8-1996

The effect of thyroid hormone on the expression of A7 myosin heavy chain mRNA in xenopus laevis

Bradley Thomas Butkovich

ABSTRACT

THE EFFECT OF THYROID HORMONE ON THE EXPRESSION OF A7 MYOSIN HEAVY CHAIN mRNA IN XENOPUS LAEVIS

Bradley Thomas Butkovich

Master of Science in Biology

University of Richmond

1996

Dr. Gary P. Radice

It has long been known that thyroid hormone (TH) is responsible for metamorphosis in tadpoles. It is also known that A7 MHC, an adult muscle isoform, is expressed in adult *Xenopus laevis* frogs, but not in larvae. Furthermore, the appearance of A7 corresponds with the onset of metamorphosis. The present study investigated the relationship between TH and the expression of A7 MHC mRNA in *Xenopus laevis*. Isolated tails in culture and 6-n-propyl 2-thiouracil (PTU) treated tails were treated with TH and the presence of A7 mRNA analyzed with *in-situ* hybridization, and RT-PCR. It was found that TH caused substantial regression of tails. It was also found that A7 was expressed in tails treated with TH and was located primarily in a region rich in slow twitch fibers near the base of the tadpole tail. Finally, A7 is also expressed in PTU treated tadpoles, which suggests a novel mechanism is involved.

University of Richmond

Graduate School of Arts and Sciences

This is to certify that the thesis prepared by Bradley Thomas Butkovich entitled "The effect of thyroid hormone on the expression of A7 myosin heavy chain mRNA in *Xenopus laevis*" has been approved by his committee as satisfactory completion of the thesis requirement of Master of Science.

Gary P. Radice, Ph. D., Thesis Advisor

Francis B. Leftwich, Ph. D., Committee Member

(IN ABSENTIA)
Rafael O. de Sá, Ph. D., Committee Member

Examining Faculty

THE EFFECT OF THYROID HORMONE ON THE EXPRESSION OF A7 MYOSIN HEAVY CHAIN mRNA IN XENOPUS LAEVIS

By

BRADLEY THOMAS BUTKOVICH

University of Richmond, 1994

A Thesis

Submitted to the Graduate Faculty

of the University of Richmond

in Candidacy

for the degree of

MASTER OF SCIENCE

in

Biology

August, 1996

Richmond, Virginia

ACKNOWLEDGEMENTS

I would like to express my many thanks and gratitude to Dr. Gary Radice, he has to be the most patient man I have ever known. Through all of the ups and downs this research experience has brought me, he was only a phone call or a door away, ready to drop everything, such that I could ask his advice or just talk about life. Everything I know about my research I learned from him. I would also like to thank Dr. Frank Leftwich, who kept me going all six of my years here at the University of Richmond, and was always quick with a joke or a helping hand. I would also like to express my appreciation to the biology faculty, I think have taken at least one class from each of you, or have made an attempt to know you out of class. I would like to thank my fellow graduate students, especially Rachel Van Gilder, for all of their assistance and good humor.

My sincere and great appreciation is extended to my mother and father and to my two sisters, Malissa and Jennifer, whose support I could not have done without.

Finally, this thesis is for Debbie Jane Blades, who is always at my side, and always will be.

TABLE OF CONTENTS

LIST OF FIGURES

BACKGROUND

Overview of Amphibian Metamorphosis

The term metamorphosis is derived from the roots 'meta' meaning across and 'morph' meaning shape, thus it is defined as a "change in shape." In Anura, such as *Xenopus laevis*, it is the process which transforms the free-swimming, gilled, legless tadpole into an air breathing, four-limbed, sexually mature adult frog. Metamorphosis induces a cascade of interesting changes, both drastic and subtle. In fact, a staging series (Figure 1), can be followed which outlines the development of *Xenopus* from birth through adulthood (Nieuwkoop and Faber, 1975). It is relatively simple to note some of the visible anatomical changes, including the loss of aquatic features such as the tail and gills and the appearance of more terrestrial features like legs and lungs. However, at the cellular and molecular level, it becomes apparent that virtually every tissue and organ in the body is transformed.

Regressive changes include the tadpole's loss of internal gills, as well as the destruction of the tadpole's tail. At the same time, constructive processes, such as the completion of limb development and dermoid gland construction, are evident. The filter feeding apparatus notable in the young *Xenopus* disappears and the jaw and mouth take a new shape (Alley, 1989). The large intestine,

characteristic of herbivores, shortens to suit the carnivorous diet of adult frogs (Ishizuya-Oka and Shimozawa, 1992). The gills regress and the gill arches degenerate, and the lungs, which have already formed, begin to change greatly to adapt the frog to an air-breathing lifestyle. New connective tissues are formed in the mesenchyme which separates the dense collagen lamella from the basement membrane. These play an important role in the appearance of epidermal basal cells that are the progenitor cells of germinative basal cells of the adult skin (Kawai *et al.*, 1994). In turn, changes in the skin occur, such that the larval epidermis, which is bilayered and unkeratinized, begins to stratify and keratinize (Nieuwkoop and Faber, 1975; Reeves, 1977). The muscles reconfigure and switch fiber types, and cartilage begins to transform into bone (Gilbert and Frieden, 1981). The eyes undergo significant changes. In tadpoles, as in freshwater fishes, the major retinal photopigment porphyropsin, changes to rhodopsin, the characteristic photopigment of terrestrial and marine vertebrates (Gilbert, 1994). Furthermore, the middle ear develops as does the tympanic membrane characteristic of adult frogs (Duellman and Trueb, 1994). At metamorphosis, red blood cells switch production of tadpole hemoglobin to an adult form, which binds oxygen more slowly and releases it more quickly than the tadpole-specific form (McCutcheon, 1936; Broyles, 1981). Finally, tadpoles, like most freshwater fishes, excrete ammonia. Adult frogs obtain the ability to excrete

urea which is due to the emergence, during metamorphosis, of urea cycle enzymes in the liver (Cohen, 1970).

Hormonal Regulation of Amphibian Metamorphosis

Many of the changes seen in metamorphosis are thought to be brought about by the increased secretion of the hormones thyroxine (T_4) and triiodothyronine (T_3) from the thyroid gland (Figure 2). T_3 is the primary metabolite of T_4 and is believed to be the active hormone responsible for the induction of metamorphosis in tadpoles (Kistler *et al.*, 1977). In fact, evidence for the control of metamorphosis by thyroid hormones (TH) was originally discovered by Gudernatsch (1912), who found that tadpoles metamorphosed prematurely when fed powdered sheep thyroid gland. Allen (1916) found that when the thyroid gland was removed from tadpoles, the larvae never metamorphosed, becoming giant tadpoles instead. Subsequently, it was shown that tadpoles treated with anti-thyroid agents failed to undergo metamorphosis unless they were given appropriate amounts of TH (Allen, 1929; Weber, 1967). Also, when isolated tails were cultured independently and experimental tails treated with thyroid hormone, the experimental tails 'curled' and showed significant signs of regression. The control tails, without the addition of thyroid hormone, remained healthy and intact (Weber, 1964; Niki *et al.*, 1984).

Thyroid hormone does play an active role in metamorphosis, but other hormones, including prolactin (PRL), thyroid stimulating hormone (TSH), and even some glucocorticoids, also help to control metamorphosis. In young tadpoles, prior to metamorphosis, T_3 and T_4 are produced in small amounts, but their effects are inhibited by the secretion of a significant amount of prolactin from the anterior pituitary (Figure 3). In amphibians, prolactin acts as a larval growth hormone and inhibits metamorphosis (Bern *et al.*, 1967; Etkin and Gona, 1967). During premetamorphosis, the hypothalamic portion of the brain is under-developed, so there is little control of the anterior pituitary. In turn, with no hypothalamic regulation of the pituitary, there is no release of TSH from the anterior pituitary. Thus, T_3 levels remain low until further brain development occurs. As the hypothalamus develops and begins inducing the anterior pituitary to release TSH, thyroid hormone levels increase gradually until the first changes of metamorphosis, defined as prometamorphosis, occur (Etkin, 1968). At this point, the rising thyroid hormone concentrations cause a further increase in TSH, and consequently TH, via a positive feedback loop. In addition, development also results in the secretion of dopamine from the hypothalamus that inhibits the pituitary synthesis of prolactin (White and Nicoll, 1981). The reduced prolactin levels accompanied by the rapid increase in thyroid hormone concentration,

increase the ratio of TH to prolactin and metamorphic climax is reached, whereby full-scale metamorphic changes are induced (Dent, 1988).

Each tissue responds differently to the changing hormone ratio, and the basis of this tissue specificity is a matter of great curiosity. The response seems to rest with thyroid hormone receptor activity. There has been extensive research conducted on the thyroid hormone receptors (TRs) in mammals, yet the research on amphibian receptor function is still relatively young. It is known that the extent of metamorphosis in tadpoles is dependent on the TH concentration to which the tadpole is exposed (Kollross, 1961). This is best observed in thyroidectomized tadpoles treated with exogenous TH. At low concentrations of TH, only intestinal shortening and hindlimb growth occur. At high concentrations of TH, tail regression is seen before hindlimbs are even generated. Coupling this observation with the fact that levels of TH are the same throughout the tadpole (Leloup and Buscaglia, 1977), provides evidence for a model in which some other factor, such as TR concentration or TR affinity for TH, enables different tissues to have varying TH sensitivity. It has, in fact, been shown that the concentration of TRs vary in *Xenopus*, where high concentrations of TRs are seen in areas undergoing significant changes during metamorphosis such as the brain, spinal cord, intestinal epithelium, tail and hindlimb buds (Kawahara *et al.*, 1991). Furthermore, Galton (1992) has shown that the timing of metamorphic events are

regulated by the affinities of TRs for TH, such that hindlimbs have a higher affinity for TH and tail tissues have a lower affinity, explaining Kollross' observations. In light of all this, an autoinduction threshold model has been proposed, whereby the greater the TH concentration, the greater the increase in autoinduction of receptors and the more complete metamorphosis becomes for tissues with more receptors with higher affinities (Chatterjee and Tata, 1992). Furthermore, it is thought that some tissues might have a "head start" on other tissues based on the concept that they are prematurely exposed to 'maternal' TRs. In essence, It is thought that the initial TRs required for autoinduction in these tissues are derived from maternal messenger in the oocyte (Kawahara, *et al.,* 1991). TH binds to a small amount of the maternal TRs, which leads to activation of genes for more receptor and other genes with high affinity sites for the TH receptor. Higher concentrations of receptor then induce transcription from genes encoding adult-specific changes (Tata *et al.*, 1991). Eventually, metamorphosis in the other tissues catches up and the tadpole is completely transformed into a frog. Finally, Banker *et al.* (1991), Baker and Tata, (1992), Brooks, *et al.* (1989), Baniahmad, *et al.* (1990), have begun to define the role of the thyroid hormone receptor, known as *c-erb* A, in the metamorphic process of *Xenopus laevis*. The *c-erb* A proto-oncogene encodes the thyroid hormone receptor, and is a ligand-dependent transcription factor which plays an important role in vertebrate growth and

development. They have shown that substantial increases in the levels of *erb* A-alpha RNA were noted at stages well after the onset of zygotic transcription at the mid-blastula transition, with accumulation of *erb* A-alpha transcripts reaching maximum levels well in advance of metamorphosis. Also, the *erb* A-alpha RNA's are expressed unequally, with greater expression in the head than the body, across *Xenopus* neural tube embryos. This differential expression continues through later stages of development, including metamorphosis and suggests that these thyroid hormone receptors play roles in tissue-specific processes across all of *Xenopus* development. These observations coupled with other studies and conclusions reached so far show that TRs, in concert with prolactin and T_3, do indeed seem to play a vital role in metamorphosis in the tadpole.

Normal metamorphosis of *Xenopus* tadpoles is only initiated after developmental stage 54 (30-35 days after fertilization) when the tadpole thyroid gland begins to secrete thyroid hormones (Leloup and Buscaglia, 1977). Yet, it has been shown that bullfrog tadpoles acquire a response to exogenous thyroid hormone in the first week after fertilization (Moskatis *et al.*, 1989). This suggests that the receptor is expressed well before the target cells would be exposed to endogenous thyroid hormones. Furthermore, *in-situ* hybridization analysis confirmed the presence of TR mRNAs in early developmental stages of *Xenopus* tadpoles (Kawahara *et al.*, 1991). Within 1 week after fertilization (stage 44), TR

mRNAs were found predominantly located in the tadpole brain, spinal cord, intestinal epithelium, tail and liver. At mid-metamorphosis strong hybridization signals were also found in the hind limb buds.

With the cloning of *Xenopus* TR-α and TR-ß cDNAs it has been possible to establish how TR genes are expressed during development (Marsh, 1993). Results obtained by Kawahara *et al.* (1991), using RNAse protection assays, showed that TR-α and TR-ß transcripts accumulated rapidly with development, reaching their maximum levels by metamorphic climax (stages 58-62), then dropping to almost undetectable levels upon completion of metamorphosis. This pattern is compatible with the differential and rapidly increasing sensitivity of tadpole tissues to thyroid hormones as metamorphosis progresses, as well as the virtual absence of response of adult amphibia to the hormone, possibly due to the fact that receptor levels decline (Tata, 1968; Gilbert and Frieden, 1981). In addition to these findings, it has been shown that thyroid hormone has the capability to autoinduce the TR genes, such that the presence of TH induces the synthesis of the TR for thyroid hormone (Kawahara, *et al.*, 1991). Exposure of tadpoles at premetamorphic stages (48-52) to exogenous T_3 substantially increased the accumulation of TR mRNA, which could explain the increase in sensitivity of tadpoles to thyroid hormones at the onset of natural metamorphosis. Furthermore, experiments involving the urea cycle enzyme, ornithine

transcarbamoylase (OTC), which is only found in adult frogs, have established that TH, in the absence of any other hormonal influence, stimulates transcription of mRNAs for its own TR-ß and consequently OTC mRNAs during prometamorphosis (Helbing *et al.*, 1992). This provides further evidence suggesting TH's influence on expression of adult characteristics. It is also known that prolactin disappears very rapidly at the onset of metamorphosis, the kinetics of its disappearance exhibiting a close reciprocal relationship to the rapidly increasing appearance of thyroid hormones in blood (Duellman and Trueb, 1994). When prolactin was added to organ cultures of *Xenopus* limb buds and tails, it prevented both morphogenesis and cell death induced by T_3 in these two tissues (Kawahara, *et al.*, 1991). Baker and Tata (1992) were in fact able to show that exogenous prolactin administered to stage 50-54 *Xenopus* tadpoles or to tail organ cultures completely abolished the rapid T_3 induced autoinduction of TR-α and TR-ß mRNAs. One could conclude that prolactin inhibits the auto-induction of TR and thus prevents T_3 from effectively working or vice-versa. In essence, without the drop in prolactin, neither T_3 nor the TRs could induce metamorphosis.

A working model (Figure 4) has been proposed (Yaoita and Brown, 1990; Chatterjee and Tata, 1992; Marsh, 1993; Tata, 1993) to explain the significance of the events surrounding autoinduction of TRs, T_3 increase, and prolactin

inhibition and decline. At the onset of prometamorphosis, trace amounts of thyroid hormone bind to the small amount of TR, probably derived from maternal messenger RNA in the oocyte (Kawahara, *et al.*, 1991), leading to upregulation of its own receptor and other early response genes with high-affinity sites for the receptor. The combined effect of rapidly increasing amounts of circulating thyroid hormone and release from the inhibition of autoinduction due to loss of prolactin in blood would lead to higher concentrations of receptor. Transcription from 'late' genes is then induced, encoding adult-specific proteins. Specifically, one would see high levels of TR-α and TR-ß during metamorphic climax and induction of preprogrammed genes (in our case, myosin heavy chains) to produce tissue specific phenotypic changes.

