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Developmental Biology

Expression of the *Xenopus laevis* prolactin and thyrotropin genes during metamorphosis

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ABSTRACT **The cDNAs encoding *Xenopus laevis* prolactin (PRL) and the α and β subunits of thyroid-stimulating hormone (TSHα and TSHβ, respectively) have been cloned from a pituitary library. Results of developmental RNA blot analysis contradict the long-held biological role for PRL as a juvenilizing hormone in amphibia. The pituitary gland of a premetamorphic tadpole expresses PRL mRNA at very low levels. The abundance of PRL mRNA increases late in metamorphosis as a response to thyroid hormone (TH), suggesting that PRL is more likely to have a function in the frog than in the tadpole. TSHα and -β mRNA levels increase through prometamorphosis; this rise does not appear to be regulated directly by TH. At climax, both TH and TSH mRNA levels drop. The sequential morphological changes that characterize prometamorphosis depend upon the gradual increase of endogenous TH, which peaks at climax. This increase in TH in turn depends upon the lack of a traditional thyroid–pituitary negative-feedback loop throughout prometamorphosis.**

The thyroid (1) and pituitary (2) glands are known to be essential for amphibian metamorphosis. Thyroid hormone (TH) initiates metamorphosis of target tissues in the tadpole. Removal of either gland inhibits metamorphosis. The expected relationship of the two glands is presumed to occur by analogy with the well-known thyroid–pituitary negative-feedback loop, in which the pituitary secretes thyrotropin (thyroid-stimulating hormone; TSH), which stimulates TH production by the thyroid gland, which, then in turn, down-regulates TSH synthesis by the pituitary. There is considerable evidence that the first part of the feedback loop exists in tadpoles because inhibition of TH production causes goiter formation. However, the endogenous TH level increases during prometamorphosis, reaching its highest concentration at climax. Apparently during this period, the negative part of the loop—namely, the shutdown of TSH production by high TH levels—does not occur. At the end of climax, when metamorphosis is complete, TH drops to a low level, presumably signifying the establishment of the classical feedback loop. Therefore, the complete thyroid–pituitary feedback loop may itself be a developmental phenomenon of metamorphosis, as are so many other metabolic events.

Another well-known pituitary hormone, prolactin (PRL), has long been implicated in metamorphosis as a juvenilizing hormone (3, 4). The experimental evidence leading to this conclusion is as follows: (*i*) anti-TH effects are caused by treating metamorphosing tadpoles with large doses of mammalian PRL and amphibian pituitary fractions containing PRL activity (5) but not with growth hormone (GH) (3, 6, 7) and (*ii*) the injection of antibodies to PRL in tadpoles stimulates metamorphosis (7, 8). However, there is no biological evidence for the existence of a juvenilizing hormone in amphibians, and reported levels of PRL in tadpoles do not correlate with the expectation that the hormone exerts a juvenilizing effect (9). This conclusion was also reached by Takahashi *et al.* (10), who cloned bullfrog PRL cDNA and demonstrated an increase in PRL mRNA late in metamorphosis.

To study these events in greater detail, we have cloned cDNAs encoding TSH α and β subunits (TSHα and -β, respectively) and PRL from a *Xenopus laevis* pituitary cDNA library. Using these homologous probes, we describe the levels of their mRNAs during metamorphosis. We conclude that, although TSHα and -β mRNAs follow the expected developmental profile of unrestrained synthesis until just before metamorphic climax, this is not so for pituitary PRL mRNA. In fact, our results indicate that TH induces, although indirectly, the synthesis of PRL mRNA during metamorphosis.

MATERIALS AND METHODS

Animals. *X. laevis* embryos and tadpoles were raised in dechlorinated tap water and fed with nettle powder and freeze-dried salmon. Developmental stages were assigned according to Nieuwkoop and Faber (11). Five nanomolar 3,3′,5-triiodo-L-thyronine (T_3) was added to the water. Tadpoles were raised in the presence of 1 mM methimazole, an inhibitor of TH synthesis (12). Tadpoles and frogs were anesthetized by chilling in ice water before surgery.

