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EFFECT OF SEASONAL ACCLIMATIZATION ON THE EXPRESSION OF THE CARP TRANSCRIPTION FACTOR PIT-1

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SUMMARY

We isolated a clone comprising four exons of the carp Pit-1 gene. Using synthetic oligonucleotide probes derived from the carp Pit-1 sequence Pit-1 expression was assessed by *in situ* hybridization in pituitary sections from summer- and winter-acclimatized carp. Semi-quantitative analyses of the hybridization signals revealed a significant higher Pit-1 expression in the proximal pars distalis (PPD) and pars intermedia (PI) of the pituitary glands from summer-acclimatized carp, compared to the winter-acclimatized fish. In both adaptive states, relative to the PPD and PI, only a basal Pit-1 expression was detected in the rostral pars distalis. Thus, during seasonal acclimatization of an eurythermal fish, Pit-1 seems to be involved in the mechanisms that underlie the compensatory response. Key words: Pit-1, carp, acclimatization, fish, gene expression.

INTRODUCTION

Seasonal changes in the environmental factors demand compensatory responses in eurythermal fish both at the cellular and molecular level. In the carp (*Cyprinus carpio*) the mechanisms that underlie this natural cyclical adjustment (*i.e.* acclimatization process) involve the modulation of transcription in distinctive gene expression processes which take place in several tissues [1-6].

To assess whether the pituitary gland could be a central coordinating node, supplying molecules according to the homeostatic requirements imposed by the changing environmental signals, we previously studied the expression of prolactin (PRL) during the acclimatization of the carp and observed a high expression of PRL mRNA in the rostral pars distalis (RPD) of summer-acclimatized carp while a negligible level of transcription was detected in the pituitaries of the

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winter-acclimatized fish [7]. Furthermore we established that photoperiod constitutes a particularly relevant modulator in the neuroendocrine cascade that activates PRL transcription in the carp [8].

It is well known that the tissue-specific transcription factor Pit-1 is implied in the control of PRL expression [9,10]. The transcription factor Pit-1 plays an important role in the precise temporal and spatial patterns of gene expression in the anterior pituitary gland. Pit-1 controls the development and maintenance of the differentiated state of lactotrophs, somatotrophs and thyrotrophs, which are the cell lines producing PRL, growth hormone (GH) and the β subunit of the thyroid stimulating hormone (TSH) respectively [11]. In addition, somatolactin (SL), a PRL-related hormone identified in the pars intermedia (PI) in fish [12,13], also seems to be controlled by Pit-1 [14]. Apart from the autoregulation of Pit-1 expression [15] several transactivating factors are involved in the transcription of the Pit-1 gene [16].

To determine if Pit-1 plays a role in regulating prolactin gene expression in carp during acclimatization, we have isolated and characterized part of the carp Pit-1 gene and assessed its expression in pituitary cells in summer- and winter-acclimatized carp. In this article we report a genomic sequence which codes for carp Pit-1, the first corresponding to a teleost. Also we show that a strong seasonal differential transcription of Pit-1 occurs in the GH and SL producing cells of carp pituitary glands and that only a negligible amount of Pit-1 transcripts could be detected in both acclimatized states in the PRL producing cells.

MATERIALS AND METHODS

Male carp (*Cyprinus carpio*) weighing about 1000 - 1500 g were caught during winter and summer and maintained in a fixed 3 x 4 m cage submerged 2 m in an affluent of the same river. The temperatures of the water in winter and in summer were 8-10°C and 18-20°C, respectively. Pituitary glands and other tissues, from winter- and summer-acclimatized carp, were dissected and either frozen in liquid nitrogen and stored at -70°C for RNA extraction or fixed immediately for *in situ* hybridization studies, as reported elsewhere [8]. The fixed sections were kept at -70°C and processed together.