Hormonal Regulation of Muscle-Specific Genes

The tissue specific changes in which we are most interested are those dealing with the myosin heavy chains in skeletal muscle of the *Xenopus* tadpole and the switch that occurs from the embryonic isoforms, E3 and E19, to the adult isoform A7. Previously, E3 and E19 were isolated from a cDNA library from young (stage 37) *Xenopus* tadpoles while A7 was isolated from a separate cDNA library of adult *Xenopus* leg muscle (Radice and Malacinski, 1989).

It has been shown that both E3 and E19 are initially expressed in late gastrulation and accumulate through the beginning of metamorphosis in both trunk and tail muscle. During metamorphosis these transcripts decline in both the degenerating tail and in the growing hind limbs and are not present in adult skeletal muscle. In contrast, A7 is not expressed in embryos and does not appear until early metamorphosis when it can be detected in developing limbs and in the degenerating tail (Radice and Malacinski, 1989; Radice, 1995). Research on the control of myosin heavy chains (MHCs) in amphibians is still a relatively young field. However, the evidence that TH regulates MHC expression, growth and differentiation has been extensively studied in rats, mice, rabbits, chickens and even humans. In mammals, cardiac-specific myosin heavy chains are known to be regulated by TH. There are two cardiac isoforms: ß-MHC is normally expressed predominantly in young animals and is replaced by the α–MHC isoform as the animal ages and TH levels increase. Knowing this, Lin *et al.* (1989), first induced rabbits to fully express embryonic ß-MHC via a diet supplemented with 6-n-propyl 2-thiouracil (PTU), which is a thyroid hormone blocker. The rabbits were then given varying levels of TH with the use of an osmotic pump. It was found that the exogenous thyroid hormone induced expression of adult α–MHCs. In support of this, Franklyn *et al* . (1989) and Dillmann *et al.* (1989) also found significant upregulation of α–MHC in rat cardiac

muscle with an increase in TH. Furthermore, it has been shown that there is a substantial increase in myocardial β-MHC mRNA and decrease in α–MHC mRNA in hypothyroid rats (Green *et al.*, 1989). When these rats were given TH supplements, a dose-dependent increase in α–mRNA was evident within 6 hours after administration of TH. Similarly, a decrease in β-mRNA was observed 72 hours after TH administration. Winegrad *et al.* (1990) showed that MHCs are primarily α–isoforms in rats when induced by TH, and these patterns of expression are consistent with those seen in MHCs during cardiac growth.

Further evidence of transformation of embryonic muscle into adult analogs via TH induction has been obtained in other species. Van Horn *et al.* (1989) found that MHCs in chickens changed from embryonic isoforms *in-ovo*, to adult isoforms, once nerves were established in correlation with TH secretion. Substantial growth (more proteins per muscle cell) of adult muscles was seen in human fetal quadricep muscle, when embryonic developmental isozymes were replaced, immediately after birth, by adult isozymes. Specifically, it was observed that excessive amounts ($>10^{-8}$ M) of TH act directly on the muscle and result in a precocious accumulation of adult MHCs and maturation of muscle (Butler-Browne *et al.*, 1990).

Connections between increased TH, growth, and adult α–cardiac MHC expression are well substantiated. Interest now centers on the mechanism of TH

regulation of muscle growth. A commonly accepted model is that TH binds to TR, and the complex enters the nucleus where it binds to DNA and interacts with specific regulatory sequences called thyroid response elements (TREs). Gustafson *et al.* (1987) have observed that TH directly regulates expression of an α-MHC gene which was transfected into fetal heart cells, causing production of adult myosin in a region that is otherwise devoid of adult myosin until later in development. Morkin *et al.* (1991) provide further insight into TH mediated molecular regulation of cardiac MHC gene expression via a study of cultured fetal rat heart cells. They observed that thyroid hormone-receptor complexes interacted with certain upstream elements, most notably TREs. These TREs in turn, conferred TH responsiveness to gene promoters. The promoters were, in turn, shown to be linked to the downstream expression of the α–MHC genes. Conversely, TREs have been identified in relationships with a suppressor element which is located immediately upstream to the β–MHC gene. In essence, TREs alliance with either suppresser or promoter elements causes inhibition or expression of MHC genes, respectively. This scenario strongly supports observations related to the decline of ß-MHC mRNA and an increase of α–MHC mRNA when cells are exposed to TH. In addition, Flink and Morkin (1990) have observed an interaction of TREs with *cis*-acting elements in the human α–MHC gene promoter. This suggests that the TREs are not solely responsible for

conferring promotion or inhibition gene expression, but that they act in concert with other "enhancer" or "restrictive" elements located adjacent to TRE regions. Finally, Downes *et al.* (1993) have shown that TREs are present in the mouse myogenin gene (which expresses MHC), where expression is thought to be controlled by TH. In essence, TH plays a major role in developmental processes by regulating MHC expression and growth in a variety of organisms besides amphibians through the unified interaction of receptors, promoters, TREs, and *cis*-elements.

Evidence of TH action on gene expression in amphibians has been observed by Ray and Dent (1986), who reported that newly synthesized adult MHC proteins appeared in bullfrog tadpoles treated with TH. Also, changes in protein synthesis and composition in the liver of *Xenopus* larvae with manipulation of TH in hypothyroid and hyperthyroid animals have been observed (May and Knowland, 1981). Some of the most significant evidence for TH effects on MHCs are presented by Chanoine *et al.* (1987). Their results show that, in salamanders (Urodeleans), a moderate increase in the level of TH is sufficient to induce the differentiation of skeletal muscle as assessed by changes in the myofibrillar ATPase profile and a transition of myosin isoforms from embryonic to adult types. Furthermore, it was found that the regulatory activity of TH during

amphibian metamorphosis was comparable to that described in the mammalian postnatal period (d'Albis *et al.*, 1987).

While studies have not been conducted to evaluate whether TH regulates MHCs in *Xenopus*, there have been extensive studies focusing on keratin gene expression and TH activity. In much the same way as adult isoform expression changes in muscles, 63-kDa keratin gene expression appears in the larval epidermis and accumulates during metamorphosis to become the most abundant of proteins in adult epidermis (Ellison *et al.*, 1985; Sargent *et al.*, 1986). It has been observed that although TH is not required for the initial activation of the 63-kDa keratin genes, high-level expression of keratin mRNA (Mathisen and Miller, 1987) and protein (Nishikawa *et al.*, 1992) is absolutely dependent on TH and is prevented by PTU treatment (which prevents the thyroid from secreting TH).

All in all, there is strong evidence from other species to support the hypothesis that the embryonic to adult transition of MHC gene expression in *Xenopus* is regulated either directly or indirectly by changing levels of TH. A working model explaining this scenario suggest that low levels of TH and TRs are required for expression of embryonic isoforms E3 and E19, but are insufficient to express the adult isoform A7. In contrast, high levels of TH and TR turn on A7 expression and perhaps turn off E3 and E19 expression. In adults, the A7 expression is then maintained in the absence of TH by other mechanisms. In light

of this, a strong foundation has been established to justify research regarding the effects that TH has on MHCs and their expression in *Xenopus laevis* during metamorphosis.

INTRODUCTION

One of the notable cellular changes during amphibian metamorphosis is the transformation of skeletal muscle fiber type, in which embryonic muscle cells transform into adult muscle cells (Chanoine *et al.*, 1987; Diffee *et al.*, 1991). This change in muscle fiber type is probably caused by a change in expression of muscle-specific genes. For example, in *Xenopus*, embryonic myosin heavy chain genes E3 and E19 turn off transcription during metamorphosis and adult myosin gene A7 turns on (Radice and Malacinski, 1989).

The mechanisms that turn off embryonic genes and turn on adult specific genes in *Xenopus* are unknown. However, several different explanations are possible. For example, changes in hormone concentrations (Regard *et al.*, 1978; Galton and St. Germain, 1985), innervation of muscle cells (Salmons and Sreter, 1976; Gauthier, 1987), or a biological clock that measures age of development (Erickson, 1993) all have been proposed as possible signals that cause changes in expression of these myosin genes and other genes controlling metamorphosis (Grove, 1989; Galton, 1992). There is little direct experiment-al evidence in amphibians to indicate whether one or all of these explanations are necessary for the transformation of muscle from embryonic to adult form (Lompre *et al.*, 1991).

This study examines the mechanism by which and adult specific

myosin gene is turned on with the onset of metamorphosis. Specifically, the aim is to determine whether TH levels regulate expression of the adult myosin heavy chain gene A7 (A7 MHC), as the tadpole undergoes metamorphosis.

There are several lines of evidence that support TH-induced metamorphosis in the frog. First, it is known that as the tadpole grows the hypothalamus and pituitary mature such that they begin to secrete hormones and respond to various factors and signals in the blood stream. This awakening is indirectly responsible for thyroid hormone production later in development and directly correlates with impending metamorphic changes (Etkin, 1968). Second, studies have demonstrated that premetamorphic tadpoles (< stage 40) given exogenous TH, metamorphosed prematurely (Gudernatsch, 1912). Third, TH-deficient tadpoles and cultured tails, denied exogenous TH, remained as healthy and intact tadpoles (Allen, 1929; Weber, 1964; Niki *et al.*, 1984). Finally, hypothyroid or thyroidectomized tadpoles and isolated tails and leg buds in culture, given TH, metamorphosed (Tata *et al.*, 1991; McCormick, 1993).

Since A7 MHC is turned on at the peak of metamorphosis (Radice and Malacinski, 1989) and that TH plays such a major role in the transformation of the tadpole into a frog (Galton and Hiebert, 1988), this leads one to hypothesize that TH must regulate MHC expression as well, either indirectly or directly. There is evidence that TH is a key factor in the regulation of muscle gene expression, and

experiments with rats have shown that the normal appearance and expression of MHCs in leg muscles is dependent on innervation of fetal muscles at a particular stage in development (Harris *et al.*, 1989). It is thought that the switch in MHC expression depends on innervation, yet innervation is, in fact, triggered by the rising TH concentration (Ericksen, 1993). In chickens, embryonic myosins expressed *in-ovo* are replaced by adult myosins once the chicken has hatched (Crow, 1987). In other vertebrates (rats) the switch from embryonic myosin protein is controlled by TH concentration. It is most prominent in the heart muscle, where embryonic β–cardiac MHCs regress and adult α–cardiac MHCs are expressed (Green *et al.*, 1989). Evidence for this in amphibians is provided by Chanoine *et al.* (1987). They have shown that in Urodeleans, a moderate increase in the level of TH is sufficient to induce the differentiation of skeletal muscle as revealed by the loss of embryonic MHC proteins and the appearance of adult isoforms.

Finally, in light of TH's effect on metamorphosis and skeletal muscle differentiation, it is possible that TH is acting directly on MHC gene expression. Evidence for this can be seen in mammals where DNA binding properties associated with nuclear thyroid hormone receptors reflect a mechanism by which T_3 binds to receptors and the receptor/T_3 complex binds to MHC genes (Gustafson *et al.*, 1987; Morkin *et al.*, 1989; Flink and Morkin, 1990; Morkin *et al.*,

1991) have observed an interaction of thyroid response elements (TREs) with *cis* -acting elements in the human α–MHC gene promoter. In amphibians, work with keratin genes and TH expression have found that high-level expression of the 63-kDa keratin is absolutely dependent on TH (Mathisen and Miller, 1987). It is possible that mechanisms for TH induced control of MHCs are essentially similar.

In summary, there is an abundance of evidence indicating the role that TH plays in development and amphibian metamorphosis. Furthermore, it has been demonstrated that genes encoding embryonic myosins (E3 and E19) turn off and adult myosins (A7) turn on at the onset of metamorphosis and studies on growth and development in other animals support the demonstrated effect that TH has on adult myosin expression. However, it has never been shown whether TH influences A7 myosin expression in *Xenopus laevis*. **In an attempt to test the hypothesis that thyroid hormone regulates A7 expression during metamorphosis in Xenopus laevis,** the following experiments were carried out.

1) Whole live freely swimming tadpoles were treated with PTU (an exogenous T_3 inhibitor), PTU/T_3 or remained untreated (controls). After treatment, the tadpoles were assayed for A7 mRNA activity to determine any inhibitory or stimulatory effects, or lack thereof, T_3 had on A7 myosin expression.

2) Isolated tadpole tails were manipulated in organ culture; tails were treated with T_3 or remained untreated (controls). After treatment, the tails were

assayed for A7 mRNA activity to determine whether TH stimulated A7 myosin expression.

3) A7 mRNA accumulation was assayed using *in-situ* hybridization and Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR).

MATERIALS AND METHODS

Animals-Stage 42-50 (Nieuwkoop and Faber, 1975) *Xenopus laevis* tadpoles and tails were used in all experiments. All animals were raised from birth in a container of 10% Steinberg's solution (60 mM NaCl, 0.67 mM KCl, 0.34 mM $Ca(NO_3)_2$, 0.83 mM $MgSO_4$, 10 mM HEPES, pH 7.4). The animals were obtained by injecting adult female frogs with 0.5 ml (2,000 IU) of human chorionic gonadotrophin (Sigma). The frogs were then allowed to spawn overnight. Male frogs were sacrificed and the testes were subsequently removed and the sperm was used to fertilize eggs squeezed from the females. The embryos were then allowed to mature. Tadpoles were fed Gerber's creamed spinach. Periodically, the water was changed and dead embryos were removed.

Tail Cultures-40 tadpoles were taken from their tank and dipped briefly (7-10 seconds) in 0.01% potassium permanganate solution to effectively kill any epidermal bacteria. All further manipulations of tadpoles were conducted under a sterile hood. The tadpoles were allowed to swim in sterile 10% Steinberg's containing antibiotics and antimycotics (100 IU/ml penicillin, 0.1 mg/ml streptomycin, 0.2 μg/ml Amphotericin B, 0.9% NaCl) (Sigma), over a period of 24 hours. After 24 hours the tadpoles were transferred to petri dishes containing

10% sterile Steinberg's and antibiotics. The tadpoles were over-anesthetized with 0.1% 3-aminobenzoic acid ethyl-esterase (Sigma) and the tails were removed using a sterile scalpel. The tails were then placed into another petri dish containing 0.01% potassium permangate solution in 10% sterile Steinberg's for 10 minutes. Finally, the tails were rinsed in 10% Steinberg's and antibiotics and transferred into petri dishes containing 15 ml of sterile filtered 60% L-15 Amphibian Culture Medium (Gibco) supplemented with 0.3 IU/ml of insulin (enabling cells to utilize glucose from culture). Four petri dishes were set up with ten tails per dish, one dish served as a control. The control tails were allowed to grow in the culture medium for 14 days, with medium changed every 3 days. The experimental tails received $1x10^{-8}$ M T_3 over a period of 14 days. The medium and T_3 was replaced every 3 days. The growth and regression of the tails was measured using a Hewlett-Packard scanner and NIH Image 1.54 imaging software on an Apple Quadra 700. Initial measurements were taken at day zero and follow-up measurements taken approximately every 3 days through day 14.

Beginning on day 9, tails were removed daily from control and experimental cultures, preserved in formaldehyde and stored in 100% Methanol for *in-situ* hybridization or quick frozen in liquid nitrogen and stored at -70^{o}C for RT-PCR.

PTU Inhibition of Thyroid Hormone Secretion-60 tadpoles at stage 46 were removed from the growth tank. Six tanks were set up, each containing 10 tadpoles in 1 liter of 10% Steinberg's. 2 tanks served as controls. The experimental animals received 0.01% 6-n-propyl-2-thiouracil (PTU) in dimethyl sulfoxide (DMSO) in their water, while the controls received only DMSO. Once the controls had completely metamorphosed (stage 66), half of the PTU-treated tadpoles were given $1x10^{-8}$ M T_3 to simulate endogenous T_3 action that was inhibited by the PTU. The T_3 was replenished every 3 days for one week and all animals were then euthanized and assayed. Once the controls had completely lost their tails, the tadpoles were considered metamorphosed and were either fixed in formaldehyde and stored in 100% methanol or quick frozen in liquid nitrogen and stored at -70°C for *in-situ* hybridization and RT-PCR, respectively. The tadpoles were fed weekly with Gerber's creamed spinach suspension in water. The water was changed weekly and the PTU in DMSO replenished.