Construction and Screening an *X. laevis* Pituitary cDNA Library. Twenty adult frogs were anesthetized, and their pituitaries were surgically removed. Polyadenylylated mRNA was purified directly from a tissue homogenate in guanidinium thiocyanate, as directed by the manufacturer (QuickPrep system; Pharmacia). The polyadenylylated RNA (≈2 μg) was used to prepare cDNA for directional cloning in λ Zap II (Stratagene). Immunoscreening (*pico*Blue kit; Stratagene) with a polyclonal antibody prepared against chicken PRL (from A. F. Parlow, Harbor–University of California, Los Angeles, Medical Center, Torrance) was used to identify phage expressing *X. laevis* prolactin. This antiserum reacts strongly with a single protein band by immunoblot in *X. laevis* pituitaries (E. Nelson and D.D.B., unpublished data). Other sera directed against various mammalian prolactins cross-reacted poorly with protein extracts from *X. laevis* pituitaries. These antisera were prepared under a National Institute of Diabetes, Digestive, and Kidney Diseases contract and were made available through the National Hormone and Pituitary Program.

Phage containing *X. laevis* TSHα were identified by using a cDNA probe prepared from the rat TSHα-encoding gene (13) under low-stringency hybridization conditions (described below). TSHβ-containing phage were similarly iden-

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Abbreviations: PRL, prolactin; TSH, thyroid-stimulating hormone; TSHα and TSHβ, TSH α and β subunits, respectively; TH, thyroid hormone; GH, growth hormone.

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tified with a probe derived from the bovine TSHβ-encoding gene (14).

Southern Blots. Genomic DNA from homozygous diploid frogs (15) was digested separately with restriction enzymes. Digested DNA (8 μg) along with molecular size standards (GIBCO) were electrophoresed through 0.8% agarose gels and transferred to Nytran filters (Schleicher & Schuell), as described (16). DNA was cross-linked to the membrane by UV irradiation. Labeled DNA probes were prepared by hexamer oligonucleotide random priming (Amersham). Southern blot hybridization was done overnight at 42°C by using standard hybridization conditions {50% (vol/vol) formamide/5× SSPE [1× SSPE = 0.18 NaCl/10 mM NaH_2PO_4 (pH 7.4)/20 mM EDTA]/5× Denhardt's solution/denatured salmon sperm DNA at 50 μg/ml}. For low-stringency blots the formamide concentration was reduced to 25%. Blots were incubated three times for 15 min with an excess of 2× SSPE/1% SDS at room temperature, followed by 15 min in 0.2× SSPE/0.1% SDS at 65°C. Low-stringency blots were washed the same way, except with 0.5× SSPE/0.25% SDS at 52°C.

RNA Isolation and RNA Blots. Total RNA was prepared by the acid guanidinium thiocyanate single-step isolation method (17). RNA and molecular-size markers (GIBCO) were fractionated by electrophoresis through formaldehyde-agarose gels (1.2–1.4%) and transferred to GeneScreen (DuPont), as described (16). RNA was cross-linked to the membrane by UV irradiation. Labeled DNA probes were prepared by hexamer oligonucleotide random priming. Blot-hybridization and washing conditions were as described above for Southern blots. Blots that were reused were stripped of probe by boiling 5 min in an excess of buffer (10 mM Tris, pH 7.5/1 mM EDTA/0.1% SDS). Probe removal was confirmed by autoradiography. RNA blot data are expressed on a per pituitary basis; there is no other obvious way to standardize such data because the relative abundance of various cell types, as well as pituitary size, changes during metamorphosis. Pituitary size appears (visually) to increase proportionately with the size of the tadpole or frog.

The pituitary of a long-term methimazole-treated tadpole, such as the one used for the experiment of Fig. 4, is not visible as a discrete structure under a dissecting microscope. For this reason, the entire surrounding brain tissue was included in the sample.

Sequence Analysis. cDNAs were sequenced by the dideoxynucleotide chain-termination method with an automated system and fluorescent dye terminators (Applied Biosystems), as recommended by the manufacturer. The European Molecular Biology Laboratory and GenBank data bases were searched with the deduced amino acid sequence by using the FASTA program (18).

