Developmental and thyroid hormone-dependent regulation of pancreatic genes in *Xenopus laevis*

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We isolated and sequenced a cDNA encoding *Xenopus laevis* pancreatic trypsin, which has ~70% amino acid sequence identity to mammalian trypsinogen. Northern blotting analysis shows that the trypsin gene is activated just before the tadpole starts to feed, reaches peak activity in the swimming tadpole (premetamorphosis), and is then repressed during prometamorphosis, attaining its lowest activity at the climax of metamorphosis. The same gene is then activated again in frogs but to a much lower level. The pattern of the changes in trypsin gene expression is followed by at least two other pancreas-specific genes and marks the remodeling of the pancreas of the animal at metamorphosis. Thyroid hormone, which is the causative agent of metamorphosis, can down-regulate trypsin gene expression prematurely.

[Key Words: Thyroid hormone; *Xenopus*; pancreas; trypsin; metamorphosis]

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The metamorphosis of amphibian tadpoles to frogs is initiated by and dependent on thyroid hormone [Weber 1967; Dodd and Dodd 1976; Cohen et al. 1978; Fox 1981; White and Nicoll 1981; Galton 1983]. During metamorphosis, some tissues, such as limbs, are formed from undifferentiated blastema cells, resembling the formation of adult structures from imaginal discs in insects. Others, such as liver, skin, and the nervous system, do not change as much morphologically but express different genes after metamorphosis. Cell death is the major mechanism for tadpole tail regression. There is still another kind of change, that is, as exemplified by tadpole intestine, a remarkable remodeling that shortens the intestinal tract to a fraction of its previous length. The pancreas has also been reported to decrease in size (relative to total body weight) and change its morphology and cell structure during metamorphosis [see also Leone et al. 1976]. These visceral changes are associated with the transition from a herbivorous tadpole to a carnivorous frog. In this paper we describe how the onset of pancreatic development, growth of the pancreas in tadpoles, and subsequent remodeling as a result of metamorphosis of the pancreas are reflected in the expression of genes specific to the exocrine pancreas.

Results and discussion

Isolation and characterization of *Xenopus laevis* trypsin cDNA

We screened a cDNA library derived from stage 49 tadpole mRNA with cDNA probes prepared from stage 44 and stage 49 mRNA to search for genes that are regulated during early tadpole development. [All developmental stages are classified according to Nieuwkoop and Faber 1956.] Tentative clones after secondary screening were subcloned, and the cDNA inserts were used as probes of Northern blots. Two clones (PR22 and PR23) gave identical developmental regulation patterns [see below] and cross-hybridized. Where they overlap, the two cDNAs have the same restriction enzyme sites and DNA sequence except for alternative polyadenylation [Fig. 1]; an open reading frame encodes 243 amino acids, with >70% amino acid identity to rat trypsinogen [Fig. 2].

Trypsin is synthesized in the pancreas, secreted in an inactivated form, and activated later by a Ca\(^2+\)-dependent autocleavage process. The *Xenopus* protein contains the hydrophobic prepeptide necessary for secretion, as well as the activation peptide [Fig. 2], even though the peptides are not well conserved among different trypsinogens. In addition, 12 conserved cysteine residues involved in disulfide bond formation are located at the same positions, as in most other trypsinogens. *X. laevis* trypsin also conserves other key amino acid residues important for enzymatic activity, for example, the catalytic site [Barratt 1986] and amino acid residues 194–197 and 215–220, which form the substrate binding pocket. A summary of conserved features of various trypsinogens is given by MacDonald et al. [1982a].

Southern blotting analysis of homozygous diploid genomic DNA at high stringency suggests that a single gene encodes this particular trypsin cDNA [Fig. 3A]. At lower stringency, the cDNA probe [PR22] hybridized to...
many bands with different intensities (Fig. 3B), suggesting the existence of other trypsin or trypsin-related genes with different degrees of homology to PR22, as is the case in mammals (Craik et al. 1984).