A7 Hybridization Probe-The probe was constructed as described by Radice and Malacinski (1989). The A7 probe was labeled with anti-digoxigen for *in-situ* hybridization.

In-situ Hybridization-Modified from methods as described by Harland (1991). Each tail (after fixation in formaldehyde and storage in 100% methanol at -20°C) was assayed for A7 activity using the following *in-situ* protocol: Tissue

was rehydrated in successive 5 min incubations in methanol:water 75:25, 50:50, then methanol:0.1M phosphate buffer containing 0.01% Tween (PTw) 25:75, followed by three washes of PTw. Tails were then treated with Protease K (1 µl of 10 µg/ml in 1 ml PTw, 30 min at room temperature (RT) to allow for proper probe penetration into the cells. Tissues were then rinsed 2X 5 min each in 0.1 M triethanolamine (TEOLA) at pH 7-8, then treated with 12.5 µl acetic anhydride in 5 ml TEOLA, 5 min. After 5 min another 12.5 µl of acetic anhydride was added to the same solution and incubated 5 min more. tissues were then washed twice for 5 min each in PTw.

After postfixation in 4% paraformaldehyde in PTw for 20 min, tissues were washed 5X, 5 min each in PTw, and pre-hybridized in hybridization buffer (50% formamide, 5X SSPE, 1mg/ml *Torula* RNA, 100ug/ml Heparin, 1X Denhart's, 0.1% Tween 20, 0.1% CHAPS, 5 mM EDTA) without probe at 60°C for 3-6 hr. Tissues were hybridized with fresh hybridization buffer containing 1 µg/ml of probe (usually 10 µl of a probe synthesis that results in 0.1µg/ml in 100 µl). Hybridization proceeded overnight at 60°C.

Tissues were then washed in hybridization solution without probe, 30 min at 60°C., then a mix of hybridization solution and 2X SSPE 0.3% CHAPS for 10 min at 60°C, then 3 times in 2X SSPE/CHAPS, 10min each at 37°C, and 2X SSPE/CHAPS plus RNAse A at 20 µg/ml, 30 min at 37°C. The final washes

were 2 times in 2X SSPE/CHAPS, 10 min each at RT, 2 times in 0.2X SSPE/CHAPS, 10 min at 60°C. Tissues were then rinsed in PTw 2X, 10 min and stored for antibody incubation.

For antibody incubation and detection, tissues were incubated in phosphate buffered saline containing 20% lamb serum for 1 hr to block non-specific binding, then incubated with a 1:2000 dilution of alkaline phosphatase linked anti-digoxigen antibody (Boehringer Mannheim) and incubated overnight at 4°C. After washing at least 5X, 1 hr each in PBT at RT, the antibody was detected using the protocols and color reagents of the Genius™ detection system (Boehringer Mannheim). After the color developed, tissues were fixed in 4% formaldehyde, dehydrated in graded methanol and cleared in benzyl benzoate/benzyl alcohol (BB/BA) 2:1. Following the *in-situ* hybridization, the tails were analyzed under a dissecting scope and photographed.

RNA Isolation-Tadpole tails (quick frozen in liquid nitrogen and stored at -70°C) were ground up using a mortar and pestle (autoclaved and chilled at -20°C) and then homogenized and RNA was isolated by acid guanidinium thiocyanate-phenol-chloroform extraction as described by Chomczynski and Sacchi (1987). The resulting RNA was then analyzed at 260 nm and 280 nm to determine a 260 nm/280 nm ratio for purity and concentration. This was done using a DU-65 spectrometer.

Reverse Transcriptase-Polymerase Chain Reaction-RT-PCR was performed on mRNA samples extracted from treated tadpole tails and legs to determine the presence or absence of A7 activity. Using OLIGO, a computer based primer construction program, two sets of primers (Genosys Biotechnologies, Inc.) were constructed based on the A7 specific region of the 1.5kB cDNA MHC A7 template (Figure 5). The first primer set (primer set X) consisted of a 3' anti-sense 20-mer primer with a sequence of 5'-CGCGCAAGGAGTCGAAGGAG-3' and a 5' sense 16-mer primer with a sequence of 5'-GACGTCCTGGACCACC-3'. It produced a product length of 140 bp in the presence of A7. The second primer set (primer set Y) consisted of a 3' anti-sense 17-mer primer with a sequence of 5'-GTTATAGTCGCGCAAGG-3' and a 5' sense 20-mer primer with a sequence of 5'-GATGTTCTCCGTCTGTCTTC-3'. It produced a product length of 107 bp in the presence of A7.

RT-PCR was carried out using a kit supplied by Promega and a Perkin-Elmer Cetus thermocycler. The production of a DNA template from A7 mRNA and the subsequent replication of that DNA template were carried out via the following protocol modified from methods as described by Kawasaki (1990):

STEP 1-To 0.65 ml PCR tubes the following reagents were added: 7.5 μl DEPC water, 2 μl RNA (1μg/μl), and 1 μl (1.0 μg) 3' primer. These tubes were

placed in a thermocycler at 70°C for 10 minutes to equilibrate the nucleic acid, followed by a 4°C soak for 10 minutes.

STEP 2-During the 4°C incubation, the following reagents were added to initiate the RT reaction (final concentrations in 20 μl are in parentheses): 4μl of 25mM $MgCl_2$ (5mM); 10X reverse transcription buffer (1X= 10mM Tris HCL (pH 8.8 at 25°C), 50mM KCl, and 0.1% Triton X-100); 2 μl 10 mM dNTP mixture (1 mM each dNTP); 0.5 μl rRNasin ribonuclease inhibitor (1 IU/μl); and 1 μl AMV reverse transcriptase (15 IU/μg). The tubes were then incubated at 42°C for 30 minutes to allow for cDNA template production from mRNA, then 94°C for 5 minutes to halt the reaction, and finally 4°C for 10 minutes.

STEP 3-During the 4°C chill the following reagents were combined to initiate the PCR reaction (final concentrations in 100 μl are in parentheses): 20 μl of first strand cDNA reaction (<10 ng/μl); cDNA reaction dNTP's (200 μM); 4 μl of 25mM $MgCl_2$ (2mM); 8μl of 10X reverse transcriptase buffer (1X=10 mM Tris-HCl (pH 8.8 at 25°C) 50 mM KCl, and 0.1% Triton X-100); 1 μl 5' primer (1.0 μg); 0.5 μl AMPLITAQ DNA polymerase; 66.5 μl of DEPC treated water. One drop of mineral oil was added to each tube to prevent evaporation of sample. The final volume was taken though 35 cycles of 94°C X 30 seconds; 55.6°C X 30 seconds for primer set X or 53.1°C X 30 seconds for primer set Y; and finally, 72°C for 30

seconds. After 35 cycles the samples were held at a 4^{o}C soak cycle, then stored at -20^{o}C and subsequently analyzed on a gel.

Following the RT-PCR reaction, a 1.5% agarose gel was prepared using 0.75g agarose in 50 ml of 5X TBE buffer. The samples were loaded onto the gel and electrophoresed at 80 volts for 90 minutes in 1X TBE buffer. The gel was stained with ethidium bromide and washed as described by Sambrook *et al.* (1989). The gel was photographed using a Polaroid camera.

RESULTS

Thyroid Hormone Induces Metamorphosis in Cultured Tails-It is well established that isolated tails (Tata *et al.*, 1991; Weber, 1964; Niki *et al.*, 1984) and tail explants (Ray and Dent, 1986) maintained in tissue culture undergo metamorphic resorption when exposed to thyroid hormone. A significant decrease in length and resorption of stage 42 and stage 48-50 cultured tails exposed to 10^{-8} M T_3 was also seen in our experiments. The data shown is based on the average length of all the tails either treated with T_3 or not (controls). The standard error is based on 95% confidence. In experiment 1 (Figure 6a), stage 42 tadpole tails treated with T_3 were seen to have 35.1% resorption by day 7, where control (nontreated) tails showed only 7.1% resorption. By day 12, T_3-treated tails showed resorption of 61.9%, where controls showed resorption of 11.4%. On the final day of the experiment (day 14), the T_3-treated tails showed resorption of 71.8% and the controls showed resorption of 32.8% (which in the controls, seems to be attributed more to necrosis than resorption, due to the fact that by day 14 the culture is no longer able to sufficiently sustain the tails). In experiment 2 (Figure 6b), stage 48 tadpole tails treated with T_3 were seen to have 21.5% resorption by day 7, whereas controls showed only 5.0% resorption. By day 12, T_3-treated tails showed resorption of 42.0%, where controls showed

resorption of 16.0%. On day 14 of the experiment, the T_3-treated tails showed resorption of 47.0% and the controls showed resorption of 16.3%. In experiment 3 (Figure 6c), stage 50 tadpole tails treated with T_3 were seen to have 25.4% resorption by day 7, where controls showed resorption of 2.0%. By day 12, T_3-treated tails showed resorption of 30.5% and controls showed resorption of only 7.4%. There were no measurements taken on day 14. Overall, one can see the effect that TH has on tail resorption. At day one in the cultures, both the experimental and control tails were approximately the same size (Figure 7 a, b). By day 7, substantial regression in the tails treated with TH is observed. The experimental tails begin to show darkening on the ventral surface, and tend to curl (Figure 7 c, d). By days 12-14 the T_3 tails are shriveled and have lost a great deal of their muscle mass. The controls on days 12-14 were still quite healthy and, for the most part, were comparable to their condition on day 0 with some minor regression, if any (Figure 7 e, f).

PTU Inhibits Metamorphosis of Whole Tadpoles-PTU is a thyroid hormone inhibitor that halts TH production by inhibiting the thyroid organic binding and coupling reactions, as well as the conversion of T_4 to T_3 in the peripheral tissues. As a result of this, tadpoles treated with PTU tend to develop large thyroid goiters due to the constant stimulation and growth of the follicle cells by thyroid stimulating hormone (TSH) (Ingbar, 1985). In essence, it becomes a

futile attempt by the thyroid follicle cells to produce TH and satisfy the negative feedback loop with the anterior pituitary. As a result, with TH adequately blocked in the organism, one is allowed to manipulate TH levels exogenously. Previous research has shown that PTU inhibits metamorphosis in the tadpole while allowing growth (Allen, 1929; Weber, 1967). The current research replicates previous findings by demonstrating that PTU does significantly inhibit metamorphosis in tadpoles (Figure 8). As stated previously, growth is still seen in PTU treated/TH deficient unmetamorphosed tadpoles. In fact, growth is so marked that severe hypertrophy of the dorsal and ventral muscles in the tail is observed. The tadpole becomes grossly disproportionate and oversized as compared to younger tadpoles and those animals allowed to metamorphose.

Exogenous supplements of T_3 were given to PTU-treated animals once the controls had completely metamorphosed (stage 66). It was observed that an exogenous supplement of 10^{-8} M T_3, simulating the amount of T_3 present at metamorphosis in the normal growing tadpole, caused extremely rapid and significant metamorphosis in the PTU treated tadpole. Within seven days, the tadpole had acquired full hind legs and forelimbs prior to regression of the tail. The tail begins to show darkening both dorsally and ventrally and curling occurs similar to that seen in isolated tails in culture, treated with T_3 (Figure 9).

Prometamorphic Tail Muscle does not Synthesize Adult MHC A7- Based on *in-situ* analysis of cultured control tails not treated with T_3, it can be observed that prometamorphic tail muscle shows no hybridization for the MHC A7 mRNA signal (Figure 10). Furthermore, RT-PCR analysis of isolated mRNA from cultured control tails, not treated with T_3, shows no A7 cDNA replicated transcript (Figure 11). This corresponds with results seen regarding *in-situ* analysis.

Exogenous T_3 Induces Expression of A7 MHC in Cultured Tails and Whole Tadpoles- Based on *in-situ* analysis of cultured tails, it can be seen that the addition of exogenous T_3 induces expression of A7, such that ventral somites stain for the A7 mRNA message (Figure 12). PTU treated/ T_3 inhibited whole tadpoles, supplemented with 10^{-8} M T_3, simulating the amount of T_3 present at metamorphosis in the normal growing tadpole, showed A7 mRNA message both in ventral and dorsal somites (Figure 13). RT-PCR of mRNA isolated from T_3 tails in culture and PTU/T_3 treated whole tadpoles shows a positive band for A7 cDNA replicated transcript, corresponding with results observed in the *in-situ* analysis.

Characterization of A7 Expression in Metamorphosing Tadpoles- Although characterization of other myosins, specifically the embryonic myosins, E3 and E19, has been done in tadpoles at earlier stages (Radice and Malacinski,

1989; Radice, 1995), A7 has never been characterized in metamorphosing tadpoles. These experiments show that A7 expression seems to reside in the periphery of the ventral and dorsal somites near the base of the tail, but not in the tip (Figure 13). The regions in which A7 is expressed in the metamorphosing tadpole tail correspond with regions consisting of slow twitch fibers.

Although PTU Inhibits Metamorphosis, It does not Inhibit A7 MHC Expression-In PTU-treated whole tadpoles, *in-situ* analysis shows A7 expression not only in those animals treated with T_3, but in the animals treated with PTU only (Figure 14). As in the T_3 treated/PTU animals, expression of A7 was observed in the periphery of the ventral and dorsal somites at the base of the tail, while the tip shows no A7 expression. Furthermore, RT-PCR of mRNA isolated from PTU treated tadpoles shows a band for A7 cDNA replicated transcript. This suggests that A7 is indeed present in the PTU treated tadpoles despite the absence of exogenous T_3 and signs of metamorphosis.

DISCUSSION

The results described in this research provide insight into the T_3-mediated expression of A7 in the metamorphosing tail of the *Xenopus laevis* tadpole. This study had four main goals, of which the first was to establish a reliable culturing system for isolated tails. Secondly, the effects that T_3 and PTU had on metamorphosis were to be observed. Thirdly, the expression of A7 during metamorphosis was to be characterized. Finally, the effects on expression of A7 when manipulated with T_3 and PTU were to be evaluated.

A System to Regulate Culture of Tadpole Tails-As others have shown (Tata *et al.*, 1991; Weber, 1967; Derby and Etkin, 1968), the benefits of using a system utilizing the culturing of tails *in-vitro* are numerous. With the use of this culturing system, it is possible to isolate tail tissue from any and all of the endogenous effects and substances that might be encountered in whole tadpoles and manipulate desired treatments exogenously. In essence, we have a model system, in which tails cultured *in-vitro* could be exposed to T_3 without the possibility that some other factor such as endogenous hormones, innervation or cell to cell interaction might be interfering with the results regarding A7 expression.

The health and integrity of the controls throughout the experiments and the absence of bacterial and fungal infection demonstrated that the culture was working sufficiently to support the tails and allow for consistent data. Furthermore, the culturing of tails was shown to be possible up through day 14 (with the minor exception of experiment 1, in which the culture began to deteriorate prematurely on day 13), which provided an ample period of time to allow for the comparison of TH-treated experimental tails versus non-treated control tails in culture.

Exogenous T_3 Induces Metamorphosis-The results of the cultured tail experiments indicate that the tadpole tails, regardless of their premetamorphic stage (42, 48 or 50), are sensitive to thyroid hormone. All three experiments show significant regression of T_3 tails compared to control tails (Figure 6). The controls maintained their integrity and remained intact and healthy (Figure 7e). They were not subject to the characteristic regression, curling and darkening demonstrated by the metamorphosing T_3-treated tails (Figure 7f). These results demonstrate findings similar to those found by Weber (1967); Etkin and Gona (1967); Niki *et al.* (1984); and Tata *et al.* (1991), that show that T_3 does cause significant regression of tadpole tails *in-vitro*.