RESULTS

Isolation of an *X. laevis* PRL cDNA Clone. A full-length PRL cDNA was cloned by expression screening with an antibody raised against chicken PRL. Three differently sized immunopositive clones were isolated and found by partial sequencing to be derived from the same gene. The longest of the three cDNA clones has an insert of 924 nt and was sequenced in both directions (GenBank accession no. L07620). The sequence revealed an open reading frame encoding a protein of 230 amino acids. This *X. laevis* cDNA clone has its highest amino acid homology with PRL genes from chicken (67%), pig (66%), human (60%), and bull frog (80%).

Isolation of *X. laevis* cDNAs Encoding TSHα and -β. The *X. laevis* TSHα cDNA was cloned with a heterologous probe derived from the rat TSHα-encoding gene (13) under low-stringency hybridization conditions. Positive clones were isolated, and the longest one, of 578 nt, was sequenced in both directions (GenBank accession no. L07619). The sequence contains an open reading frame encoding a polypeptide of 122 amino acids. The predicted amino acid sequence was searched against the data base. Its highest homology is to TSHα from several species, including mouse (72%), rat and turkey (both 71%), sheep, and cow (both 69%).

A similar strategy was used to clone the *X. laevis* TSHβ cDNA with a heterologous probe derived from bovine TSHβ cDNA (14). The longest clone, 445 nt, was sequenced in both directions (GenBank accession no. L07618). An open reading frame encoding a polypeptide of 129 amino acids was identified. The highest amino acid homology was to TSHβ from human, mouse (both 62%), cow, and rat (both 60%).

GH and PRL Genes. GH and PRL are structurally related hormones that arose by gene duplication 350 million years ago (for review, see ref. 19). Lactotrophs are derived from somatotrophs during development of the pituitary in mammals. The percentage of cells producing both hormones can be as high as 95% in neonatal rats (for review, see ref. 20). Because of this relatedness, we have compared expression of PRL and GH genes to better understand their roles during metamorphosis.

A partial cDNA clone for *X. laevis* GH (21) has been isolated. Because of the relatedness between GH and PRL, we first showed by Southern blot that the two cDNAs hybridize with entirely different sets of DNA bands (data not shown). Martens *et al.* (21) demonstrated that there are at least two genomic copies of the GH gene (A and B). Our Southern blots show that there are probably two, but not more than three, genomic genes each encoding GH and PRL. We carried out a low-stringency Southern blot with *X. laevis* PRL cDNA in an attempt to detect additional diverged genes that might encode a PRL-like protein. The hybridization patterns of the low- and high-stringency blots were essentially the same (data not shown). Therefore, no gene family members related to PRL can be detected in the *X. laevis* genome.

Developmental Expression of PRL and GH mRNAs. To account for the antimetamorphic effects of PRL, it has been hypothesized that endogenous levels of PRL should be greatest in premetamorphosis, decreasing toward the end of prometamorphosis and climax (3, 6). Pituitaries were isolated from tadpoles at stages spanning premetamorphosis to climax (stages 52–65), as well as from small frogs. Total RNA was fractionated by agarose gel electrophoresis for RNA blots. A single RNA species of about the length of the PRL cDNA (1 kb) hybridized with the PRL cDNA (Fig. 1*A*), indicating that the isolated cDNA is close to, if not, full-length. The autoradiograms were quantitated by densitometric scanning (Fig. 1*B*). This analysis demonstrated that PRL mRNA is expressed at very low levels throughout premetamorphosis and early prometamorphosis. Expression increases dramatically in late prometamorphosis (stage 58/59) and continues to rise thereafter. This pattern of expression resembles that described for PRL protein (9, 22) and PRL mRNA (10) observed in ranid frogs, which also increases throughout prometamorphosis, peaking near climax.

