Regulation of Prolactin Gene Expression in Fishes

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Synopsis

To date, little research has been done to consider the process involved in regulating prolactin (PRL) gene transcription in fish species. Comparative work carried out shows that 5'-flanking sequences of PRL genes in mammals differ quite extensively from those in two teleostean fish species: the tilapia and chinook salmon. Nevertheless, several potential binding sites for the transcription factor Pit-1, which are known to be involved in PRL gene expression in mammals, are found in the 5'-flanking regions of the salmon and tilapia PRL genes. This suggests the factor may possibly play a role in fish PRL gene expression. Recent data indicate that Pit-1 is conserved quite extensively among vertebrates. Studies on the salmon and tilapia PRL genes point to the conservation of part of the molecular mechanisms involved in pituitary-specific expression of the PRL genes from teleosts to mammals and, more particularly, to the role of Pit-1. However, the species-specific patterns of PRL gene expression that do exist might involve species-specific factors that have yet to be identified. Or, alternatively, they could concern species-specific interactions of Pit-1 with the transcription machinery.

In vertebrates, prolactin (PRL) is involved in many physiological processes such as reproduction, osmoregulation, growth, differentiation, metabolism, and immunity (for a review, see Clarke and Bern, 1980). In teleostean fish, the primary role of PRL and, for that matter, the one
that has been most researched, is to regulate water and electrolyte homeostasis. PRL is well established as a freshwater-adapting hormone for euryhaline fish, even though in some species it is not essential for their survival in freshwater (Lorez and Bern, 1982; Hirano, 1986; Wendelaar Bonga and Pang, 1988, Prunet et al., 1990). Several studies have shown that PRL acts on virtually all osmoregulatory surfaces, i.e., gills, opercular membrane, skin, gut, kidney, and urinary bladder, to conserve ions and to minimize hydration.

PRL has been isolated from different fish species, including tilapia (Specker et al., 1985a), chum, chinook, and Atlantic salmon (Idler et al., 1978; Kawauchi et al., 1983; Prunet and Houdebine, 1984; Andersen et al., 1989), common carp (Yasuda et al., 1987), and eel (Suzuki et al., 1991). The teleostean PRLs show 60–80% sequence identity among themselves, and only 20–30% with mammalian PRLs. The fish PRLs all lack the first 12 amino acid residues present in mammalian PRLs and, consequently, lack the first disulfide loop to be found in mammalian PRLs. Two forms of PRL, with high sequence identity (more than 97%), have been observed in salmon (Onchorynchus keta) and carp (Cyprinus carpio) (Yasuda et al., 1986; 1987). In tilapia (Oreochromis mossambicus), two PRLs have also been isolated from pituitaries (Specker et al., 1985; Yamaguchi et al., 1988) and the corresponding cDNAs have been cloned and sequenced (O. niloticus, Rentier-Delrue et al., 1989). However, these two PRLs have different molecular weights and share only 69% amino acid identity. The larger one (tPRL I, 188 amino acids) has more in common with other fish PRLs than does the smaller one (tPRL II, 177 amino acids). Both tPRL I and tPRL II have been produced as recombinant proteins (Sweeneen et al., 1991) to investigate their proper biological activities (Auperin et al., 1994a, 1994b; Eik et al., 1994; Auperin et al., 1995).

In most teleostean fish, PRL cells are located in the rostral pars distalis of the adenohypophysis. In accordance with its key role in freshwater osmoregulation, PRL cell activity and plasma PRL levels are higher in freshwater than in seawater in some teleostean species (Bharatam and Nishioka, 1968; Abraham, 1971; Nagahama et al., 1975; Oliverereau et al., 1981; Prunet et al., 1990; Borski et al., 1992; Ayson et al., 1993; Yada et al., 1994). In some teleosts, including tilapia, PRL cell activity responds directly to changes in osmotic pressure (Nishioka et al., 1988; Grau et al., 1994).