One would think that T_3, which is known to initiate metamorphosis (Gudernatsch, 1912; Allen, 1929) would immediately cause regression in the

experimental tails. However, this is not the case. The results from these experiments also show that the T_3-treated tails remain approximately the same size until about day 4-5, at which time the T_3-treated tails begin to regress (Figure 6). Since it is not possible for prolactin or some other agent to be causing the delay (the tails are *in-vitro*), there must be some other mechanism responsible for the delaying effect of T_3 on tail regression.

This assumption correlates directly with what Kollross (1961) observed, whereby different tissues respond to different concentrations of T_3. Specifically, it takes a higher concentration of T_3 to induce metamorphosis in tails, as opposed to limbs, which require a lower concentration of T_3. In essence, what we might be seeing is a delay due to concentration build up to meet the T_3 threshold required to induce metamorphosis in the tails. The T_3 that is added to the culture on day one of the experiment, causes immediate autoinduction of TRs, which further induce other TRs as described by Chatterjee and Tata (1992) and Galton *et al.* (1992). It is this autoinduction of TRs that takes time. Once there are enough TRs to bind the exogenous T_3 and reach the required threshold, which is day 4-5 in our experiments, the tails undergo metamorphosis.

PTU Inhibition of Metamorphosis-The effects of PTU on metamorphosis in the whole tadpole are quite drastic, as demonstrated from the results (Figure 8), which supports what others have shown (Allen, 1929; Weber, 1967).

Specifically, the results indicate that the tadpole never responds to endogenous T_3 and fails to undergo metamorphosis. Severe hypertrophy can be seen in the dorsal tail muscle, probably due to the persistence of prolactin in the absence of T_3, which acts as a juvenile growth hormone in tadpoles and induces embryonic muscle to continue growing. As a result, the tail continues to grow, while the rest of the body remains relatively small. The legs and forelimbs never appear and the animal becomes a massively oversized tadpole. It is almost as if the tadpole is literally bursting to metamorphose and that the slightest amount of T_3 will push it over the 'threshold'.

Overall, these experiments demonstrate that PTU indeed acts as a sufficient inhibitor of metamorphosis, and provides another model for study of the addition of exogenous T_3 to examine expression of A7. Although unlike isolated tail culture, in which hormone levels can be manipulated free from the tadpoles own sources, PTU inhibition of T_3 in whole tadpoles does have its advantages. It allows for long term experimentation not permissible with cultures. It allows for one to observe whole body changes during metamorphosis and correlate those with metamorphic changes in the tail. Finally, it is as close to the natural system in the tadpole that one can achieve, aside from thyroidectomy.

T_3 Causes Rapid Metamorphosis in PTU Treated Tadpoles-The results in this experiment indicate that an exogenous supplement of T_3 at a

concentration found in a normal prometamorphic tadpole causes rapid and substantial metamorphosis in the PTU treated tadpole (Figure 9). Similar results were seen in thyroidectomized tadpoles supplemented with T_3 (Hoskins and Hoskins, 1917). With the addition of T_3 at normal physiological levels (10^{-8} M) to PTU treated whole tadpoles, the tadpoles showed immediate tail regression and completion of limb metamorphosis within four days after the addition of T_3. This was quite a marked difference when compared to the cultured T_3-treated tails which failed to show regression until 4-5 days after being exposed to 10^{-8} M T_3. This result is not consistent with findings suggesting that different tissues in the tadpole have varying affinities for T_3 (Galton, 1992), due to the fact that both the tail and legs responded with the same rate of metamorphosis at a normal, not excessive, T_3 concentration. Usually, it takes excessively high amounts of T_3 (10^{-6} M) to cause the metamorphosis of tails and legs (Kollross, 1961), normal amounts of T_3 only cause metamorphosis of legs, in regards to PTU-treated tadpoles given T_3. Once again, as in the cultured tails exposed to T_3, it seems that the threshold concept might provide some insight.

According to Kollross (1961), these findings suggest that normal concentrations of T_3 are causing drastic metamorphosis in the PTU tadpole treated with T_3, where only high concentrations should. It is more probable that the T_3 threshold in the PTU treated animals had been compromised prior to

exogenous addition of T_3 or that the threshold was already reached or near the limit once T_3 was added, in essence allowing a smaller concentration of T_3 to act as if it were causing metamorphosis characteristic of a higher concentration of T_3.

There are several scenarios to explain these findings. Perhaps, by the time the controls reached stage 66 and T_3 was added to the PTU treated tadpoles, enough autoinduction of TRs had already occurred, such that the TRs had been saturated to the point that normal levels of T_3 would cause a massive response. This could be accomplished by maternal TRs in the tadpole (Kawahara *et al.*, 1991), which work slowly, but over time, could saturate the tissues. Another explanation could be based on the idea of a biological clock, such that TRs become fully functional at a certain point in metamorphosis regardless of T_3 induction and consequent autoinduction. TRs would not respond to T_3 before a certain age of development without a lag period of autoinduction. After when the tadpole should be at stage 60, for instance, the TR gene is automatically turned on, and begins autoinducing without the aid of T_3, in turn, saturating the tissues with TRs.

If such scenarios exist, it would be possible for normal or less than normal concentrations of T_3 to induce drastic metamorphosis. This would all be due to higher concentrations of receptors which, in turn, are more capable of inducing

transcription of genes encoding adult-specific changes as suggested by Tata *et al.* (1991).

Thyroid Hormone Induces Expression of Adult Isoforms-As expected, the results of *in-situ* analysis of T_3 treated tails in culture and inhibited with PTU show expression of MHC A7 (Figures 12, 13). Corresponding RT-PCR analysis supports this as well (Figure 11) It has been demonstrated in this research that T_3 is responsible for metamorphosis in cultured tails and in PTU treated tadpoles. Previous studies have shown that A7 is expressed in adult muscle (Radice and Malacinski, 1989). The results of this research demonstrate that there is, in fact, a relationship between T_3, metamorphosis and A7 expression, such that T_3 seems to be directly responsible for the expression of A7 in metamorphosing tadpoles. This is similar to results found in rabbits where exogenous T_3-induced expression of adult α-MHC, which is an adult-specific cardiac isoform (Lin *et al.*, 1989). Also, Winegrad *et al.* (1990) showed that MHCs are primarily alpha isoforms in rats when induced by T_3, which has patterns of expression that are consistent with those of the MHC multigene families during cardiac growth in mammals. Finally, T_3 induced A7 MHC expression is similar to T_3 induction of keratin proteins in amphibians, where T_3 is necessary for the keratins to be expressed at high levels (Nishikawa *et al.*, 1992).

A7 expression is limited to the somites around the edges of the base of the tail (Figures 12, 13). These regions are known to be exclusively slow twitch fibers (Radice and Malacinski, 1989). As a result, one can easily conclude that A7 is indeed a slow fiber MHC. A7 expression does not appear in any other part of the tail, and seems to be highly conserved in the region near the base of the tail. All in all, T_3 mediated expression of A7 is quite an interesting concept, since expression persists in a tissue that is programmed for regression and cell death. Regardless, A7 expression persists in the tail until the tadpole has metamorphosed and then continues to be expressed in other slow twitch fibers throughout the froglet via other means once TH levels drop off.

PTU Treated Animals inhibited in Metamorphosis Still show A7 Expression-Results from *in-situ* analysis for PTU treated tails not given T_3, show A7 expression similar to that seen in T_3 treated tails in culture and PTU/T_3 treated whole tadpoles (Figure 14). RT-PCR analysis also supported this result (Figure 11). This is the most interesting finding regarding A7 expression in the tadpole tail. It was predicted that since tadpoles treated with PTU did not metamorphose, that A7 expression would not be apparent. Expression of A7 was not seen in cultured tails, therefore, the A7 expression in the PTU model had to be due to either PTU, or some other substance in the tadpole, that has no effect in the cultured tail.

There are three possible explanations for the A7 expression seen in the PTU treated tails. The first is that A7 is turned on when the tadpole reaches a certain age regardless of what treatment is given to it, but remains at low levels unless TH levels rise. This is similar to what was observed by Mathisen and Miller (1987) regarding keratins. They found that keratins begin expression at a low level at some point in prometamorphosis and continue to express at low levels until subjected to T_3. Although the keratins initially express at low levels, they still require TH to express consistently at high levels. A7 might be expressing at low levels, and once exposed to T_3, it expresses at higher levels. The results obtained in this experiment are consistent with this. It could be that A7 does not start low level expression until after our 14 day tail culture had expired or that the tails we use in tail culture were too young to catch the beginning of the baseline. Regardless, none of our tails at stages 42, 48 or 50 showed A7 expression. One could find out if A7 has a baseline level by running consecutive 14 day isolated tail cultures, for each stage of tail from 42-66 to determine if and when the baseline begins to appear in the tails.

A second explanation rests in the idea that although the tadpoles have been treated with PTU and did not metamorphose, they still might be secreting some level of TH. It could be that PTU works well in inhibiting most of the T_3 but that just enough manages to leak out from the follicles in the thyroid to allow for a

low level baseline of T_3, which summarily causes receptor autoinduction and has a high enough concentration for expression of A7 at low levels. This could be possible due to the fact that PTU acts by competitive inhibiton of thyroid peroxidase, blocking the coupling step that leads to the formation of THs. Since there is competitive inhibition, some thyroid peroxidase coupling might be occurring allowing low levels of TH to be produced (Ingbar, 1985). This explanation is also consistent with results found in the cultured tails, since they were never exposed to T_3. This scenario would help to explain the rapid metamorphosis seen in the PTU/T_3 tadpole. A T_3 baseline would in essence, lower the threshold and saturate the receptors for T_3 in all of the tissues. Any additional T_3 even at normal or below normal levels given exogenously would cause marked metamorphosis. A suitable experiment to test this scenario would involve thyroidectomizing tadpoles and analyzing A7 expression. This has not been done, due to the difficulty of the procedure. If A7 expression is not apparent, then the thyroid is involved; if A7 expression is still apparent, then some other substance must be involved. One could also increase the effectiveness of PTU by increasing the concentration to which the tadpole is exposed, consequently increasing the competitive inhibition whereby PTU is saturated in the coupling step. Also, in order to measure if T_3 was at all present in the tadpole it would be beneficial to run a radio-immuno assay for T_3.

The final scenario involves perhaps a compound such as prolactin, retinoic acid, or some yet unknown substance. Tata *et al.* (1991) has done some research with retinoic acid and other substances, but has failed to find any that cause metamorphosis without T_3. This is not to say that any of these would not cause expression of A7. That, however, is a matter of trial and error. One could set up a tail culture in which tails are treated with substances and hormones and combinations of substances and A7 expression analyzed.

In summary, the present investigation identified a consistent culturing system for isolated tails as well as a PTU treatment regimen. It was observed that metamorphosis occurred in cultured tails and PTU treated whole tadpoles exposed to T_3, but appeared to vary regarding response rates and mechanisms. A7 expression was seen in T_3-treated tails in culture and tails from whole tadpoles treated with PTU/T_3. A7 shows expression mainly in the base of the tail both ventrally and dorsally in muscle regions known to be exclusively slow twitch fiber. Finally, it was observed that PTU treated tails also showed A7 expression characteristic of tails treated with T_3. This finding suggests that T_3 is not totally inhibited from the thyroid gland, a clock timing the expression of A7 might be involved, or some other substance besides TH is acting to cause expression of A7.

REFERENCES

Allen, B.M. (1916) Extirpation experiments in *Rana pipiens* larva. Science 44: 755-757.

Allen, B.M. (1929) The influence of the thyroid gland and hypophysis upon growth and development of amphibian larvae. Quart. Rev. Biol. 4: 325-352.

Alley, K. (1989) Myofiber turnover is used to retrofit frog jaw muscles during metamorphosis. Am. J. Anat. 184: 1-12.

Baker, B.S., and Tata, J.R. (1992) Prolactin prevents the autoinduction of thyroid hormone receptor mRNAs during amphibian metamorphosis. Dev. Biol. 149: 463-467.

Baniahmad, A., Steiner, C., Koehne, C., and Renkawitz, R. (1990) Module structure of a chicken lysozyme silencer: involvement of an unusual thyroid hormone receptor binding site. Cell 61: 505-514.

Banker, D.E., Bigler, J., and Eisenman, R.N. (1991) The thyroid hormone receptor gene (c-erbA-alpha) is expressed in advance of thyroid gland maturation during the early embryonic development of *Xenopus laevis*. Mol. and Cell Biol.11:5079-5089.

Bern, H.A., Nicol, C.S., and Strohman, R.C. (1967) Prolactin and tadpole growth. Proc. Soc. Exp. Biol. Med. 126: 518-520.

Brooks, A.R., Sweeney, G., and Old, R.W. (1989) Structure and functional expression of a cloned *Xenopus* thyroid hormone receptor. Nucleic Acids Res. 17: 9395-9405.

Broyles, R.H. (1981) "Changes in the blood during amphibian metamorphosis". In L. I. Gilbert and E. Frieden (eds.), "Metamorphosis: A Problem in Developmental Biology". 2nd Edition. Plenum Press, New York. pp 462-490.

Butler-Browne, G.S., Barbet, J.P., and Thornell, L.E. (1990) Myosin heavy and light chain expression during human skeletal muscle development and precocious muscle maturation induced by thyroid hormone. Anat. Embryol. Berl. 181: 513-522.

Chanoine, C., D'Albis, A., Lenfant-Guyot, M., Janmot, C., and Gallien, C. (1987) Regulation by thyroid hormones of terminal differentiation in the skeletal dorsal muscle. II. Urodelean amphibians. Dev. Biol. 123: 33-42.

Chatterjee, V.K.K., and Tata, J.R. (1992) Thyroid hormone receptors and their role in development. Cancer Surveys 14: Growth Regulation by Nuclear Hormone Receptors: 147-166.

Chomczynski, P., and Sacchi, N. (1987) Single step method of RNA isolation by acid quanidium thiocyanate-phenol-chloroform extraction. Anal.Biochem. 162: 156-159.

Cohen, P.P. (1970) Biochemical differentiation during amphibian metamorphosis. Science 168: 533-543.

Crow, M. (1987) The determinants of muscle fiber type during embryonic development. American Zoologist 27: 1043-1053.

d'Albis, A., Lenfant-Guyot, M., Janmot, C., Chanoine, C., Weinman, J., and Gallien, C.L. (1987) Regulation by thyroid hormones of terminal differentiation in the skeletal dorsal muscle: I. Neonate mouse. Dev. Biol. 123: 25-32.

Dent, J.N. (1988) Hormonal interaction in amphibian metamorphosis. American Zoologist 28: 297-308.

Derby, A., and Etkin, W. (1968) Thyroxine induced tail resorption *in-vitro* as affected by anterior pituitary hormones. J. Exp. Zool. 169: 1-8.

Diffee, G.M., Haddad, F., Herrick, R.E., and Baldwin, K.M. (1991) Control of myosin heavy chain expression: interaction of hypothyroidism and hindlimb suspension. Am. J. Physiol. 261: 1099-1106.

Dillmann, W.H., Barrieux, A., and Shanker, R. (1989) Influence of thyroid hormone on myosin heavy chain mRNA and other messenger

RNAs in the rat heart. Endocr. Res. 15: 565-577.

Downes, M., Griggs, R., Atkins, A., Olson, E.N., and Muscat, G.E. (1993) Identification of a thyroid hormone response element in the mouse myogenin gene: characterization of the thyroid hormone and retinoid X receptor heterodimeric binding site. Cell Growth Differ. 4: 901-909.

Duellman, W.E., and Trueb, L. (1994) "Biology of Amphibians." Baltimore: Johns Hopkins University Press.