Developmental and thyroxine-dependent regulation of the X. laevis trypsin gene

By using the cDNA as a probe to hybridize RNA from individual tissues, we confirmed that the trypsin gene is expressed exclusively in the pancreas of a tadpole or frog (data not shown). No signal was detected in any other tissue of the frog or tadpole. Developmental Northern blotting analysis of total RNA from tadpoles (Figs. 4 and 5) first detects the trypsin mRNA at stage 44/45. The mRNA increases during tadpole growth, reaching a peak at stage 54. This period of tadpole growth is called premetamorphosis (up to stage 55; Broyles 1981), that is, the period preceding the development of a functional thyroid gland. The decrease in trypsin mRNA mirrors the changes in morphology (prometamorphosis, stages 55–61); the mRNA drops to a minimum as the tadpole reaches the climax of metamorphosis at stage 62. The mRNA drops to a minimum as the tadpole reaches the climax of metamorphosis at stage 62. The amount of trypsin mRNA decreases again to a level in a frog well below the peak attained at stage 54. The amount of trypsin mRNA is >300-fold lower at stage 62 than at stage 54, and a frog has >50-fold less trypsin mRNA than a stage 54 tadpole on the basis of measurement per milligram of total RNA. We estimate that ~0.5% of the total mRNA in a stage 54 tadpole is trypsin mRNA. The changes in trypsin mRNA level agree qualitatively with the reported enzymatic activity of trypsin between stages 56 and 66 (Kujat 1981).

Because X. laevis appears to have more than one genomic trypsin or trypsin-related gene, the dramatic reduction in the abundance of trypsin mRNA could mean either that postmetamorphic frogs have less trypsin mRNA or that a different trypsin gene(s) is expressed in frogs, the product of which hybridizes weakly with our cDNA probe (PR22), thus accounting for an apparent decrease in mRNA. To test these possibilities, we synthesized cDNA from adult and tadpole RNAs using a primer complementary to the 3'-untranslated region of the trypsin cDNA just beyond the termination codon, a region expected to be more variable than the coding region. Any trypsin mRNA with a different 3'-untranslated region should not contribute to the cDNA thus produced. Identically sized cDNAs in the predicted region of trypsin mRNA detected by PR22, even though the hybridization signal increased for both. This result supports the conclusion that the mRNA detected by PR22 in frogs is encoded by the same gene(s) as that detected by PR22 in tadpoles. These experiments indicate that frog pancreas expresses the same trypsin gene(s) as does tadpole pancreas but at a much lower level.
The development of the pancreas and the increase in trypsin mRNA coincides with the formation of the intestine and the onset of tadpole feeding. The decrease of trypsin mRNA and the reduction in the size of the pancreas occur along with the many morphological changes that characterize metamorphosis. These changes, in turn, are related to formation of the thyroid gland (see Dodd and Dodd 1976; Cohen et al. 1978) and the endogenous thyroid hormone content that peaks at the climax of metamorphosis (Fig. 5), suggesting that regulation is influenced at least indirectly by thyroid hormone. In addition, when added exogenously, either thyroxine (T4) or its monodeiodinated derivative T3 [3,5,3'-triiodothyronine] can induce precocious metamorphosis, albeit at different concentrations. Exogenous thyroxine has been reported to induce reduction in the size of the tadpole pancreas, mimicking natural metamorphosis (Blatt et al. 1969). Considering these facts, we added exogenous thyroid hormone (T4 or T3) to tadpoles at different stages of development and measured the levels of trypsin mRNA after varying periods of time. Figure 6 shows that 1 week of hormone treatment down-regulates the trypsin mRNA level. A 3- to 10-fold reduction was observed between stage 46/47 and stage 56 (referring to the stage when hormone treatment was begun). Response to T4 and T3 was indistinguishable. Although dramatic effects of hormone treatment were observed at developmental stages when the endogenous hormone level was low (see Fig. 5), little or no difference was observed both in tadpole morphology or trypsin mRNA level when stage 58 or 62 tadpoles were subjected to 1 week of thyroxine treatment [data not shown]. This is expected, because at stage 58, the endogenous thyroxine level is elevated to half of the maximum level that is present at the climax of metamorphosis (stage 62), so that exogenous thyroxine after stage 58 should have no effect. A time course of T3 treatment (Fig. 7) shows that trypsin mRNA is reduced by half after ~2.5 days. The decrease of trypsin mRNA correlates with visible morphological changes induced by thyroxine in the tadpole, including a noticeable decrease in the size of the pancreas.