The PRL Gene Family

Prolactin (PRL) belongs to a family of structurally and functionally related polypeptides, including growth hormone (GH), placental lactogen
(PL), proliferin, and somatolactin (SL). The primary structures of the GH, PRL, and PL in mammals and the structures of their genes suggest they are derived from a common ancestral gene as a result of the duplication of a smaller coding region and the insertion of introns (Niall et al., 1971; Martial and Cooke, 1980; Cooke et al., 1981; Barta et al., 1981; Miller and Eberhardt, 1983; Slater et al., 1986). In 1988, the first teleostean GH gene, the rainbow trout GH gene, was cloned and characterized (Appellon et al., 1988). Since then, the primary structure and the genomic organization of GH have been determined for several fish species (Johansen et al., 1989; Chiou et al., 1990; Ber and Daniel, 1992; Zhu et al., 1992; Hong and Schartl, 1993; Tang et al., 1993). A few PRL genes have also been sequenced in teleost species including the common carp (Chen et al., 1991), tilapia (fPRL I; Swennen et al., 1992) and chinook salmon (fPRL II; Xiong et al., 1992). All fish PRL genes contain five exons and four introns like the mammalian PRL genes (Gubbins et al., 1980; Camper et al., 1984; Truong et al., 1984). Moreover, the position and type of each exon/intron splice site are well conserved between mammals and teleosts. The length of the fish PRL genes varies between 2,600 and 3,700 base pairs (bp), whereas the mammalian PRL genes are 10–12 kilobases (kb) long. Size differences are due solely to variations in intron size. Recent research, focused on the purification of a novel pituitary glycoprotein from several teleosts (Ono et al., 1990; Rand-Weaver et al., 1991) and an analysis of its amino acid sequence, revealed that this protein belongs to the GH/PRL family. Because of its structural resemblance to GH and PRL, the protein was described as a somatolactin (SL). Cloning of the chum salmon SL gene has produced a new evolutionary model for the GH/PRL/SL family (Takayama et al., 1991; see Chen et al., 1994 for a review). These genes evolved from a common ancestral gene, produced by shuffling five exons, and diverged in the early stages of vertebrate evolution, before fish and tetrapods went their separate ways (Fig. 1). Emerging after the divergence of mammals, PL genes are derived from the GH gene in primates and PRL gene in rodents (Kawauchi et al., 1990).

Regulation of PRL Gene Expression in Mammals

The fact that the lactotroph cells of the anterior pituitary form the main site of PRL expression in vertebrates indicates that developmental mechanisms determining tissue-specific expression of PRL genes were conserved during evolution.

Research on the rat PRL gene shows that two positive regulatory regions are involved in its pituitary-specific expression: a proximal pro-
Figure 1. Evolutionary model for the PRL/GH/SL gene family (adapted from Takayama et al., 1991). Introns (lines) and exons (boxes) are drawn to scale.

The promoter between coordinates -36 and -422, and a distal enhancer between positions -1,550 and -1,830 (Nelson et al., 1986, 1988). The proximal region is sufficient to elicit pituitary-specific expression in transgenic mice. However, a synergistic interaction with the enhancer is necessary for high levels of expression in prolactin-producing cell types (Crenshaw III et al., 1989). Transient transfection experiments reveal that pituitary-specific expression of the human PRL gene is regulated by three positive regions: a proximal promoter lying on the first 250 bp 5′-flanking sequences, a distal region between coordinates -1,064 and -2,050, and a superdistal region between -4,430 and -4,777 (Lemaigre et al., 1989; Peers et al., 1990; Van de Weerdt, personal communication). In rats and human beings, these regulatory regions contain several cis-acting sequences that interact with the pituitary-specific transcription factor Pit-1 (also called GHF-1).

This factor was at first identified as a transcriptional activator of the GH and PRL genes in the rat (Bodner et al., 1988; Ingraham et al., 1988). Pit-1 is a member of the POU domain transcription factor family (reviewed by Verrijzer and Van der Vliet, 1993). The POU domain is a bipartite DNA binding domain containing a 75 amino acid POU-specific domain, a variable linker of 15-30 residues, and a 60 amino acid POU homeodomain (Herj et al., 1988; see Wright, 1994 for a review). Transcriptional activation is mediated by a less conserved domain, rich in serines and threonines, located at the N-terminal end (Theill et al., 1989; Ingraham et al., 1990).