Carp genomic DNA was prepared according to Garbutt et al. [17]. To obtain a carp Pit-1 probe, oligonucleotide primers were derived from the highly conserved POUs region of salmon Pit-1 cDNA sequence [18]. PCR amplification was performed using carp genomic DNA (80 ng) as template in a 50 µl mixture containing 25 pM of each primer (oligo1: 5'-CTCTGCTTCGTCCAGCCACTT-3'; oligo2: 5'-ACAACCATCTGTCGCTTTGA-3'), and 2.5 U *Taq* DNA polymerase (GibcoBRL), according to the manufacturer's instructions. Following a denaturation step of 3 min at 95°C, 30 cycles of amplification were performed (30 sec at 93°C, 30 sec at 55°C, 10 sec at 72°C), rendering a 93 bp fragment. High specific labeling of this fragment

was obtained during PCR amplification when $\alpha[^{32}P]$-dCTP (3000 Ci/mmol, 10 μCi/ml, Amersham) was used as described by Mertz and Rashtchian [19]. To isolate Pit-1 genomic sequences a λFixII carp genomic library (Stratagene) was screened with the 93 bp fragment according to Sambrook et al. [20]. Subcloning as well as sequencing of DNA by the dideoxy chain-termination method were performed as described [20].

Total RNA was prepared with Trizol (GibcoBRL) according to the supplier instructions. Northern blotting was performed using total RNA (10-12 μg) from different carp tissues, which were then fractionated on a 1.2% denaturing agarose gel containing 2.2 M formaldehyde, and transferred to a nylon membrane (Amersham Hybond). Hybridization was carried out overnight at 42°C using as probe the 1700 bp fragment of GP5, labeled with $[^{32}P]$ by the random primer method (GibcoBRL). The blot was washed twice for 15 min under stringent conditions (0.2 x SSC, 0.1% SDS at 60°C) and subjected to autoradiography for 48 h at -70°C using a Kodak X-Omat film.

In situ hybridization was performed in the fixed summer- and winter- pituitary gland sections simultaneously [8]. Carp Pit-1 specific oligonucleotide hybridization probes were derived from the furthermost 5' coding region of $pGP5_{1700}$. The antisense 5'-GTAAGCTGTGGGTCAAAG-3' and the complementary sense oligonucleotide were labeled at the 3'-end with digoxigenin using terminal deoxynucleotidyl transferase (GibcoBRL) [7].

Quantification of the label in the pituitary sections was carried out as previously described [8] using an automated image digitizing system (UN-SCAN-IT, Silk Scientific, Inc.). In the PI and in the proximal pars distalis (PPD) of each pituitary section a square of approximately 0.2 mm^2 was defined. Five regions were randomly selected in the less reactive areas of the pituitary sections to obtain the background number of pixels. In the PI and the PPD, 5 areas were marked, their density measured and corrected for the average background value. These analyses were performed on sections of different individuals, *i.e.* winter- and summer-acclimatized carp fish, using the Student *t*-test. $P<0.01$ was considered significant.

RESULTS

By screening about 1 million recombinants from a λFixII carp genomic library two clones (GP1 and GP5) containing Pit-1 coding sequences were isolated based on their hybridization to a POUs-specific carp 93 bp DNA fragment obtained by PCR. Restriction mapping and Southern blot analyses revealed that both clones contain the carp POUs sequence. GP5 was selected for further analysis. A *Xba*I-*Pst* I fragment comprising approximately 1700 bp of GP5 was subcloned in pUC19 and sequenced.

Comparison to the sequence data bank of the primary structure of the subclone, $pGP5_{1700}$, confirmed that it corresponds to sequences coding specifically for Pit-1. Moreover, comparison to the gene structure of mouse Pit-1 reveals that the clone GP5 encompasses exons III to VI, thus starting in intron II (Fig.1). As the *Xba*I-*Pst*I fragment of pGP5 corresponds to the 5'-end of the lambda recombinant, GP5 misses the 5'region of the carp Pit-1 gene, however, comprises the coding sequence for the entire POU-domain and part of the amino-terminal domain. The