Developmental profile of the expression of pancreatic-specific genes

The genes for many pancreatic-specific enzymes have been cloned and characterized, mainly from mammalian sources. Dr. R. MacDonald kindly gave us cDNAs encoding rat elastase and carboxypeptidase. These cDNAs cross-hybridize to X. laevis RNAs of about the same size as their respective rat mRNAs [1.1 kb for elastase I (MacDonald et al. 1982b); 1.5 kb for carboxypeptidase B (Clauser et al. 1988)]. The developmental pattern of gene expression [Fig. 8] resembles that of trypsin, peaking at

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**Figure 2.** Comparison of the amino acid sequence of X. laevis and rat trypsinogen. The prepeptide (amino acids 1–15) is shown in italics, and the activation peptide (amino acids 16–23) is underlined. The 12 conserved cysteine residues involved in disulfide bond formation are indicated by carets.

**Figure 3.** Genomic Southern blotting analysis of the trypsin gene. Two micrograms of homozygous diploid DNA per lane was used. [Lanes 1–5] BamHI, BamHI + HindIII, HindIII, PstI, and EcoRI digests, respectively. [A] A blot, washed twice in 0.05 × SSC, 0.1% SDS, at 65°C for 30 min. [B] A blot, washed twice in 1 × SSC, 0.1% SDS at 65°C for 30 min. The bars at right of A and B indicate the positions of λ/HindIII DNA size markers.

**Figure 4.** Developmental Northern blotting analysis of the cDNA clone PR22. One microgram of total RNA was used in each lane and hybridized with 32P-labeled cDNA insert. The sizes of the mature mRNA (0.8 kb) and two higher-molecular-weight RNA bands that could be precursors (2.8 and 5.4 kb) were determined by use of an RNA size ladder [BRL]. [The sizes of 18S and 28S RNAs are 1.8 and 4.1 kb, respectively.] (Lanes 1–7) Total RNA from ovary, tadpoles at stages 41, 44/45, 54, 58, and 62, and 6-month-old frog (~4 grams).
Figure 5. Quantification of trypsin mRNA at different developmental stages plotted with the known endogenous thyroid hormone level (Leloup and Buscaglia 1977). Each point is the average of four different measurements that were reproducible within a factor of two. The mRNA was quantified by slot-blot hybridization of total RNA from ovary, 6-month-old frog, or different stage tadpoles with known amounts of PR22 cDNA as a standard and PR22 eDNA as a probe. The hybridization signal was measured by Cerenkov counting. (Inset) The trypsin mRNA increase plotted at stages 62, 64, and 66 and frog (ng mRNA/mg total RNA) on an expanded scale. The amount of RNA used was determined by UV absorption and verified after blotting by methylene blue staining (Herrin and Schmidt 1988). Standardization was also monitored by hybridization with the cDNA PR28 whose mRNA does not change (within threefold) in abundance during development from ovary to frog (data not shown).

The trypsin mRNA increase plotted at stages 62, 64, and 66 and frog (ng mRNA/mg total RNA) on an expanded scale. The amount of RNA used was determined by UV absorption and verified after blotting by methylene blue staining (Herrin and Schmidt 1988). Standardization was also monitored by hybridization with the cDNA PR28 whose mRNA does not change (within threefold) in abundance during development from ovary to frog (data not shown).

Stage 54, dropping at metamorphosis climax, but present at varying levels in adult frogs (cf. Figs. 8 and 1). Elastase mRNA is about as abundant in frog as it is in tadpole, whereas carboxypeptidase mRNA is intermediate between trypsin and elastase mRNAs in frogs compared to tadpoles. Leone et al. (1976) have reported similar developmental changes for the enzymatic activity of amylase and lipase during metamorphosis. Thus, it appears that the genes for exocrine pancreatic enzymes are regulated, at least in part, through a common mechanism characterized by peak activity in actively feeding tadpoles and down-regulation during metamorphosis when the tadpole stops feeding and the digestive system is remodeled, reaching the lowest level at the climax of metamorphosis followed by variable increases in gene activity in the frog pancreas. Presumably, a variety of biochemical mechanisms, including gene expression, account for the levels of digestive enzymes suited for a frog, which has a completely different diet from that of a tadpole.