In mammals, Pit-1 is expressed in thyrotroph, somatotroph, and lactotroph cell types. It has been shown to activate the expression of GH, PRL, TSHβ, growth hormone-releasing factor receptor genes and its own gene (Mangalam et al., 1989; Fox et al., 1990; Chen et al., 1990; Mason
et al., 1993; reviewed by Rhodes et al., 1994). Snell and Jackson dwarf mice, defective in the Pit-1 gene, are characterized by a lack of lactotroph, thyrotroph, and somatotroph cells. This suggests that functional Pit-1 is required for the proliferation and maintenance of these cell types as well as for the transcriptional activation of the GH, PRL and TSHβ genes (Li et al., 1990). Pit-1 may mediate the transcriptional regulation of these genes, but more work still needs to be done to discover exactly what mechanisms restrict their expression to their respective cell types. Possible mechanisms are active suppression of target genes in heterologous cells and/or different forms of Pit-1 with different activities and/or synergistic interaction between Pit-1 and other transcriptional factors (see Andersen and Rosenfeld, 1994; Rhodes et al., 1994 and references therein).

Regulation of PRL Gene Expression in Fish Species

Analysis of the 5'-flanking region of the PRL genes

The 5'-flanking sequences of PRL genes have been cloned and characterized in two teleostean species: the tilapia PRL I (tiPRL I; Swennen et al., 1992; Poncelet et al., submitted) and chinook salmon PRL II (sPRL II; Xiong et al., 1992) genes. An analysis of these regions reveals several potential Pit-1 binding sites, which differ by no more than one or two nucleotides from the mammalian Pit-1 motif, A (A/T) (A/T) TATNCAT (Nelson et al., 1988). Only 30% identity is found between the 5'-flanking sequences of the tilapia and salmon PRL genes. In the 140 bp proximal promoter regions, however, the sequence identity is 63% (Fig. 2). Our DNase I footprinting experiments on the tiPRL I proximal promoter revealed two footprints in the presence of rat pituitary cell extracts, P1 (-73, -68) and P2 (-110, -111). This is attributed to the binding of the rat Pit-1 factor. The potential Pit-1 binding sites P2 are identical in the two species, whereas the tilapia P1 site, containing a degenerate Pit-1 consensus, partially overlaps with the TATGCAT motif (-63, -57) to be found in the chinook salmon PRL gene.

Comparison of the 5'-flanking sequences of the rat, human, and bovine PRL genes shows that several regions are highly conserved (70% identity) among mammals. The first region corresponds to the proximal promoter. Located on the first 250 bp, the proximal promoter contains four and three Pit-1 binding sites in rat and human, respectively, as revealed by DNase I footprinting experiments (Nelson et al., 1988; Lemaigre et al., 1989). The second region corresponds to the rPRL enhancer between coordinates -1,800 and -1,530, and is highly homolo-
Figure 2. Comparison of the proximal promoters of the tilapia and chinook salmon PRL genes. The potential Pit-1 binding sites are shaded and the TATA boxes are in italics.
gous to the bovine PRL enhancer (-1,124 and -985; Wolf et al., 1990) and to the 5'-flanking region of the hPRL gene between coordinates -1,400 and -1,150 shown to contain four distal Pit-1 binding sites (Peers et al., 1990). A comparison of the regulatory sequences of mammalian PRL genes with the 5'-flanking regions of the tilapia and salmon PRL genes points to a major difference between mammals and teleosts (less than 30% identity). Although the proximal promoter regions of the mammalian and teleostean PRL genes are fairly dissimilar, the positions of the first Pit-1 binding sites are conserved (Fig. 3). This suggests that the sites could be involved in the transcriptional regulation of the teleostean PRL genes.

**Functional analysis of fish PRL promoters**

Xiong et al. (1992) set about investigating the pituitary-specific expression of the salmon PRL-II gene by performing transfection experiments on rainbow trout pituitary cells with constructs containing the sPRL-II promoter. Preliminary functional analysis suggests the presence of a proximal promoter lying on the first 109 bp 5'-flanking region and containing one putative Pit-1 binding site, and a negative region between coordinates -109 and -2,400. In our laboratory, we investigated the pituitary-specific expression of the tPRL-I gene. We conducted transient expression experiments on hybrid genes containing tPRL-I 5'-flanking sequences linked to the luciferase reporter gene (tPRL-I-LUC constructs) in rat and tilapia pituitary cells and in nonpituitary cells. The experiments showed that three regulatory regions are involved in the pituitary-specific expression of the tPRL-I gene: a proximal positive region between coordinates -50 and -550, a negative region spanning coordinates -550 to -2,600 and a slightly activating region between positions -2,600 and -3,400 (Poncelet et al., submitted). The first 160 bp of the tPRL-I promoter is sufficient for pituitary-specific expression to occur. This activity correlates with the presence of binding sites for the pituitary-specific factor Pit-1 (sites P1 and P2), which was identified in footprinting experiments. This situation is similar to that observed in the rat and human PRL genes insofar as their proximal promoter regions are sufficient to elicit pituitary-specific expression and contain four and three Pit-1 binding sites, respectively, (Nelson et al., 1988; Lemaigre et al., 1989; Peers et al., 1990). Involved, in particular, in basal activity, the two promoters also feature a highly conserved sequence, named BTE (rat) or A (man), spanning coordinates -85 to -115 between the first two Pit-1 sites (Peers et al., 1991, 1992; Jackson et al., 1992). That the same sort of sequence is not found in the tilapia and salmon PRL promoters suggests it is not conserved in teleosts.
Figure 3. Comparison of the first 250 bp of the 5′ flanking sequence of the human, rat, tilapia, and salmon PRL genes. The proximal Pit-1 binding sites of the mammalian PRL genes and the potential Pit-1 sites in the teleostean genes are indicated (P, dark boxes) as well as the BTE/A sequences (light boxes) binding a 100 kDa ubiquitous factor (see text for description).
Figure 3, contd.