Ellison, T.R., Mathisen, P.M., and Miller, L. (1985) Developmental changes in keratin patterns during epidermal maturation. Dev. Biol. 112: 329-337.

Erickson, P. (1993) The effect of innervation on adult muscle production in the hindlimbs of *Xenopus laevis* tadpoles. Unpublished research. University of Richmond.

Etkin, W. (1968) "Hormonal control of amphibian metamorphosis". In W. Etkin and L. I. Gilbert (eds.), "Metamorphosis: A Problem in Developmental Biology". Appleton-Century-Crofts, New York, pp. 313-348.

Etkin, W., and Gona, A. (1967) Antagonism between prolactin and thyroid hormone in amphibian development. J. Exp. Zool. 165: 249-258.

Flink, I.L., and Morkin, E. (1990) Interaction of thyroid hormone receptors with strong and weak cis-acting elements in the human alpha-myosin heavy chain gene promoter. J. Biol. Chem. 265: 11233-11237.

Franklyn, J.A., Green, N.K., Gammage, M.D., Alhquist, J.A., and Sheppard, M.C. (1989) Regulation of alpha- and beta-myosin heavy chain messenger RNAs in the rat myocardium by amiodarone and by thyroid status. Clin. Sci. 76: 463-467.

Galton, V.A. (1992) The role of thyroid hormone in amphibian metamorphosis. Trends In Endocrinology And Metabolism 3: 96-100.

Galton, V.A., and Hiebert, A. (1988) The ontogeny of iodothyronine 5' monodeiodinase activity in *Rana catesbeiana* tadpoles. Endocrinology 122: 640-645.

Galton, V.A., and St. Germain, D.L. (1985) Putative nuclear triiodothyronine receptors in tadpole liver during metamorphic climax. Endocrinology 117: 1-5.

Gauthier, G. (1987) Vertebrate muscle fiber types and neuronal regulation of myosin expression. American Zoologist 27: 1033-1042.

Gilbert, S.F. (1994) "Developmental Biology." New York: Sinauer Assoc.

Gilbert, L.I., and Frieden, E. (1981) "Metamorphosis: A Problem in Developmental Biology". Plenum Press, New York.

Green, N.K., Franklyn, J.A., Ahlquist, J.A., Gammage, M.D., and Sheppard,M.C. (1989) Differential regulation by thyroid hormones of myosin heavy chain

alpha and beta mRNAs in the rat ventricular myocardium. J. Endocrinol. 122: 193-200.

Grove, B.K. (1989) Muscle differentiation and the origin of muscle fiber diversity. Crit. Rev. Neurobiol. 4: 201-234.

Gudernatsch, J.F. (1912) Feeding experiments on tadpoles. I. The influence of specific organs given as food on growth and differentiation. A contribution to the knowledge of organs with internal secretion. Wilhem Roux Arch. Entwicklungsmech. Org. 35: 457-483.

Gustafson, T.A., Markam, B., Bahl, J.J., and Morkin, E. (1987) Thyroid hormone regulates expression of a transfected alpha-myosin heavy-chain fusion gene in fetal heart cells. Proc. Natl. Acad. Sci. USA 84: 3122-3126.

Harland, R.M. (1991) *In-situ* hybridization: An improved whole-mount method for *Xenopus* embryos. Meth. in Cell Biol. 36: 685-695.

Harris, A.J., Duxson, M.J., Fitzsimons, R.B., and Rieger, F. (1989) Myonuclear birthdates distinguish the origins of primary and secondary myotubes in embryonic mammalian skeletal muscles. Development 107: 771-784.

Helbing, C., Gergely, G., and Atkinson, B.G. (1992) Sequential up-regulation of thyroid hormone beta receptor, ornithine transcarbamoylase, and carbamyl phosphate synthetase mRNAs in the liver of *Rana catesbeiana*

tadpoles during spontaneous and thyroid hormone-induced metamorphosis. Developmental Genetics 13: 289-301.

Hoskins, E.R. and Hoskins, M.M. (1917) On thyroidectomy in amphibia. Proc. Soc. Exp. Biol. Med. 14:74-75.

Ingbar, S.H. (1985) "The Thyroid Gland". In Wilson, J.D. and Foster, D.W. (eds.), "Williams Textbook of Endocrinology". 7th Edition. W.B. Saunders Co., Philadelphia, Pa. pp. 682-815.

Ishizuya-Oka, A., and Shimozawa, A. (1992) Connective tissue is involved in adult epithelial development of the small intestine during anuran metamorphosis *in-vitro.* Roux's Arch. Dev. Biol. 201: 322-329.

Kawahara, A., Baker, B.S., and Tata, J.R. (1991) Developmental and regional expression of thyroid hormone receptor genes during *Xenopus* metamorphosis. Development 112: 933-944.

Kawai, A., Ikeya, J., Kinoshita, T., and Yoshizato, K. (1994) A three-step mechanism of action of thyroid hormone and mesenchyme in metamorphic changes in anuran larval skin. Dev. Biol. 166: 477-488.

Kawasaki, E.S. (1990) "Amplification of RNA". In "PCR Protocols: A Guide to Methods and Applications". Academic Press, New York. pp. 21-27.

Kistler, A., Yoshizato, K., and Frieden, E. (1977) Preferential binding of tri-substituted thyronine analogs by bullfrog tadpole tail fin cytosol. Endocrinology 100: 134-137.

Kollross, J.J. (1961) Mechanisms of amphibian metamorphosis: Hormones.Amer. Zool. 1: 107-114.

Leloup, J., and Buscaglia, M. (1977) La triiodothyronine, hormone del la metamorphose des Amphibiens. C. R. Acad. Sc. Paris 284: 2261-2263.

Lin, Z.Y., Wenderoth, M.P., and Eisenberg, B.R. (1989) Individual rabbit cardiac myocytes have different thresholds for alpha myosin heavy chain regulation by thyroid hormone. Am. J. Anat. 185: 455-61.

Lompre, A.M., Lambert, F., Lakatta, E.G., and Schwartz, K. (1991) Expression of sarcoplasmic reticulum Ca(2+)-ATPase and calsequestrin genes in rat heart during ontogenic development and aging. Circ. Res. 69: 1380-1388.

Marsh, J. (1993) Transforming frogs and flies. Nature. 361.

Mathisen, P.M., and Miller, L. (1987) Thyroid hormone induction of keratin genes: A two step activation of gene expression during development. Genes & Development 1: 1107-1117.

May, F.E.B., and Knowland, J. (1981) Patterns of protein synthesis in livers of *Xenopus laevis* during metamorphosis: Effects of estrogen in normal and thyostatic animals. Dev. Biol. 82: 343-349.

McCormick, C. (1993) Effects of thyroid hormone on A7 expression in *Xenopus laevis*. Unpublished research. University of Richmond.

McCutcheon, F.H. (1936) Hemoglobin function during the life history of the bullfrog. J. Cell. Comp. Physiol. 8: 63-81.

Morkin, E., Bahl, J.J., and Markham, B.E. (1989) Control of cardiac myosin heavy chain gene expression by thyroid hormone. Cell and Mol. Biol. of Muscle Dev. 34: 381-389.

Morkin, E., Edwards, J.G., Tsika, R.W., Bahl, J.J., and Flink, I.L. (1991) Regulation of human cardiac myosin heavy chain gene expression by thyroid hormone in cultured fetal rat heart cells. Acta. Cardiol. 46: 425-426.

Moskatis, J.E., Sargent, T.D., Smith, L.H., Jr., Pastori, R.L., and Schoenberg, D.R. (1989) *Xenopus laevis* serum albumin: Sequence of the complementary deoxyribonucleic acids encoding the 68- and 74-kilodalton peptides and the regulation of albumin gene expression by thyroid hormone during development. Mol. Endocrinol. 3: 464-473.

Nieuwkoop, P.D., and Faber, J. (1975) "Normal table of *Xenopus laevis* (Daudin)." Amsterdam: North-Holland.

Niki, K., Yoshizato, K., Namiki, H., and Kikuyama, S. (1984) *In-vitro* regression of tadpole tail by thyroid hormone. Development, Growth, and Differentiation 26: 329-338.

Nishikawa, A., Keiko, S., and Miller, L. (1992) Spatial, temporal, and hormonal regulation of epidermal keratin expression during development of the frog, *Xenopus laevis*. Dev. Biol. 151: 145-153.

Radice, G.P. (1995) Spatial expression of two tadpole stage specific myosin heavy chains in *Xenopus laevis*. Acta Anatomica 153: 254-262. .

Radice, G.P., and Malacinski, G.M. (1989) Expression of myosin heavy chain transcripts during *Xenopus laevis* development. Dev. Biol. 133: 562-568.

Ray, L.B., and Dent, J.N. (1986) An analysis of the influence of thyroid hormone on the synthesis of proteins in the tail fin of bullfrog tadpoles. J. Exp. Biol. 240: 191-201.

Reeves, R. (1977) Hormonal regulation of epidermis-specific protein and messenger RNA synthesis in amphibian metamorphosis. Dev. Biol. 60: 163-179.

Regard, E., Taurog, A., and Nakashima, T. (1978) Plasma thyroxine and triiodothyronine levels in spontaneously metamorphosing *Rana catesbeiana* tadpoles and in adult anuran amphibia. Endocrinology 102: 674-684.

Salmons, S., and Sreter, F.A. (1976) Significance of impulse activity in the transformation of skeletal muscle type. Nature 263: 30-34.

Sambrook, J., Fritsch, E.F., and Maniatis, T. (1989) "Molecular Cloning." Cold Spring Harbor: Cold Spring Harbor Press.

Sargent, T.D., Jamrich, M., and Dawid, I. (1986) Cell interactions and the control of gene activity during early development of *Xenopus laevis*. Dev. Biol. 114: 238-246.

Tata, J.R. (1968) Early metamorphic competence of *Xenopus* larvae. Dev. Biol. 18: 415-441.

Tata, J.R. (1993) Gene expression during metamorphosis: an ideal model for post-embryonic development. BioEssays 15: 239-248.

Tata, J.R., Kawahara, A., and Baker, B.S. (1991) Prolactin inhibits both thyroid hormone-induced morphogenesis and cell death in cultured amphibian larval tissues. Developmental Biology 146: 72-80.

Van Horn, R., Crow, M. T. (1989) Fast myosin heavy chain expression during the early and late embryonic stages of chicken skeletal muscle development. Dev. Biol. 134:279-88

Weber, R. (1964) Ultrastructural changes in regressing tail muscles of *Xenopus* larvae at metamorphosis. J. Cell Biol. 22: 481-487.

Weber, R. (1967) "Biochemistry of amphibian metamorphosis". In R. Weber (ed.), "The Biochemistry of Animal Development". New York: Academic Press.

White, B.A., and Nicoll, C.S. (1981) "Hormonal control of amphibian metamorphosis". In L. I. Gilbert and E. Frieden (eds.), "Metamorphosis: A Problem in Developmental Biology". 2nd Edition. New York: Plenum Press. pp. 363-396.

Winegrad, S., Wisnewsky, C., and Schwartz, K. (1990) Effect of thyroid hormone on the accumulation of mRNA for skeletal and cardiac alpha-actin in hearts from normal and hypophysectomized rats. Proc. Natl. Acad. Sci., USA 87: 2456-2460.

Yaoita, Y., and Brown, D.D. (1990) A correlation of thyroid hormone receptor gene expression with amphibain metamorphosis. Genes & Development 4:1917-1924.

FIGURES

FIGURE 1. Representative stages from Nieuwkoop and Faber (1975) correlated with their approximate chronological age at 22°C.

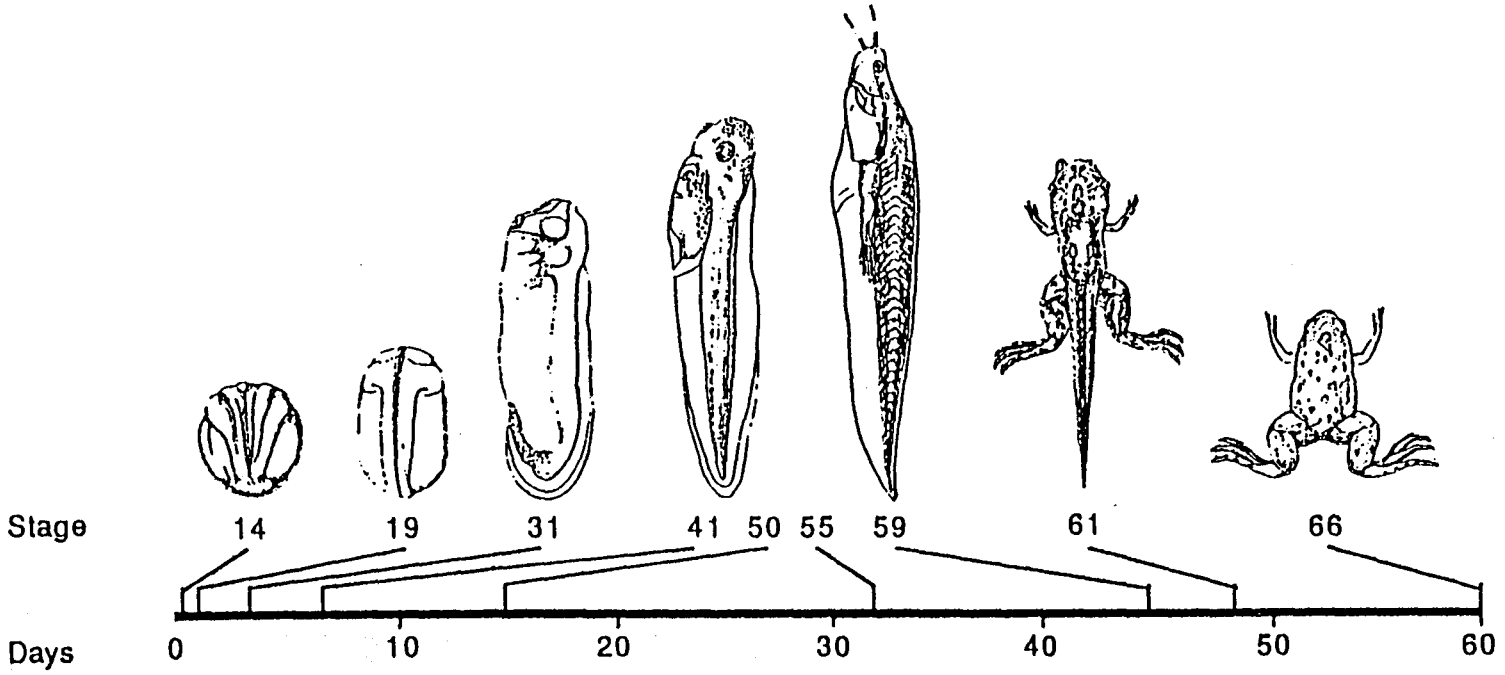
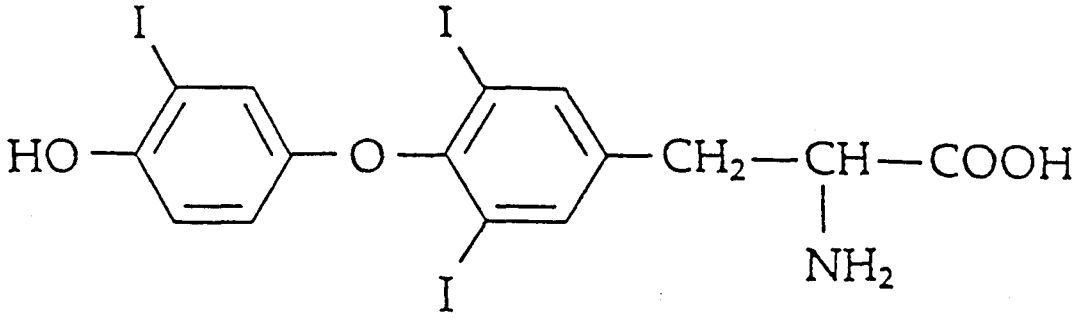
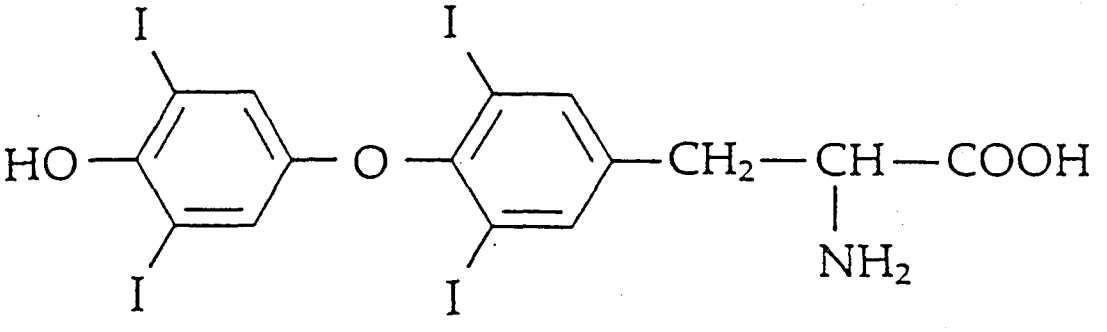

Stage
14
19
31
41
50
55
59
61
66
Days
0
10
20
30
40
50
60

FIGURE 2. Structures of T_3 and T_4. T_3 is the metabolite of T_4 and is the active form of thyroid hormone in the frog. 6-n-propyl 2-thiouracil (PTU) blocks the production of TH in the follicles of the thyroid and prevents the metabolism of T_4 into T_3 in peripheral tissues.