GH has been tested extensively over the years, showing little effect on metamorphosis except as a modest stimulant of tadpole growth (3, 6). Because of its relationship to PRL both in protein structure and in the proximity and close developmental history of the pituitary cells that synthesize each hormone, we compared GH A mRNA expression with that of PRL (Fig. 1*A*). Unlike PRL, GH mRNA is expressed at significant levels in stage-52/53 tadpoles. The GH and PRL cDNAs were labeled to similar specific activities, so that, accounting for exposure times (Fig. 1), the abundance of GH mRNA is at least two orders of magnitude greater than PRL mRNA in premetamorphic tadpoles, but, by the end of metamorphosis, their absolute levels are similar. The GH

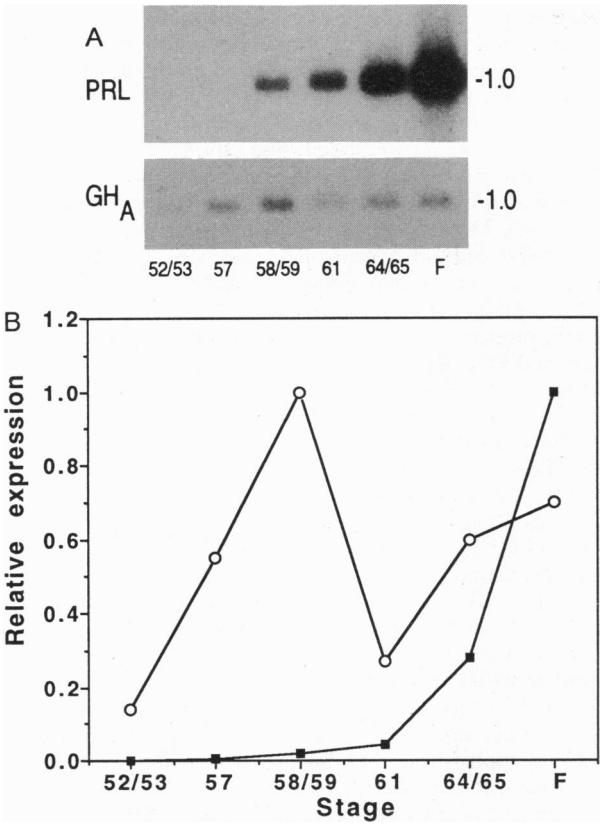

FIG. 1. Developmental expression of PRL and GH mRNAs. (*A*) Pituitaries were isolated from animals of the stages indicated (F, frog). Total RNA was prepared for RNA blot analysis, and each lane represents one pituitary RNA equivalent. *X. laevis* ^{32}P PRL cDNA was the probe. The autoradiogram (3-day exposure) is shown above; the filter was stripped of PRL probe and reprobed with ^{32}P-labeled GH cDNA (6-hr exposure, shown below). The molecular size of the two RNAs was estimated by comparing their mobility with known standards and is indicated at right (in kilobases). (*B*) Multiple exposures of the autoradiograms in *A* were quantitated by scanning laser densitometry. Results for PRL (■) and GH (○) are given relative to the highest value in each set, arbitrarily defined as 1.0.

mRNA level remains high throughout prometamorphosis, decreases at climax (stage 61), then increases again at the end of metamorphosis and remains at that level in the young frog. The expression pattern of the GH-encoding gene correlates with periods of tadpole and frog growth. Expression of GH mRNA is reduced during prometamorphosis when the tadpole is not feeding due to intestinal remodeling.

Effect of Exogenous TH on Expression of GH and PRL mRNAs. Stage 52 (premetamorphic) tadpoles were treated with 5 nM 3,3′,5-triido-L-thyronine, and pituitaries were collected daily for 5 days. Duplicate RNA blots were probed with PRL and GH cDNAs (Fig. 2). A transient decrease in expression of the pituitary hormone mRNAs was seen between 1 and 3 days after beginning TH administration (see Fig. 2 for GH and PRL; data not shown for TSHα and -β mRNAs). GH mRNA continues to decline at a time when PRL mRNA increases ≈5-fold. Both TSHα and -β mRNAs return to their control levels by day 5. The up-regulation of PRL mRNA occurs after a 3-day lag, demonstrating that it is a delayed or late response to TH.