Esholtz et al. (1992) found that the strength of the salmon PRL II gene promoter was much lower than that of the rat PRL gene in rat pituitary cells. However, the tilapia and human proximal promoter regions elicit approximately the same level of expression in rat pituitary cells. In tilapia cells, the human PRL promoter is expressed at a low level (Puncelet et al., submitted). The contrast between mammals and teleosts might result from species-specific differences in the Ftb-I function. Nev-
ertheless, several pieces of data indicate that the Pit-1 factor is conserved to a high degree among vertebrates (Elsholz et al., 1992; Argenton et al., 1993; Ono et al., 1994; Poncelet et al., submitted). There are grounds for supposing the Pit-1 function is modulated by factors that differ according to concentration, type or activity, depending on the species. In fact, several studies have shown synergistic interactions between Pit-1 and other transcription factors. For example, transcriptional synergism between Pit-1 and the thyroid hormone receptor, or the Zn15 protein has been observed in the activation of mammalian GH genes (Voz et al., 1992; Schauffele et al., 1992; Lipkin et al., 1993). Cooperative interactions between Pit-1 and the estrogen receptor are required for estrogen responsiveness of the rat PRL gene (Day et al., 1990). Pit-1 and the ubiquitous factor Oct-1 can also cooperate to induce expression of this promoter (Voss et al., 1991). An explanation for the species-specific expression of the PRL genes might be the interactions of the Pit-1 protein and species-specific factors binding to diverged regulatory sequences. It has been shown that binding of a 100-kDa ubiquitous factor to sequence A is required for full basal and hormonal regulation of human PRL promoter activity (Peers et al., 1992). This factor might differ in the tilapia pituitary or may not be expressed at all. This could offer an explanation for the low activity of the human PRL promoter observed in tilapia pituitary cells. The A sequence might not be required for tilapia PRL I gene expression.

In salmon (Xiong et al., 1992) and tilapia (Poncelet et al., submitted), negative regulatory regions are found upstream from the proximal promoter, spanning coordinates -109 to -2,400 (salmon) and -550 to -2,600 (tilapia). Distal negative regions are also described in the 5′ flanking sequence of the rat (Nelson et al., 1986; 1988) and human (Peers et al., 1990; Van de Weerd, personal communication) PRL genes, between coordinates -1,800 and -2,000 (rat) or -2,000 and -2,600 (man). In the rat, the negative control region contains an alternating purine-pyrimidine sequence, sequence APP (Manner, 1985). Able to adopt a left-handed DNA (Z-DNA) conformation in vitro, this APP sequence has an inhibiting effect on transcription of the rat PRL gene in transient expression assays (Naylor and Clark, 1990). There is also an APP-like sequence in the salmon and tilapia inhibitory regions, between coordinates -1,910 and -1,867 (salmon) and -837 and -812 (tilapia). These sequences might play a role in the negative regulation as shown in the rat PRL gene. A second APP sequence has been identified, lying on the proximal promoter region, between coordinates -146 and -179, in the tilapia PRL I gene (Swinnen et al., 1992). What functional role these sequences play in the dPRL I gene still has to be investigated, as some APP sequences have also been related to the activation of genes (Hamada
et al., 1984; Krajewski, 1995). In both teleostean PRL genes, potential Pit-1 binding sites are found in the negative regulatory region. Similarly, Pit-1 binding sites exist in the distal negative region of the human PRL gene. It would be interesting to use mutational analysis as a means of deciding whether these sequences actually play a part in negative control.