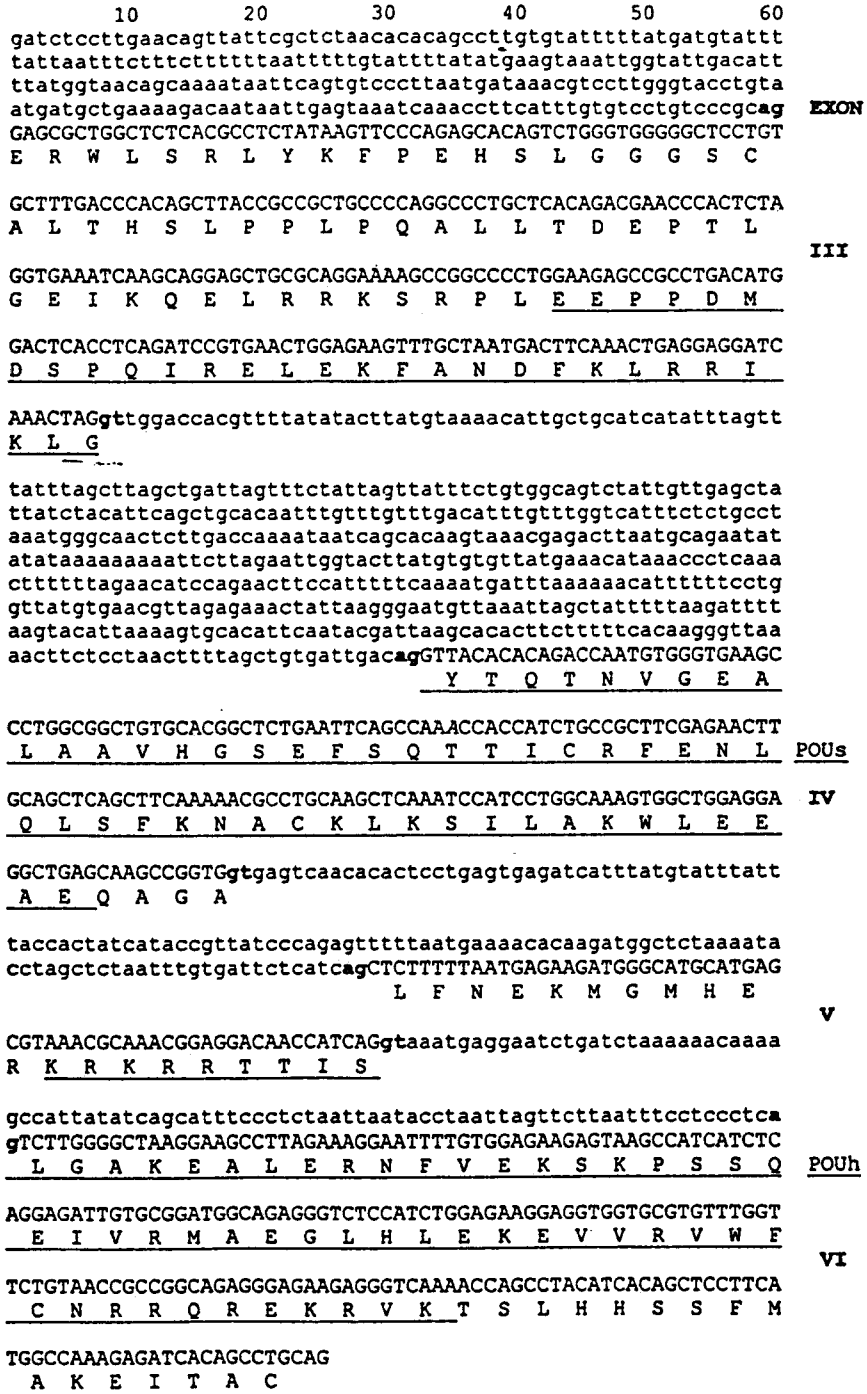

Fig. 1 Nucleotide and derived amino acid sequence of the insert contained in $pGP5_{1700}$. Coding sequences are indicated in upper case letters and exon-intron boundaries in bold type. From the several possible AG-sites of the furthermost 5' intron, only one is shown. The POUs and POUh-domain are underlined.

sequence of $pGP5_{1700}$ is deposited in the GenBank database, accession No.U92542. Alignment of the derived amino acid sequence of carp Pit-1 with rainbow trout Pit-1 reveals 61% identity in the amino terminal part and 94% identity in the POU-domain.