Expression of TSHα and TSHβ mRNAs. Expression of mRNA for both TSH subunits by RNA blot was detected at all developmental stages examined (Fig. 3*A*). A single mRNA species of ≈700 bases was detected for TSHα, whereas TSHβ mRNA consists of three hybridizing species of 4.4, 2.4, and 0.7 kb. The relative abundance of the three mRNAs for

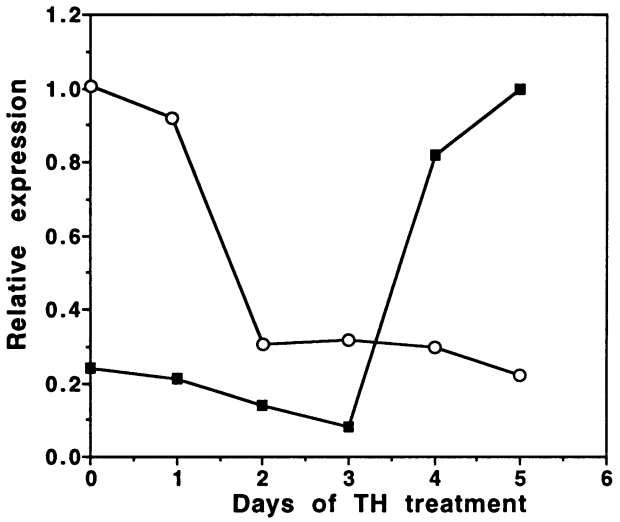

FIG. 2. Effect of TH on PRL and GH mRNA expression in premetamorphic tadpoles. Stage-52 to -53 tadpoles were chosen to examine the response of pituitary GH and PRL to exogenous TH treatment (5 nM 3,3′,5-triiodo-L-thyronine). Pituitaries were collected each day for 5 days. Total RNA was prepared for RNA blot analysis with the PRL (■) and GH (○) cDNA probes, as described for Fig. 3.

TSHβ was always the same. Expression of TSH mRNA is low, but detectable, in premetamorphic tadpoles (stages 52–53), increases throughout prometamorphosis, and peaks at about stage 59. Quantitation of the autoradiograms suggests that TSHα and -β are coordinately regulated during metamorphosis, and their expression increases ≈100-fold between stage 52 and 59 (Fig. 3*B*). TSHα mRNA is ≈20 times more abundant than TSHβ mRNA. Presumably this is from the fact that TSHα is a common subunit for the gonadotropins, as well as for TSH. In fact, we have identified a cDNA encoding *X. laevis* luternizing hormone β; its developmental expression is similar to that of TSH mRNAs (data not shown). The developmental profile of TSH mRNA mirrors the rise in TH concentration (23, 24), except that the TSH peak (stage 59) precedes the highest level of TH, which is reported to be at stage 61.

The Two Larger TSHβ mRNAs Are Extensions at the 3′ End. Rescreening the pituitary cDNA library with the original 528-bp cDNA clone (TSHβ5), we isolated and sequenced a 2.2-kb polyadenylylated TSHβ cDNA (TSHβ7). The 5′ end of the longer clone initiates at the same guanosine residue as the shortest cDNA (data not shown). Except for a 5-nt difference in its 5′ untranslated region, the two cDNAs contained identical coding and untranslated sequences up to the poly(A) tail of the small cDNA. A third longer cDNA clone is identical with the 2.2-kb clone and extends beyond the poly(A) tail, suggesting that the 4.4-kb clone differs only by the extent of sequences in the 3′ untranslated region (data not shown). There are two potential polyadenylylation signals, AGTAAA and AATAAA, at ≈18 nt upstream of the poly(A) tails of both the 0.7-kb (TSHβ5) and the 2.2-kb cDNA (TSHβ7) (data not shown). This analysis indicates that the three TSHβ mRNAs differ only in choice of the poly(A) site selection.