Triiodothyronine (T_3)



Thyroxine (T_4)

FIGURE 3. The hypothalamus-pituitary-thyroid axis during different stages of anuran metamorphosis. As the hypothalamus develops, it stimulates the pituitary to secrete thyroid stimulating hormone (TSH) which in turn stimulates thyroid hormone (T_3, T_4) secretion. The hypothalamus also secretes dopamine, which inhibits prolaction (PRL) secretion to allow the thyroid hormones to induce metamorphosis (Gilbert, 1994).

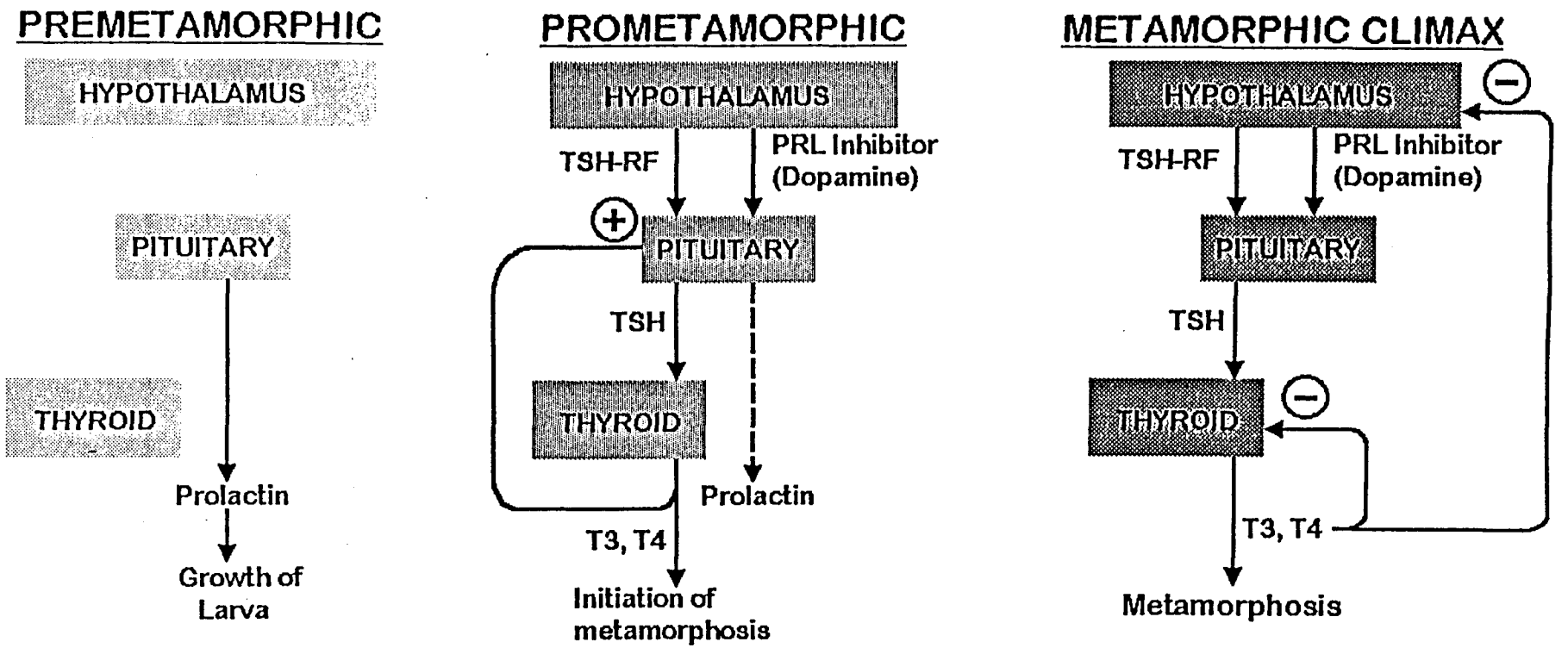

PREMETAMORPHIC
HYPOTHALAMUS
PITUITARY
THYROID
Prolactin
Growth of Larva
PROMETAMORPHIC
HYPOTHALAMUS
TSH-RF
PRL Inhibitor (Dopamine)
PITUITARY
TSH
THYROID
Prolactin
T3, T4
Initiation of metamorphosis
METAMORPHIC CLIMAX
HYPOTHALAMUS
TSH-RF
PRL Inhibitor (Dopamine)
PITUITARY
TSH
THYROID
T3, T4
Metamorphosis

FIGURE 4. A working model to explain the significance of the inhibition of auto induction of TRs by prolactin during amphibian metamorphosis. At the onset of prometamorphosis, the first traces of thyroid hormone bind to the small amount of TR to upregulate its own receptor (1) and induce expression of TR early genes (2). The combined effect of rapidly increasing amounts of circulating T_3 and release from the inhibition of autoinduction due to loss of prolactin (3) in blood would lead to high levels of TR-α and TR-ß during metamorphic climax (4). At high concentrations, TR-α and TR-ß will induce different sets of preprogrammed genes to produce tissue specific phenotypic changes (5) (Chatterjee and Tata, 1992).

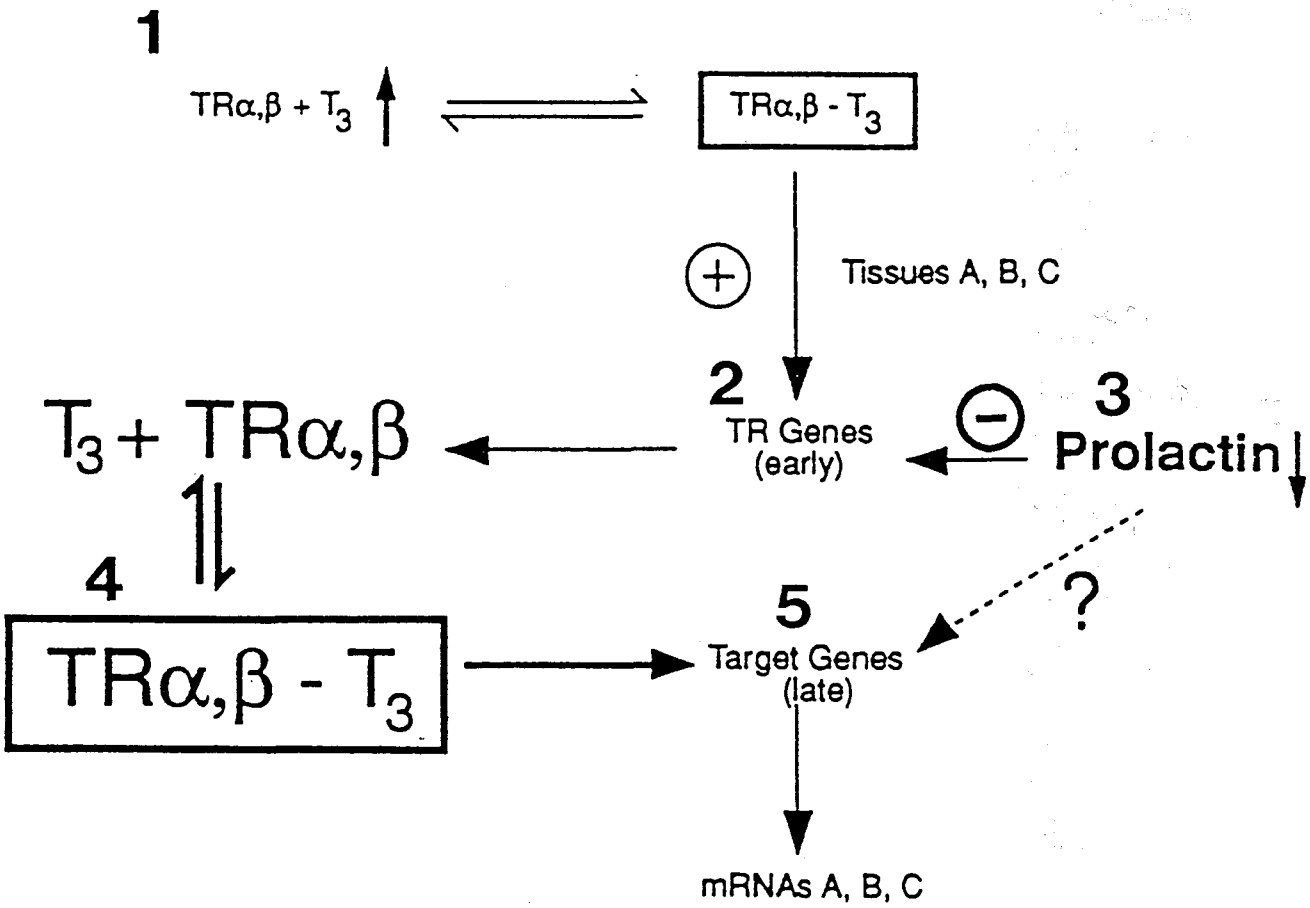

1
TRα,β + T3
TRα,β - T3
Tissues A, B, C
2
TR Genes
(early)
3
Prolactin
T3 + TRα,β
4
TRα,β - T3
5
Target Genes
(late)
?
mRNAs A, B, C

FIGURE 5. A7 specific region of the 1.5 kb A7 MHC DNA, showing primer set X and primer set Y, used during RT-PCR.

MYOSIN A-7 DNA SEQUENCE

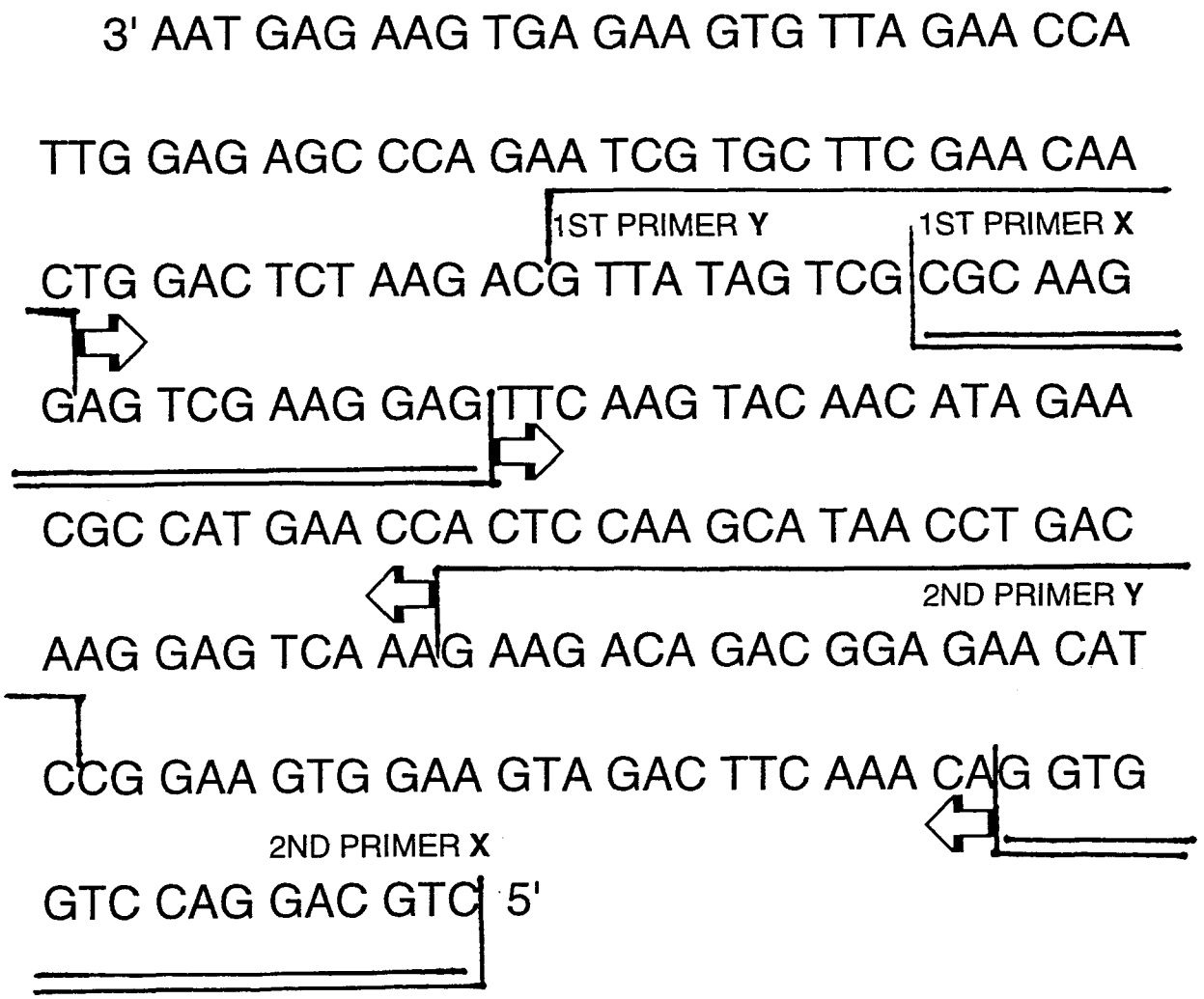


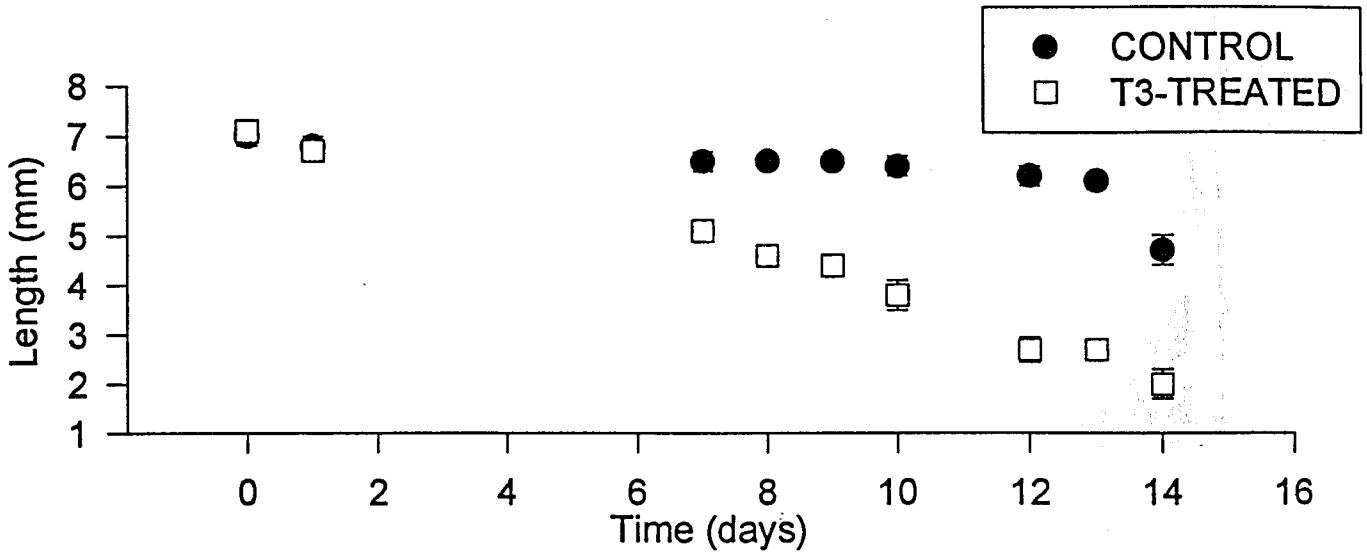
FIGURE 6. Regression of cultured tails exposed to T_3 versus controls. The younger tails tended to regress more in 14 days compared to the older ones. The mass of the tails might be a factor in this observation, such that it takes more T_3 the larger the targeted tissue becomes.

a. T_3 treated cultured tail regression of stage 42 tadpoles.