When the insert of $pGP5_{1700}$ was used as probe to hybridize RNA from different carp tissues (Fig. 2), only pituitary RNA reacted rendering two bands of 2.2 kb and 4.2 kb. No expression of Pit-1 was observed in the brain, kidney, heart and liver of the carp.

To determine Pit-1 expression during acclimatization, *in situ* hybridization of pituitary gland sections was performed. Signals for Pit-1 mRNA were localized mainly in the PPD and PI and, to a relatively low extent, in the RPD (Fig. 3). Molecular hybridization did not occur when the digoxigenin-labeled sense oligonucleotide probe, derived from the carp Pit-1 gene, was used. Semi-quantitative analyses of the *in situ* hybridization signals confirmed that a significant higher reaction was attained in the PPD and PI from fish acclimatized to summer as compared to the signal attained in the cold-season-acclimatized carp (Fig. 3)

DISCUSSION

Although some Pit-1 cDNAs from teleosts have been identified [18, 21-24], its genomic organization is unknown. To approach the expression of transcription factor Pit-1 during the acclimatization of the carp, we isolated and characterized a partial genomic sequence of Pit-1 from this fish. The sequence comprises exons III to VI, thus starts within intron II. The deduced partial carp Pit-1 amino acid sequence compared to rainbow trout is consistent with the expected extent of homology [25] in the POU-domain (94% identical). The exon-intron organization of the carp Pit-1 sequence analyzed, resembles the genomic structure of the mouse Pit-1 gene POU-domain [26].

The insert of the genomic clone $pGP5_{1700}$, comprising about two thirds of the estimated carp Pit-1 coding sequence, hybridizes with two RNAs (4.2 kb and 2.2 kb), depicting a similar pattern to that observed in chum salmon [18]. As in the case of rat and salmon [18] the carp Pit-1 RNA is expressed in the pituitary gland, but not in the liver, heart, kidney or brain. Because only a few tissues of the carp were tested by Northern hybridization, it is not possible to state that Pit-1 is specifically expressed in the pituitary gland.

In situ hybridization offers, in complex tissues, the possibility to pin point gene expression in specific cell types. This is particularly relevant in the case of Pit-1 which is known

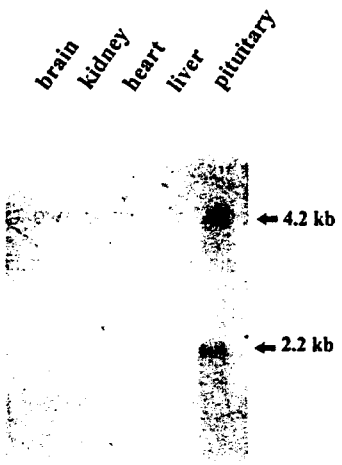

Fig.2: Northern blot hybridization of RNA from different carp tissues. RNA of various carp tissues was hybridized with the insert of $pGP5_{1700}$ as Pit-1 specific probe. The fragment sizes were estimated by a comparison with *Hind* III digests of phage λ DNA.

to transactivate at least three important hormones. Pit-1 appears to be necessary but, on itself, not sufficient to account for the precise cell type restriction of GH, PRL and SL expression [11].

As reported by Takeo et al. [27], Pit-1 protein colocalizes with the hormones whose synthesis is characteristic in the RPD, PPD and PI of rainbow trout. In the present study the results obtained by *in situ* hybridization reveal a remarkable Pit-1 expression in the PPD and PI and a much lower expression in the RPD in carp.