Expression of PRL, but Not of TSH, Depends on TH. Up-regulation of PRL mRNA was induced by TH (Fig. 2). In addition, we observed that TSH mRNA and TH levels rise in parallel during metamorphosis. These findings led us to investigate further the relationship of TH to expression of these pituitary hormones. Tadpoles were rendered functionally athyroid by rearing them from stage 50 in the constant presence of 1 mM methimazole (12). Animals treated in this way do not undergo morphological changes associated with

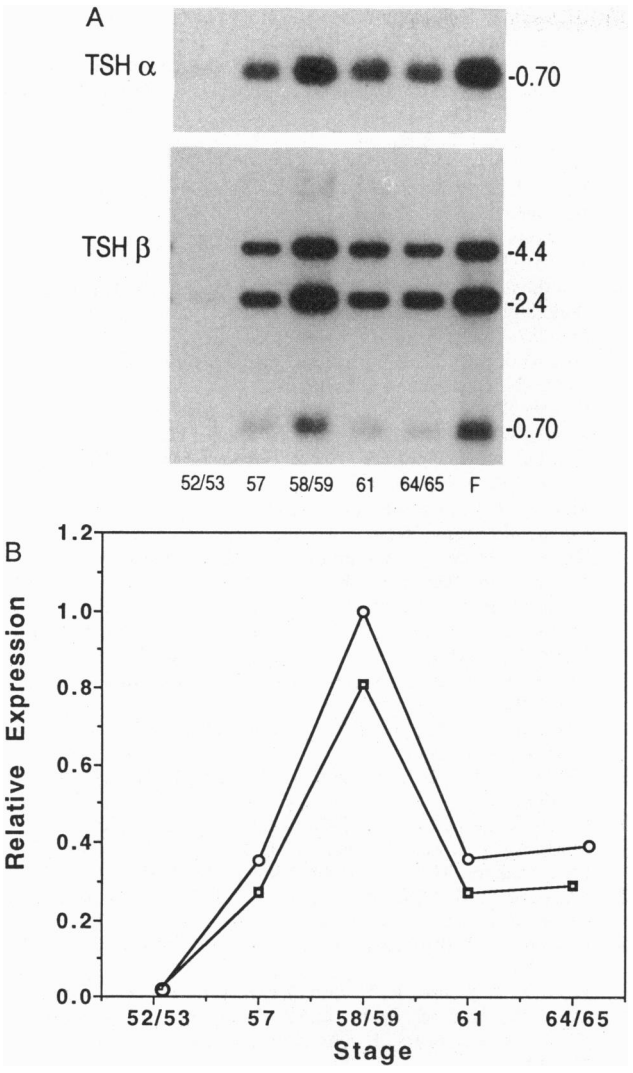

FIG. 3. Developmental expression of TSH mRNAs. (*A*) The RNA blot used in Fig. 3 was stripped and reprobed with TSHβ cDNA (3-day exposure; shown below). After the blot was stripped, it was reprobed with TSHα cDNA (1-day exposure, shown above). RNA size was estimated by comparing migration to molecular size standards, as indicated at right in kilobases. (*B*) Autoradiograms in *A* were quantitated by scanning laser densitometry. Results for TSHα (□) and TSHβ (○) are given relative to the highest value, arbitrarily defined as 1.0.

metamorphosis but continue to grow in size. An animal chosen for these studies was 10-months old and weighed >20 times a normal stage-50 animal; its siblings had undergone metamorphosis 8 months earlier. It had developed a huge goiter. In agreement with the results of Fig. 1, we detected a strong PRL signal by RNA blot from the pituitaries of frogs and stage-58/59 prometamorphic tadpoles (Fig. 4). However, PRL mRNA expression was low in the methimazole-treated tadpole. These results further demonstrate that TH is required for expression of the PRL-encoding gene. Conversely, when we analyzed levels of TSHα and -β, we noted strong expression of both subunit mRNAs in the methimazole-treated tadpole, as well as in the pituitaries from frog and stage-58/59 tadpole (Fig. 4). The high level of TSH mRNA in a methimazole-treated animal is consistent with the role of TSH as a positive effector of TH synthesis.