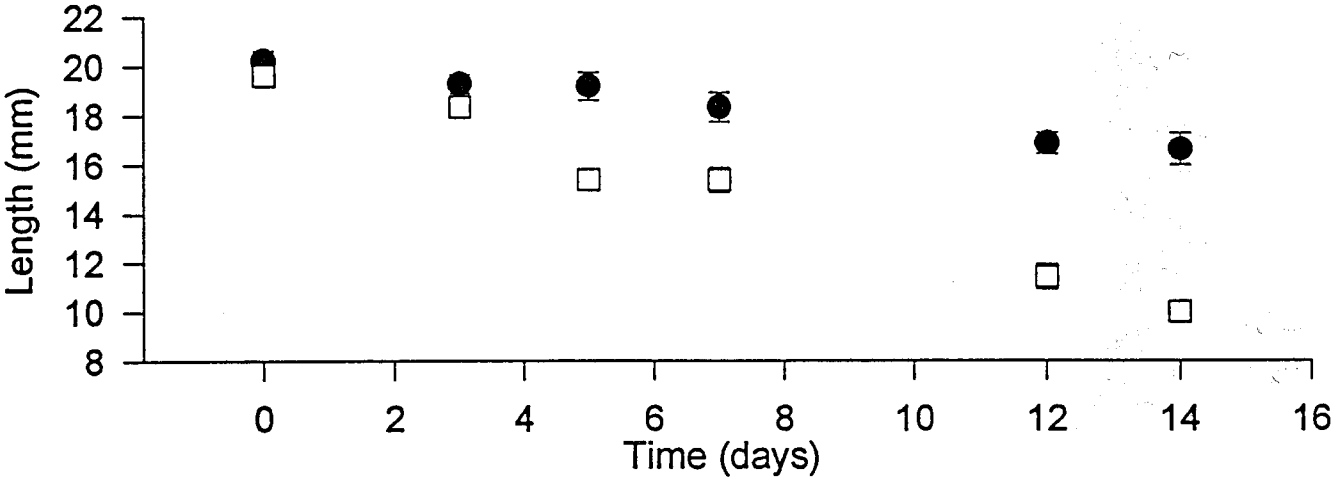
b. T_3 treated cultured tail regression of stage 48 tadpoles.

c. T_3 treated cultured tail regression of stage 50 tadpoles.

STAGE 42 TAIL REGRESSION



STAGE 48 TAIL REGRESSION



STAGE 50 TAIL REGRESSION

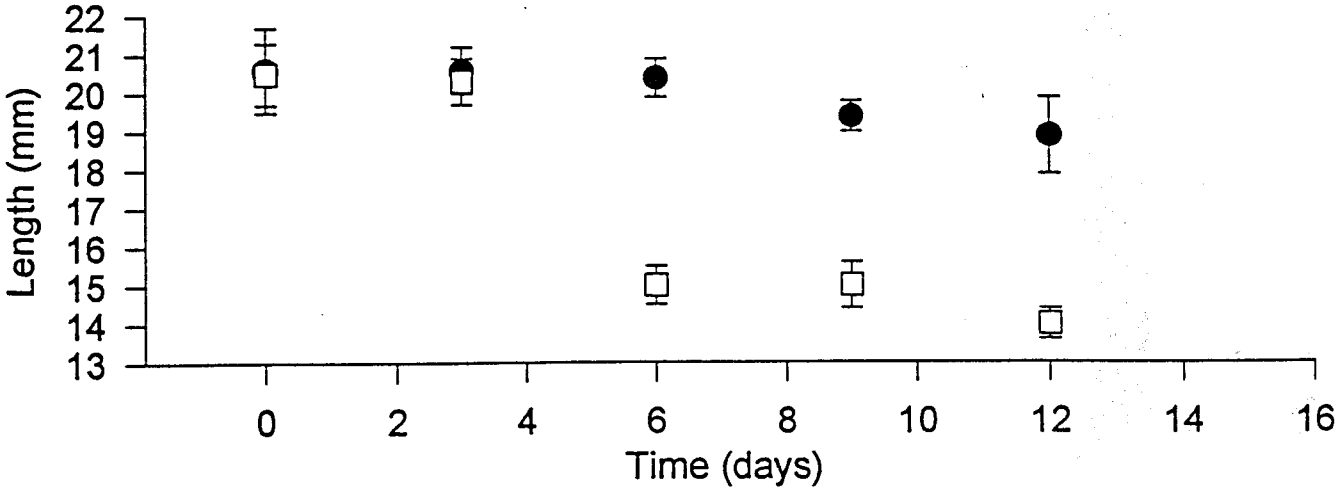


FIGURE 7. T_3 versus non-treated control tails in 14-day culture (stage 48). Notice the difference in T_3 treated tails vs. controls by day 12. Controls on day 12 are nearly as large as they were on day 0. The curling and darkening of the tips of the tails treated with T_3 is very apparent on day 12 (arrows).

a. Control tail at day 0

b. TH treated tail at day 0

c. Control tail at day 7

d. TH treated tail at day 7

e. Control tail at day 12

f. TH treated tail at day 12

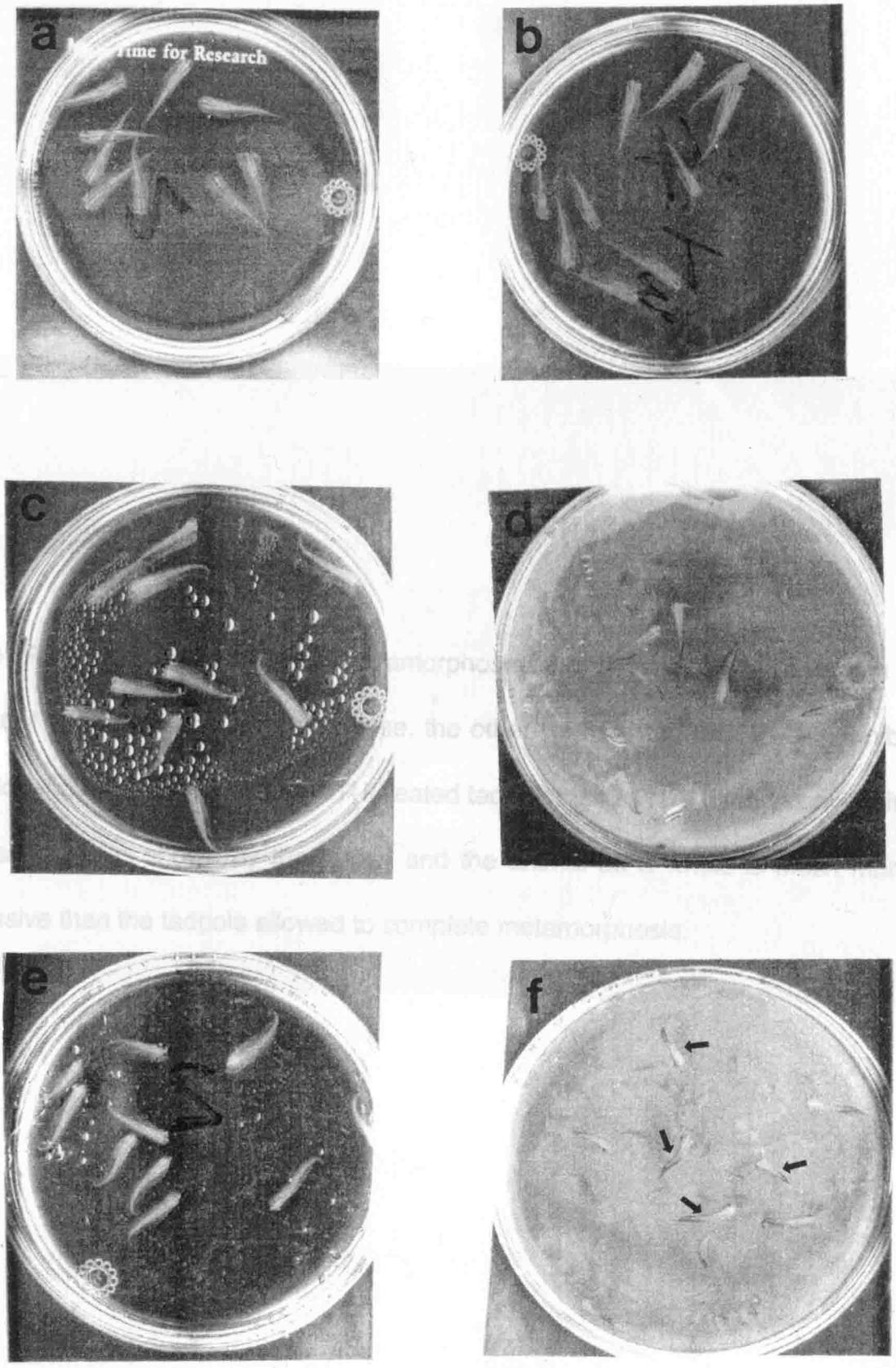

a
b
c
d
e
f

FIGURE 8. Effect of PTU on metamorphosis. 2 tadpoles shown, one was a control and allowed to metamorphose, the other was treated with PTU to inhibit endogenous levels of TH. The PTU-treated tadpole has severe hypertrophy on its dorsal side (as shown by the arrow) and the animal as a whole is much more massive than the tadpole allowed to complete metamorphosis.

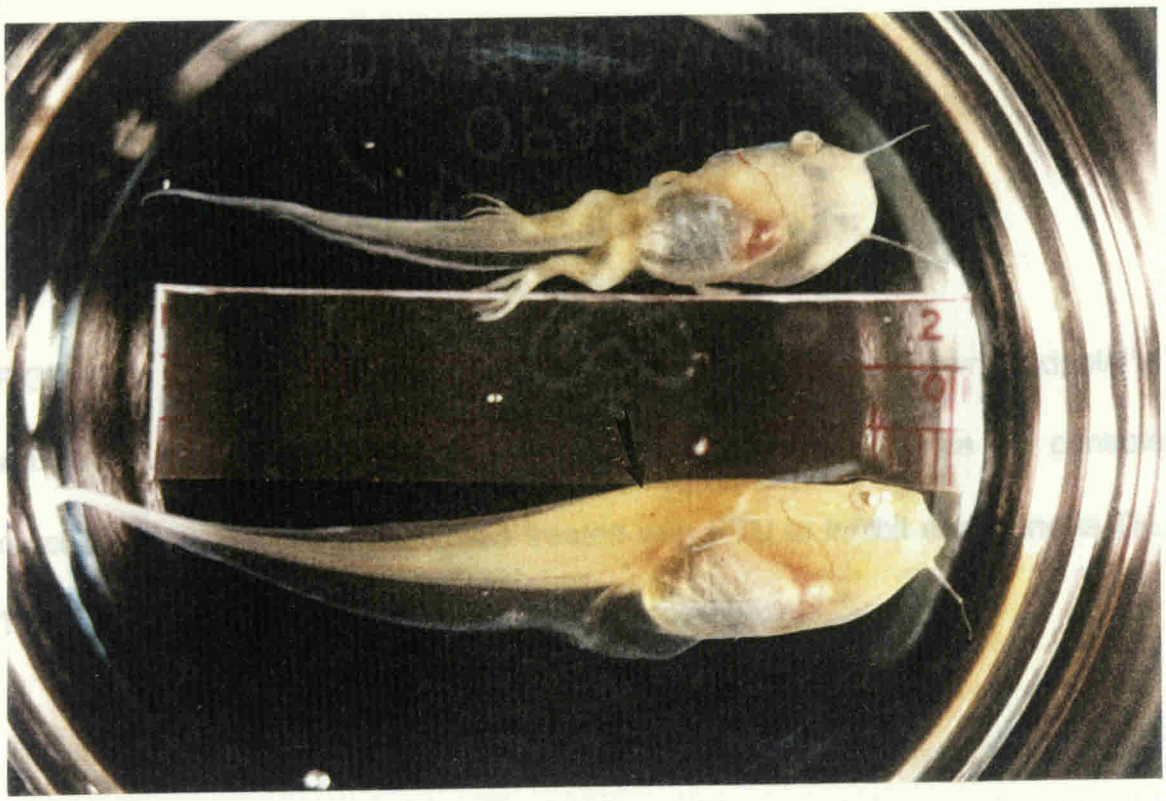


FIGURE 9. Effects of T_3 on PTU treated tadpoles. 3 tadpoles shown. Tadpole 'a' was a PTU treated tadpole that was supplemented with T_3 once the controls reached stage 66. Tadpole 'b' was treated with PTU to inhibit endogenous TH. Tadpole 'c' was a control and allowed to metamorphose.

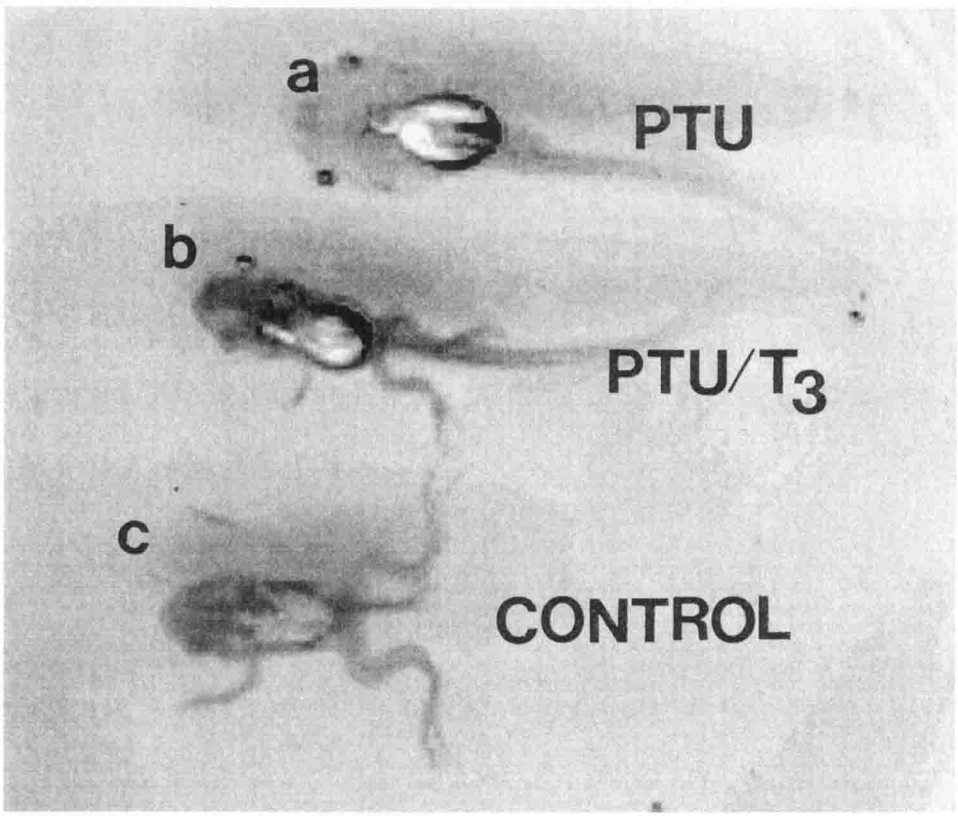

a
PTU
b
PTU/T_3
c
CONTROL

FIGURE 10. *In-situ* hybridization analysis of control tail. Tail is cleared and in 100% methanol. No expression of A7 can be seen in the periphery of somites, characteristic of that seen in tails treated with T_3 (arrows).