We observed significant differences in the amounts of Pit-1 transcripts in the PPD and PI of summer- and winter-acclimatized carp. During the warm season carp Pit-1 mRNA was 4 to 5 folds higher than in winter, implying that Pit-1 expression is involved in the compensatory response which occurs circannualy, concurrently with the seasonally environmental changes. It is of particular interest to observe that Pit-1 expression in lactotrophs exhibits a different pattern where a low basal Pit-1 transcription was observed in the RPD, both in summer- and winter-acclimatized carp. Thus, the rise in PRL mRNA and protein, which occurs in summer acclimatized male carp [7], appears to not be associated with a concomitant increase of Pit-1 expression in lactotrophs. To avoid hormonal changes associated with the reproductive cycle, all experiments were performed with male carp.

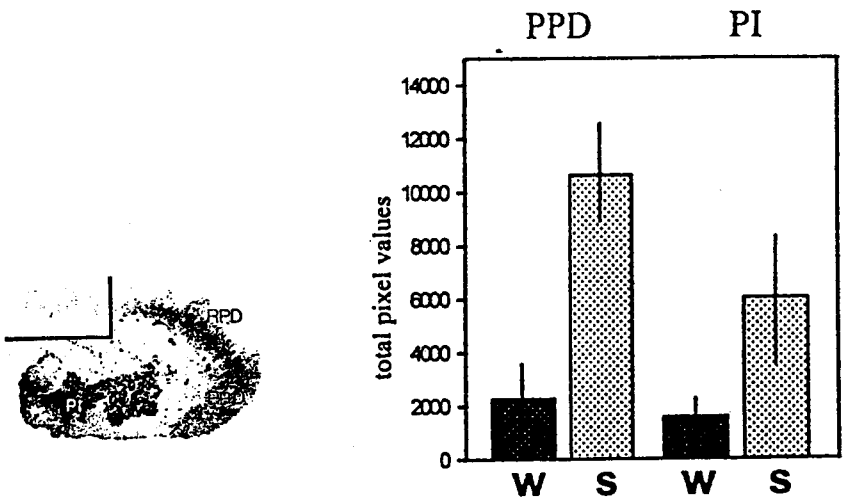

Fig. 3. *In situ* hybridization of a sagittal section of a pituitary gland from a summer-acclimatized carp. Molecular hybridization was obtained with a digoxigenin labeled carp Pit-1 specific antisense oligonucleotide probe (x30). The insert (x15) shows the results obtained when the oligonucleotide sense probe was used. RPD, rostral pars distalis; PPD, proximal pars distalis; PI, pars intermedia. The histogram depicts the level of Pit-1 expression of winter (W)- and summer(S)-acclimatized carp. Bars represent the mean ± standard deviation of the total pixel values measured in five areas of carp pituitary sections. (Student's t-test $P<0.01$; n=5 different fish corresponding to each season). The proximal pars distalis (PPD) and pars intermedia (PI) were analyzed separately.

In mammals there is evidence that the PRL distal enhancer requires the estrogen receptor in addition of Pit-1 for extensive activity [11]. Although in the male carp the steroid-binding affinity to hepatic estrogen receptors is not affected by seasonal acclimatization, the concentration of receptors is increased 2.5 fold in estrogen treated summer-acclimatized fish when compared to the cold-season-adapted carp [3]. Thus, it is possible to conceive a high PRL expression in summer-acclimatized fish [7] in the absence of changes in Pit-1 transcription. Clearly, the expression of Pit-1 target genes which are also modulated by systemic regulators such as cAMP, steroids and thyroid hormones, is still poorly understood [28].

In summary, we have cloned the first gene sequences of Pit-1 from a teleost fish and showed that its expression is seasonally modulated in the PI and PPD while in the RPD it does not show to be significantly affected. Therefore, it appears that Pit-1 is involved in the compensatory response towards the changes of the habitat of an eurythermal fish. Cloning of the entire carp Pit-1 gene is needed to gain knowledge on the regulatory sequences which could be involved in this differential expression. We are currently undertaking this study.

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