Figure 6. Effect of thyroid hormone on the level of trypsin mRNA. One microgram of total RNA was used in each lane and probed with PR22 cDNA. [Lanes 1–7] RNA isolated from control tadpoles and those treated for 1 week with hormone starting at the developmental stage given. [Lanes 1 and 2] T4-treated (26 nM) and control tadpoles at stage 46/47, respectively; [lanes 3–5] T4 (26 nM), T3 (5 nM), and control tadpoles at stage 50, respectively; [lanes 6–8] T3 (5 nM), T3 (1 nM), and control tadpoles at stage 54, respectively; [lane 9] stage 58 tadpole; [lane 10] 6-month-old frog. When the filter was reprobed with the control clone PR28, no thyroxine-dependent regulation of its mRNA was observed (data not shown).

Figure 7. Time course of the effect of thyroid hormone treatment on the level of trypsin mRNA. Stage 51–53 tadpoles were treated with 5 nM T3 and RNA was isolated from the tadpoles at the time indicated and analyzed by slot-blot hybridization. The radioactive slots were counted. The level of trypsin mRNA in control tadpoles does not change appreciably during this period (see Fig. 5, stages 51–55).
Pancreatic gene regulation in *Xenopus*

Factors affecting the reduction of trypsin mRNA

The size of the pancreas decreases during metamorphosis (Janes 1937; Race et al. 1966; Dodd and Dodd 1976). In fact, up to 70% loss of the relative weight of the pancreas has been observed in *Rana pipiens* as a result of metamorphosis (Race et al. 1966). A quantitative analysis of trypsin mRNA in pancreas from different stage tadpoles and frogs [Table 1] expressed on a cellular basis clearly demonstrates the lower amount of mRNA in frogs compared to that in tadpoles. Cell death accounts for much of the weight decrease in the pancreas at metamorphosis (Frieden and Just 1970; Dodd and Dodd 1976). Therefore at least two factors influence trypsin mRNA levels: cell death and decreased amounts of mRNA per cell. From the data in Figure 5 and Table 1, we conclude that the difference in the trypsin mRNA level between stage 56 tadpoles and small frogs (Fig. 5) is mostly due to the change in the level of mRNA per pancreatic cell [Table 1], and the remaining minor difference is caused by a drop in the number of pancreatic cells from a tadpole to a small frog.

Conclusion

One of our primary interests is to catalog genes that respond to thyroxine and order them in a cascade of interacting gene products. The trypsin gene is a late-responding gene and, therefore, is probably indirectly regulated by thyroxine. For this reason, we do not expect its cis-acting regulatory region to have thyroid hormone receptor-binding elements. We predict that thyroxine directly induces one or more genes in the tadpole pancreas, whose products, in turn, activate or repress genes like trypsin that characterize the exocrine function of the pancreas.

Materials and methods

Treatment of *X. laevis* tadpoles

Thyroid hormones T₄ and T₃, obtained from Sigma, were dissolved in 4 mM NaOH at a concentration of 0.1 mM. Prior to use, the hormones were diluted into the tadpole's water at a final concentration of 26 nM for T₄ and 1–5 nM for T₃ (Morris 1987), with daily changes of the solutions. Control tadpoles were treated identically except that an equal volume of 4 mM NaOH without hormone was added.

Isolation of RNA

RNA from whole tadpoles was isolated by using the guanidinium thiocyanate method described by Chirgwin et al. (1979). Briefly, tadpoles were homogenized in guanidinium thiocyanate solution, which was then overlaid on top of a CsCl solution. The RNA was precipitated by ultracentrifugation. The RNA pellet was dissolved in 10 mM Tris-HCl, 1 mM EDTA (pH 7.5) [1 x TE], extracted with phenol/chloroform, collected by ethanol precipitation, redissolved in 1 x TE, and stored at -70°C. Poly(A)⁺ RNA was isolated from total RNA by selection on oligo(dT)-cellulose (Pharmacia). RNA from individual tissues was isolated by using the method of Chomczynski and Sacchi (1987).

Differential screening

A ZAP II cDNA library constructed from mRNA of stage 49 tadpoles that had been treated for 1 day with T₃ (gift of Y. Yaoita) was used in the screening. Roughly 1000–2000 clones were plated on each of ten 150-mm plates. Duplicate filters Table 1. Trypsin mRNA level in *Xenopus* pancreas

| Stage | Small frog | Adult frog |
|-------|------------|------------|
| 56    | 46 ± 22    | 21 ± 11    | 2.9 ± 0.7  | 0.8 ± 0.1 |