DISCUSSION

The endocrine control of amphibian metamorphosis has been the subject of scientific investigation for 80 years. The

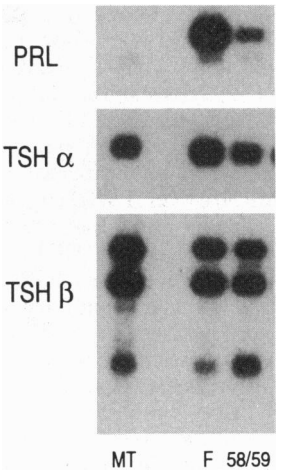

FIG. 4. TH is required for PRL but is not required for TSHα and -β mRNA expression. Pituitaries from a tadpole raised in the constant presence of 1 mM methimazole (MT), a young frog (F), or a stage-58/59 tadpole were isolated for RNA blot analysis. An equivalent of RNA from one pituitary (F and 58/59) or one-fifth of a pituitary (MT) was used in the RNA blot. The blot was probed sequentially with the following radioactive cDNAs; PRL (above), TSHα (middle), or TSHβ (below).

thyroid gland and the pituitary were implicated by Gudernatsch (1) and Adler (2). Two pituitary hormones are thought to be involved in metamorphosis—PRL as a juvenilizing hormone and TSH because of its well-known role in stimulating TH synthesis by the thyroid gland. We have cloned the cDNAs encoding these hormones and correlated the expression of their genes with metamorphosis.

PRL. There are two kinds of experiments in the literature dating back to the 1960s, suggesting that PRL is the amphibian equivalent of juvenile hormone in insects (for review, see refs. 4 and 25). PRL added to tadpoles (3, 9) or to cultured tissues (26) can prevent the effect of either endogenous or exogenous TH, thus retarding metamorphic changes. The second experiment purports to show that antiserum directed against PRL speeds up metamorphosis (7, 8). The effect of GH on metamorphosis is less dramatic and mainly reported to stimulate tadpole and frog growth (3, 6, 7).

Contradicting the proposed role of PRL as a juvenilizing hormone are published measurements of PRL protein that show a rise paralleling TH (9, 22). The mRNA levels in the pituitary (Fig. 1) resemble the protein data. Were PRL a normal juvenilizing hormone, its levels should be highest in premetamorphic tadpoles. In fact, the GH mRNA developmental profile fits better with the pattern expected of a juvenilizing hormone. TH actually appears to induce PRL formation. The delay of several days from the administration of TH until PRL mRNA levels increase (Fig. 2) suggests that TH might exert its action by inducing the differentiation of lactotroph cells in the pituitary. In mammals these cells are derived from somatotroph cells, and we suspect that this same process occurs in the amphibian pituitary during metamorphosis. These findings point to a biological role of PRL in frogs rather than in tadpoles.

TSH. The profile of TSHα and -β mRNAs closely parallels the expected expression pattern from the known endocrinology of tadpole metamorphosis. The sequential process of metamorphosis is initiated and controlled by formation of the thyroid gland. TH levels rise during prometamorphosis to peak at climax and then fall to the low level characteristic of the frog (23). The increase in TH formation depends upon the pituitary, presumably through its synthesis of TSH. The mRNAs for both TSHα and -β rise more or less in parallel with endogenous TH and then fall just before metamorphic

climax, when the drop in TH concentration occurs. The rise in TSH (Fig. 3) may signify the development of thyrotrophic cells in parallel with the thyroid gland or a gradual up-regulation of genes for TSHα and -β that is refractory to the negative feedback expected when TH levels are high. However, the increase in TSH mRNA occurs with (Fig. 3) or without (Fig. 4) the concomitant increase in TH. The great excess of TSHα mRNA over TSHβ suggests that the gonadotrophic cells of the pituitary that are known to secrete hormone glycoproteins that use TSHα as a subunit are differentiating at the same time as the thyrotrophic cells. Clearly, one part of the thyroid–pituitary feedback loop, the ability of high TH to down-regulate TSH, is inoperative until the climax of metamorphosis. The incomplete feedback loop is essential to metamorphosis because it permits exceedingly high levels of TH to be synthesized by the thyroid gland. These high levels are needed to induce the final changes, such as tail resorption. Indeed, the tadpole's ability to increase gradually its endogenous TH concentration is essential for the sequential and gradual changes that occur during metamorphosis. Thus, the tadpole escapes the steady-state regulation of TH production by its thyroid gland until metamorphosis is completed.

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