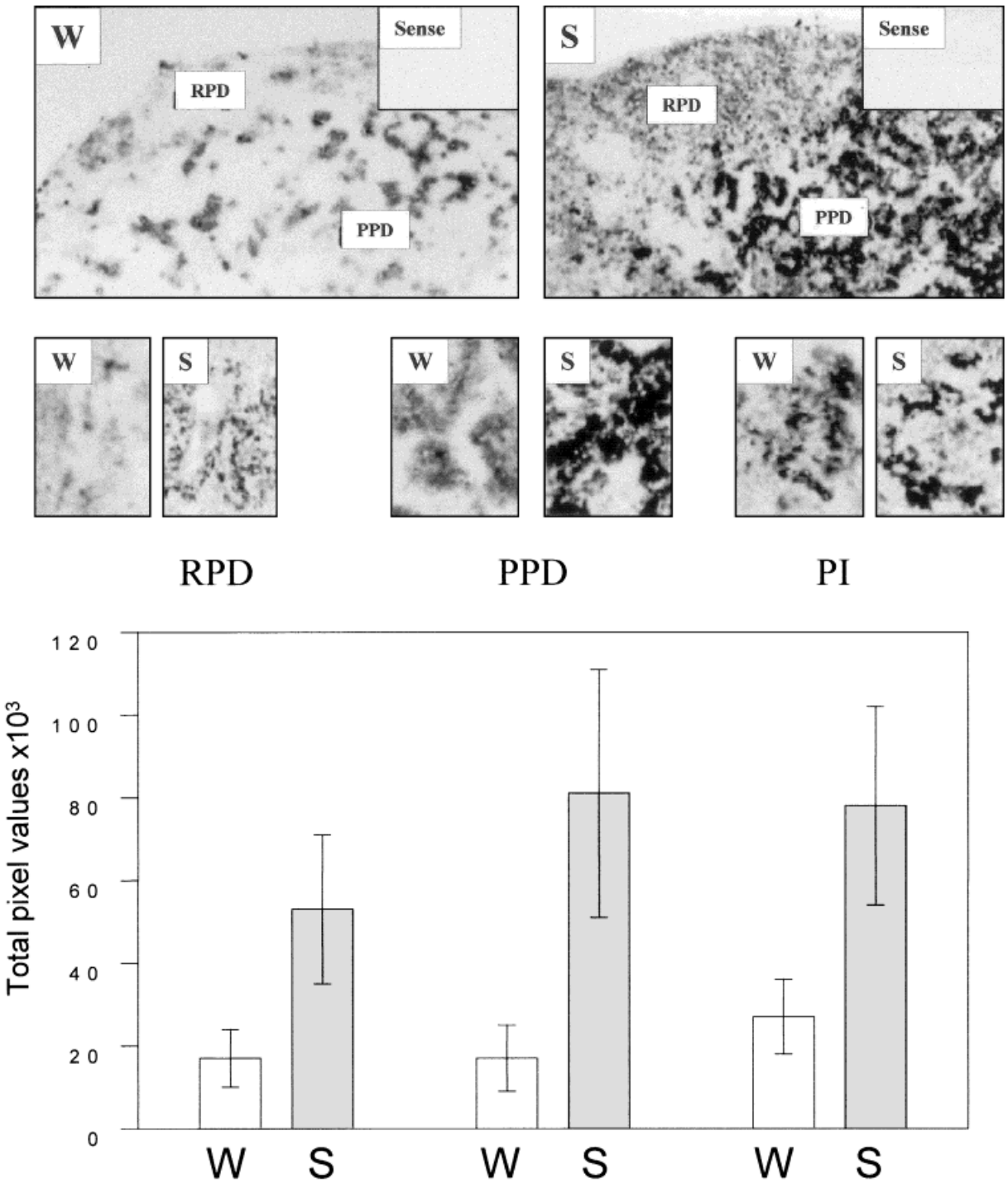

Fig. 5. In situ hybridization of sagittal sections of carp pituitary glands with riboprobes comprising the entire aminoterminal transactivating region of the transcription factor Pit-1. Molecular hybridization was attained with a digoxigenin-labeled 560-bp antisense riboprobe in winter-acclimatized (W) and summer-acclimatized carp (S), as depicted in the upper left and right micrographs (×100), respectively. The inserts show the control hybridization with the corresponding sense riboprobe that yielded the same result in each of the three regions studied (PI not shown). The hybridization pattern attained specifically in the RPD, PPD, and PI in pituitary sections from winter- and summer-acclimatized fish is depicted below (×225). Semiquantitative analyses of the in situ hybridization are represented graphically. RPD, PPD, and PI were examined separately. The histogram depicts the mean total pixel values (±SD of these analyses in three different individuals corresponding to each season (n = 3). Student's *t*-test $P < 0.03$.

not related to the nature and structure of the probe, i.e., an 18-mer oligunocleotide, we reexamined the spatial distribution of Pit-1 expression upon acclimatization of the carp, using riboprobes comprising the entire serine/threonine-rich transactivating domain (M^1-Q^{130}), derived from pCP_{560b}, the carp Pit-1–5′ cDNA clone herein reported. Although the signal in the RPD was still weaker, consistent with the pattern depicted by PRL expression, a clear difference was observed in summer-acclimatized carp compared with the winter-acclimatized fish. Furthermore, the increase in Pit-1 mRNA level in the RPD during the warm season, was similar to the one observed in the PPD and PI. In addition to Pit-1, PRL expression involves other regulators. To attain full PRL induction, cAMP or similar signal transduction pathways might be acting in combination [Xu et al., 1998]. Thus, the 3-fold increase in Pit-1 expression in the RPD may be sufficient to account for the large increase of PRL gene transcription in the pituitary of summer-adapted fish [Figueroa et al., 1994].

The semiquantitative analyses of the Pit-1 mRNA signals obtained with the riboprobe, the spatial distribution, and the seasonal differential transcription observed in cells involved with the expression of three hormones associated with the seasonal acclimatization process of the carp, suggest that indeed Pit-1 plays a role in the complex mechanism that allows ectothermal fish to adjust to environmental changes. Consistently, the quantitative RT-PCR experiments revealed an 8-fold increase of Pit-1 mRNA in the total pituitary in summer-adapted fish compared with winter-acclimatized carp.

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