Total pancreatic RNA and DNA were isolated together (Andrews and Brown 1987). The mRNA level was quantified by using known amounts of cloned cDNA as a standard, and the cell number was determined by measuring the DNA content of extracts using Hoechst 33258 fluorescence enhancement assay (Labarca and Paigen 1980), assuming 6 pg DNA per cell. Two animals were used for each stage (only one was used for the adult frog), and two measurements were performed for each pancreas sample.
were lifted from each plate. One set of the filters was hybridized with a \( ^{32}P \)-labeled cDNA probe synthesized from mRNA isolated from stage 44 tadpoles, and the other set was hybridized with a \( ^{32}P \)-labeled cDNA probe generated from mRNA isolated from stage 49 tadpoles after 1 day of \( T_3 \) treatment. Hybridization was performed overnight in Southern hybridization buffer plus 10% dextran sulfate (Sigma). The filters were then washed under stringent conditions (three times in 0.1 x SSC, 0.1% SDS, at 65°C for 30 min). Unless otherwise indicated, all hybridized filters or membranes in this study were washed two or three times under these conditions. Clones that gave different hybridization signals with the two probes were picked and subjected to a secondary screening with the same probes. From ~20,000 clones, 66 were selected and 8 were chosen after secondary screening. Two cDNA probes were also prepared from mRNA isolated from stage 49–51 tadpoles with or without 1 day of thyroxine treatment and used in secondary screening. The intention was to detect early genes regulated directly by thyroid hormone. No such clones were found, suggesting that mRNA derived from this set of genes is rare. Southern blot hybridization buffer contains 50% formamide, 5 x Denhardt's solution, 5 x SSPE, 0.1% SDS, and 100 mg/ml denatured salmon sperm DNA (Maniatis et al. 1982). 1 x SSC: 150 mM NaCl, 30 mM sodium citrate (pH 7.0); 1 x SSPE: 150 mM NaCl, 1 mM EDTA, 20 mM sodium phosphate (pH 7.4); 1 x Denhardt's solution: 0.02% Ficoll, 0.02% polyvinylpyrrolidone, 0.02% BSA.

Tentative clones isolated above were subcloned by taking advantage of the in vivo excision property of the \( \lambda \)-ZAP II cloning system (Stratagene, La Jolla, CA), which conveniently excises out the plasmid pBluescript SK(-) with the cDNA inserts at the EcoRI site of the plasmid. The inserts from the tentative clones were then purified and used in Northern blotting analysis.

Another clone (PR28) served as an internal control. Its mRNA level is not regulated in a stage-dependent manner nor is its mRNA thyroxine dependent.

Sequence determination

The DNA sequence was determined from a series of nested deletion mutants by the dideoxynucleotide method using T7 DNA polymerase (Pharmacia). Each nucleotide was read at least once from each direction, and the final sequence was obtained by compiling sequence information from all of the deletion mutants.

Northern blotting analysis

This analysis was performed by use of a standard protocol (Ausubel et al. 1988). Typically 1–10 \( \mu \)g per lane of total RNA was electrophoresed on a 1.2% agarose/formaldehyde gel. After electrophoresis, RNA was transferred directly to a GeneScreen nylon membrane (Du Pont) in 20 x SSC and fixed onto the membrane by UV irradiation (Stratalinker, Stratagene, CA). The membrane was stained with methylene blue to check the quantity and the quality of the RNA (Herrin and Schmidt 1988). The membrane was then hybridized overnight in Northern hybridization buffer [10% dextran sulfate, 50% formamide, 25 mM KPO_4 (pH 7.4), 5 x SSC, 5 x Denhardt's solution, 0.1% SDS, 100 mg/ml denatured salmon sperm DNA] with DNA fragments labeled with \( ^{32}P \)dCTP by use of either nick-translation or a random priming method.

Genomic Southern hybridization

Genomic DNA isolated from homozygous diploid \( X. laevis \) blood (Krotoski et al. 1985) was used in Southern blotting analysis (Maniatis et al. 1982). About 2 \( \mu \)g per lane of the genomic DNA was digested to completion with appropriate restriction enzymes and electrophoresed on a 0.7% agarose gel. The DNA was transferred to either GeneScreen membrane or nitrocellulose filter (Schleicher & Schuell) and fixed with UV irradiation or by baking at 80°C in a vacuum oven. The membrane was then hybridized overnight with \( ^{32}P \)-labeled DNA probe in Southern hybridization buffer plus 10% dextran sulfate.

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