**Pit-1 activation**

Several studies with rats have shown that the Pit-1 factor is required for the transcriptional activation of the PRL and GH genes (Ingraham et al., 1988; Mangalam et al., 1989; Fox et al., 1990). Rat (Bodner et al., 1988; Ingraham et al., 1988), bovine (Bodner et al., 1988), mouse (Li et al., 1990), human (Lew and Elsholtz, 1991; Tatsuno et al., 1992; Persasetti et al., 1993), turkey (Wong et al., 1992), chum salmon (Ono and Takayama, 1992), and rainbow trout (Yamada et al., 1993) cDNAs encoding the Pit-1 factor have been cloned. An analysis of these sequences indicates high evolutionary conservation (85% identity) of the POU domain containing the DNA-binding region. Both teleostean proteins (chum salmon and rainbow trout) are larger than rat Pit-1, due to two insertions of about 30 amino acids each in the N-terminal parts of these proteins.

Elsholtz et al. (1992) have shown that the rat Pit-1 factor or a chimeric rat/salmon protein containing the N-terminal part of rat Pit-1 and the salmon POU domain are both able to activate the rat and salmon PRL promoters in transient expression assays. We have conducted cotransfection experiments with mammalian nonpituitary cells involving a rat Pit-1 expression vector and plasmids containing tilapia or human PRL promoters. These experiments indicate that the rat Pit-1 factor, too, is able to stimulate the two promoters (Poncelet et al., unpublished). Two other laboratories recently showed that the Pit-1 protein from two fishes, the rainbow trout and chum salmon, can specifically activate expression of teleostean GH genes (Yamada et al., 1993; Ono et al., 1995). The suggestion is that Pit-1 may also be involved in pituitary-specific expression of the salmon SL gene (Xiong et al., 1993; Ono et al., 1994) and in the development of SL-producing cells in rainbow trout (Ono et al., 1994).

**Species-specific expression of PRL genes**

All current research suggests the Pit-1 factor plays a functional role in pituitary-specific expression of the PRL/GH/SL gene family in vertebrates and points to some conservation of the mechanisms involved in
regulating this gene family during evolution. However, species-specific patterns of PRL gene expression do exist. Esholtz et al. (1992) demonstrated that the salmon and rat genes require a phylogenetically homologous system for optimal expression. These authors also suggest that some sequences conferring species-specific expression of the salmon PRL II gene are located distally in the 5'-flanking region. Our transfection experiments have shown that discrepancies between the results obtained in rat and tilapia pituitary cells are located in the distal regulatory regions of the tilapia PRL I gene. This suggests that distal sequences might also be involved in the species-specific expression pattern of the dPRL I gene.

Even though the POU domain of Pit-1 is well conserved among vertebrates, the mammalian and teleostean factors may preferably interact with slightly different sequences. Our electrophoretic mobility shift assays point to the ability of the rat Pit-1 factor and the tilapia Pit-1-related protein to bind to similar cis-elements, albeit with different affinities (Poncelet et al., submitted).

The transactivation domains of the mammalian and teleostean Pit-1 differ in length and are less well conserved than the POU domain (55% identity). This might mean that the species-specific expression of PRL family genes are a result of differences in the transactivation activity of the Pit-1 factor. It has been shown that rat Pit-1 is more efficient in mammal cells than salmon Pit-1 for stimulating the salmon GH and SL promoter activity but not for the rat GH promoter (Oino et al., 1995). However, salmon Pit-1 possesses greater transactivation activity than rat Pit-1 towards the rat and salmon GH promoters in fish cells.

Thus, the species specificity of PRL/GH/SL gene family expression may involve (direct or indirect) species-specific interactions between the Pit-1 factor and the transcriptional machinery. Differences may also be due to synergistic interactions of Pit-1 with species-specific regulatory factors and/or a species-specific combination of factors binding to highly divergent regulatory sequences. To gain a better understanding of the molecular basis for species-specific expression of PRL genes we have to determine the target of Pit-1 and throw some light on the synergy-related mechanisms.

Effects of cAMP on PRL gene expression

In the tilapia (Oreochromis mossambicus), cyclic AMP (cAMP) plays an important role in mediating PRL secretion. Exposure to cAMP, IBMX, a phosphodiesterase inhibitor, or forskolin, a stimulator of adenylate cyclase, increases tilapia PRL release that has been inhibited previously by somatostatin or by incubation in hyperosmotic medium (Grau et al.,
1982; Grau and Helms, 1989). Several studies suggest that the cAMP second messenger pathway may be involved in the control of PRL release in response to cortisol, somatostatin and altered osmotic pressure (Borsi et al., 1991; Helms et al., 1991).

The cAMP-dependent signal transduction pathway mediates the transcriptional response of many genes (see Lalli and Sassone-Corsi, 1994 and references therein) including mammalian PRL genes. Rat and human PRL promoter activities are seen to be stimulated by cAMP (Day and Maurer, 1989; Rech and Gudernitz-Hartmann, 1989; Berta et al., 1991; Peers et al., 1991).

In work we did to investigate the effect of cAMP on the tilapia PRL I gene, tilapia pituitary primary cultures were incubated with various concentrations of chlorpromazine-cAMP for 16 or 48 hours. Total RNA from treated and control cells was prepared and analyzed by Northern blot using tPRL I and tPRL II cDNAs (Rentier-Debue et al., 1989) as probes. The β-actin cDNA was used for normalization. Northern blot analysis of the tPRL mRNAs reveals two mRNAs differing in size (1.6 and 1.3 kb) that hybridize with both tPRL I and tPRL II cDNAs. The larger mRNA produces a stronger signal with the tPRL I cDNA, the smaller one with the tPRL II cDNA. This suggests that the 1.6 and 1.3 kb mRNAs encode for the tPRL I and tPRL II, respectively. After 16 hours, low-level stimulation of the tPRL I and tPRL II mRNA synthesis, independent of the cAMP concentration, is observed, whereas a significant dose-dependent increase in tPRL mRNA synthesis is reported after a two-day incubation with cAMP (see Table 1).

These results suggest the cAMP pathway is directly involved in activating tilapia PRL genes in the pituitary. To localize the sequences required for this cAMP stimulation, we performed transfection experiments on rat pituitary cell lines with the tPRL I-LUC constructs. The results are shown in Fig. 4. A three-to-four fold increase of tPRL I promoter activity is induced by cAMP or forskolin. The negative control, the TK-LUC construct (containing the promoter of the Herpes simplex virus thymidine kinase gene), is not affected by these two factors. Deletion of regions between -3,400 and -550 does not substantially alter the fold stimulation. A progressive loss of cAMP induction is associated with deletion of sequences between -550 and -50. The tPRL I proximal promoter region spanning coordinates -550 to -50 is therefore sufficient to confer this cAMP activation. This region contains the P1 and P2 Pit-1 binding sites. However, no obvious sequence corresponding to disacting elements known to confer cAMP-response (CRE: cAMP-response element, and AP-2 binding site; reviewed by Roesler et al., 1988; Lalli and Sassone-Corsi, 1994) has been identified in the tPRL I proximal promoter region. This situation is similar to that observed in
Figure 4. cAMP induction of tilapia PRL-LUC fusion genes in rat pituitary cells GC. (A) Schematic representation of the mutant 3′-cPRL I promoters fused to the luciferase reporter gene. The thin lines indicate the consensus Pit-1 binding sites. (B) GC cells (derived from a rat pituitary tumor) were grown in a monolayer at 37°C in Ham's F12 supplemented with 10% fetal calf serum and 1% penicillin-streptomycin. For the transfection experiments, the cells were harvested with trypsin-EDTA and resuspended in the same culture medium. Twenty-four million cells were mixed with 1 µg of purified plasmid DNA in an 800 µl electroporation cuvette. The cells were then exposed to a single pulse (250 V/4 mm, 1,000 µF capacitance) delivered by a Celly electroporator (Switzerland, Belgium). The transfected cells were incubated in tissue culture dishes (85 mm) and maintained in the same culture medium. After 8 hr, half of the transfected cells were incubated for about 16 hr with 0.5 mM chlorophenylthyio-cAMP (Boehringer). The medium was then removed and the cells were washed with phosphate-buffered saline. The cells were then lysed in situ with 300 µl of lysis buffer (25 mM Tris-phosphate (pH 7.8); 8 mM MgCl₂; 1 mM EDTA; 1% Triton X-100; 1% BSA; 15% glycerol, and 1 mM DTT) according to Poncelet et al. (1990) and luciferase assays were performed. The stimulation was calculated as the ratio of the luciferase activities in cAMP-treated versus untreated cells. The results shown are averages of five separate transfection experiments and were reproduced with several preparations of each plasmid.

rat and human PRL genes. The proximal promoter regions of these genes have been shown to be sufficient to confer cAMP induction (Keech and Gutierrez-Hartmann, 1989; Iverson et al., 1990; Peers et al., 1991). Two types of cis-acting elements are involved in this activation: (1) sev-
Table 1. cAMP stimulation of the mRNAs coding for the tilapia PRL I and PRL II in tilapia pituitary cell cultures. Male tilapia hybrids (*Oreochromis niloticus* × *O. aureus*) with a body weight of 150–200 g (farm of kibbutz Ma’agan, Israel) were used to establish primary pituitary cell cultures as previously described (Levavi-Sivan and Yaron, 1992). 2.5 × 10^6 cells were plated in 1 ml of culture medium (Medium 199 containing 9 mM NaHCO₃, buffered to pH 7.4 with 10 mM HEPES, and supplemented with fetal calf serum (10% v/v), penicillin (100 U/ml), streptomycin (0.1 mg/ml), and nystatin (1.25 U/ml). Three days after plating, tilapia pituitary primary cells were incubated with various concentrations (0.1, 0.5, and 2.5 mM) of chloroform/other-cAMP for 16 or 48 hr. Total RNA from treated and control cells was prepared by the single-step guanidinium thiocyanate-phenol-chloroform extraction method according to Chomczynski and Sacchi (1987). RNA preparations were electrophoresed on a formaldehyde-agarose gel and transferred to Gene Screen Plus membrane (Amersham). Prehybridization was carried out in 1% SDS, 10% Dextran sulfate and 1 M NaCl for 4 hr. Hybridization was performed overnight at 65°C in the same buffer with 32P-labeled probe. The EcoRI/BamHI fragments of the tiPRL I and tiPRL II cDNA were labeled using the random primer labeling kit (Stratagene). The sparrus actin cDNA was used as a control of RNA preparation efficiency. The results, analyzed with the phosphorimager system (Molecular Dynamics Corporation, California), are expressed as the ratio of tiPRL I mRNA to sparrus β-actin mRNA. Total RNA from muscle was used as a negative control.

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cAMP-fold induction

evident Pit-1 binding sites, and (2) the sequence BTE (mat) or A (man), spanning coordinates −115 to −85, shown to bind a 100 kDa ubiquitous factor (Jackson et al., 1992; Peers et al., 1992). In rats, however, a sequence similar to an AP-2 binding site has been identified between coordinates −78 and −71. This might be involved in the cAMP induction of the rat PRL gene. In rats and human beings, all the signs are that Pit-1, working in conjunction with other transcription factors, plays a part in mediating both basal and cAMP-stimulated PRL gene transcription. The same might hold true for the tiPRL I gene. However, additional deletion and point mutation studies will have to be conducted to gain a more accurate understanding of the DNA elements required for cAMP induction of the tiPRL I gene. As species-specific factors could be involved in this regulation process, experiments should also be performed on tilapia pituitary cells.

Influence of salinity on PRL gene expression

PRL is thought to play a key role in the way freshwater euryhaline teleostean fishes adapt to freshwater conditions. In tilapia, small reduc-
tions in medium osmotic concentration, thought to reflect blood osmotic pressure of a fish adapting to fresh water, rapidly stimulate PRL release (Grau et al., 1981; 1987). Two forms of PRL with no more than 69% amino acid sequence identity have been found in tilapia (Yamaguchi et al., 1988; Rentier-Delrue et al., 1989). This suggests that the two PRLs could have different functions and/or be independently regulated. In addition to the growth-promoting effect of the larger PRL (Specker et al., 1985b), and the fact that the salamander integumental prolactin receptors may discriminate between the two PRL variants (Specker et al., 1989), both tPRLs behave in a similar way in restoring the hydromineral balance of hypophysectomized tilapia after transfer into freshwater (Specker et al., 1985a; Young et al., 1988).

Borski et al. (1992) recently showed that, in tilapia (O. mossambicus), the pituitary content of both PRLs is greater in freshwater compared to seawater-adapted tilapia. However, the ratio of tPRL I to tPRL II is significantly higher in the pituitary of freshwater fish than in seawater species. Moreover, no parallel change in the plasma levels and synthesis of both PRLs is noted during freshwater and seawater adaptation. This suggests that the secretion and/or metabolic clearance of the two PRLs might be regulated differently in tilapia (Yada et al., 1994; Yoshikawa-Ehesu et al., 1995). Auperin et al. (1994a) recently suggested that, in tilapia (O. niloticus), PRL I and PRL II may play different osmoregulatory roles during adaptation to the hyperosmotic environment.

By means of immunohistochemistry, both PRLs have been localized in the same secretory granules in the tilapia O. mossambicus (Specker et al., 1993). Nishioka et al. (1993) used in situ hybridization to examine expression of PRL genes in tilapia O. mossambicus. Colocalization of mRNAs for PRL I and PRL II is reported. The hybridization signals for both mRNAs in the rostral pars distalis were higher for freshwater fish than seawater species. However, the level of mRNA of PRL II seems to be less affected than that of PRL I in seawater-adapted tilapia. These results confirm our recent data. We have used Northern blot analysis to determine the pituitary content of PRL mRNAs in tilapia O. aureus x O. niloticus during adaptation to hyperosmotic medium (unpublished data). The results, shown in Table 2, indicate that the mRNA levels of both PRLs are higher in freshwater than in seawater-adapted tilapia. Moreover, the mRNA coding for tPRL II is less affected than coding for tPRL I by the transfer to hyperosmotic medium. This suggests that in the case of tilapia O. niloticus x O. aureus, expression of the PRL I and II genes might be regulated differently in response to changes in osmotic pressure. It was recently shown by in situ hybridization that the pituitary content in both PRL mRNAs decreases in seawater-adapted fish (Ayson et al., 1994) during the early development of tilapia O. mossambicus. PRL
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Table 8. Influence of salinity on tilapia pituitary content of PRL mRNAs in vivo. Twelve or fifteen fish from a freshwater (FW) stock tank were transferred to 1/3 seawater (1/3 SW; 1.1% salinity). Twenty-four hours later, half of the fish were transferred into 2/3 SW (2.2% salinity) for one or two weeks. The water temperature during experiments was maintained at 27°C and the photoperiod was 12L/12D for each group. Total RNA from pituitaries was prepared and analyzed by Northern blotting as described above. The results, analyzed with the phosphorimager system (Molecular Dynamics Corporation, California), are expressed as the ratio of tiPRL I mRNA to sparrow barin mRNA.

<table>
<thead>
<tr>
<th>transfer</th>
<th>tiPRL I 1/3 SW</th>
<th>tiPRL I 2/3 SW</th>
<th>tiPRL II 1/3 SW</th>
<th>tiPRL II 2/3 SW</th>
</tr>
</thead>
<tbody>
<tr>
<td>21 hours</td>
<td>nd</td>
<td>2.7x</td>
<td>nd</td>
<td>1.2x</td>
</tr>
<tr>
<td>1 week</td>
<td>nd</td>
<td>10x</td>
<td>nd</td>
<td>2.4x</td>
</tr>
<tr>
<td>2 weeks</td>
<td>40x</td>
<td>20x</td>
<td>3.6x</td>
<td>5.4x</td>
</tr>
</tbody>
</table>

It seems to be more highly expressed than PRL I in seawater during the early life stages.

The present data suggest that expression of the two PRL genes may be regulated differently in freshwater and seawater in embryos, larvae, and adult tilapia. Cloning of the tiPRL II gene and comparison of its 5' flanking with the regulatory regions of the tiPRL I gene will be one of the first steps in the characterization of the molecular events that regulate expression of tilapia PRL genes during adaptation to changes in medium osmolality.

Concluding Remarks

Over the past few years, the genomic structures of several genes of the PRL/GH/SL family have been characterized for some fish species. However, few studies have examined the molecular basis for their pituitary-specific expression. Further research will be necessary to determine the precise molecular mechanisms contributing to a species-specific pattern of expression in the PRL/GH/SL genes. In addition to transient expression experiments in cell culture and other molecular biology assays, gene transfer in animals may prove to be a key tool when conducting these studies. Transgenic fish technology has improved over the past years and will, therefore, be useful not only for the study of in vivo expression of the PRL/GH/SL gene family, but also for understanding the mechanisms involved in the modulation of their expression by different factors and environmental stimuli (for example, growth factors, hormones, cAMP, stress or changes of salinity).
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