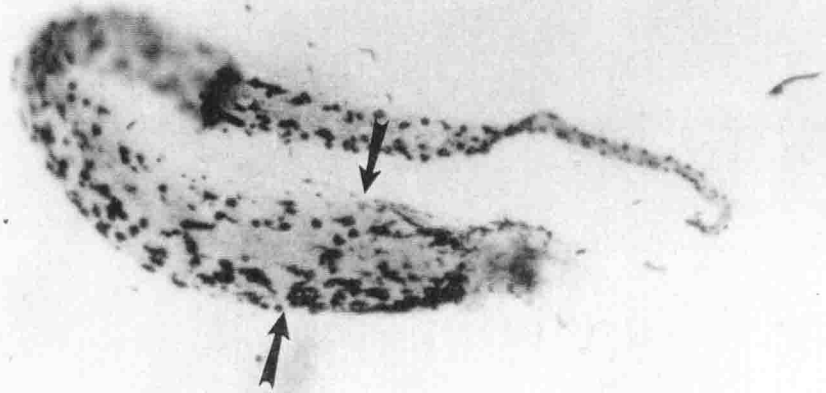


Figure 11. RT-PCR gel analysis. 1.5% agarose gel was run at 80v for 1.5 hrs. Results shown are based on one primer set (X) primer. Adult control, T_3-treated tail, control whole tadpole, PTU and T_3+PTU are positive for A7 transcript at 140 bp. Young and RT-control show no bands.

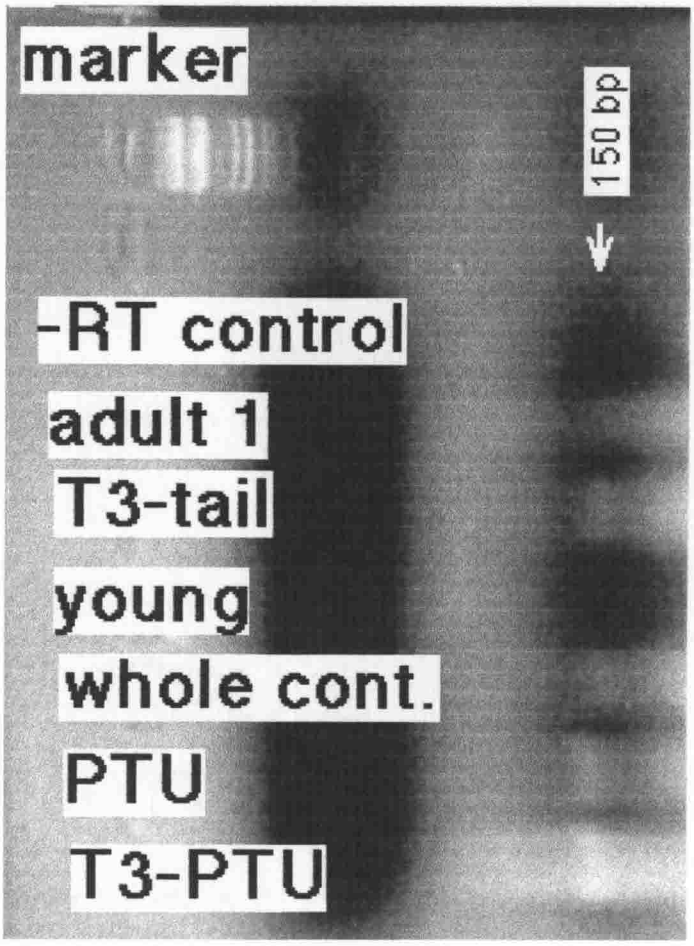

marker
150 bp
-RT control
adult 1
T3-tail
young
whole cont.
PTU
T3-PTU

FIGURE 12. *In-situ* hybridization analysis of T_3 treated cultured tail. Notice the hybridization of A7 on the ventral and dorsal side of the tadpole (arrows). The dark blue staining of the notochord is secondary background.

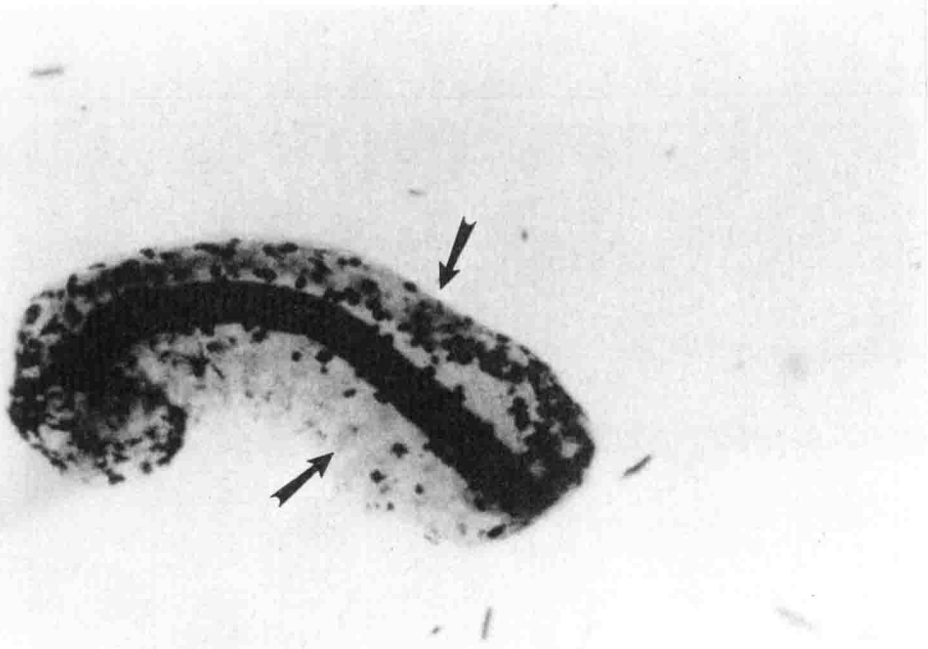
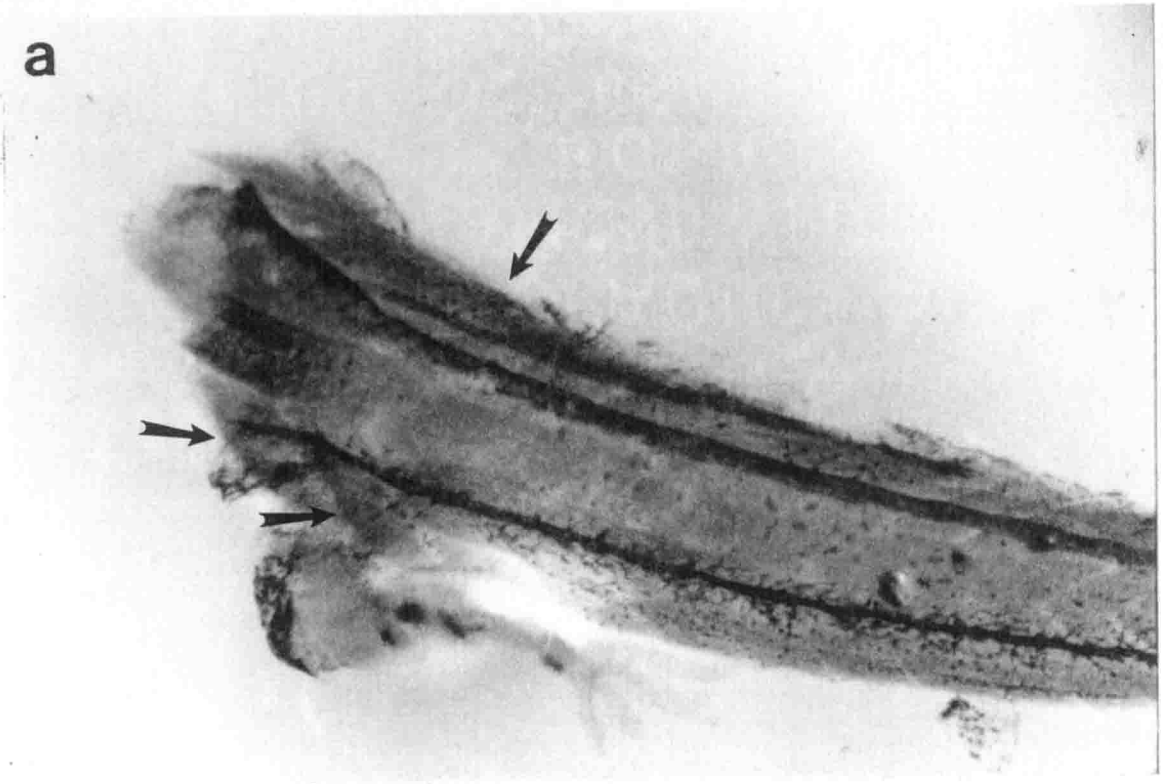


FIGURE 13 a, b. *In-situ* hybridization analysis of PTU/T_3-treated tail from whole tadpole. Notice the hybridization for A7 on the borders of the somites (arrows) near the base of the tail. The leg shows some hybridization, while the tail shows none.


a

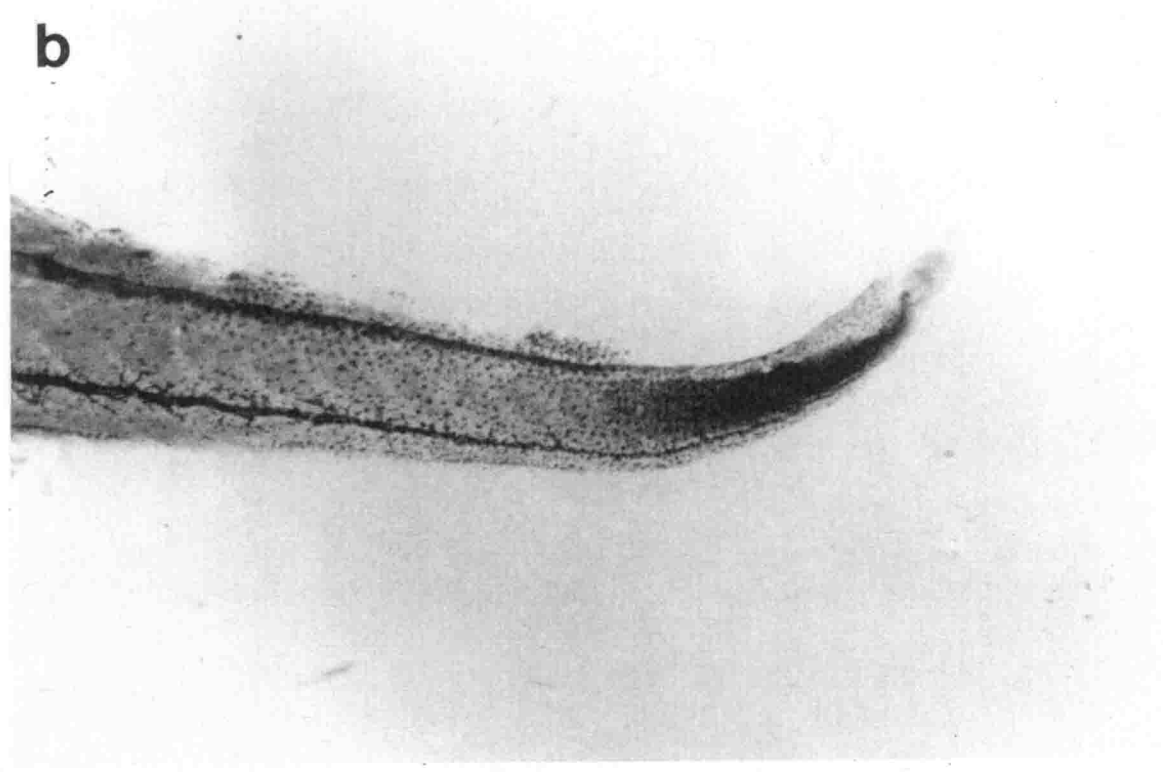
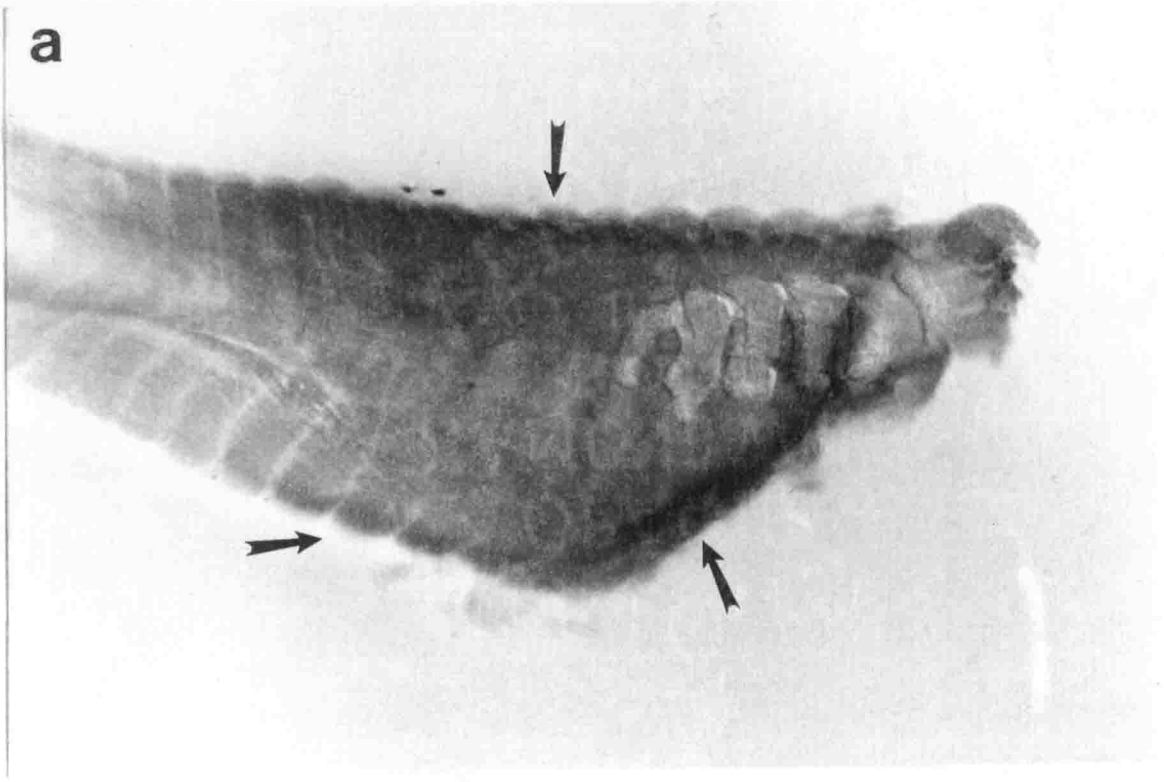
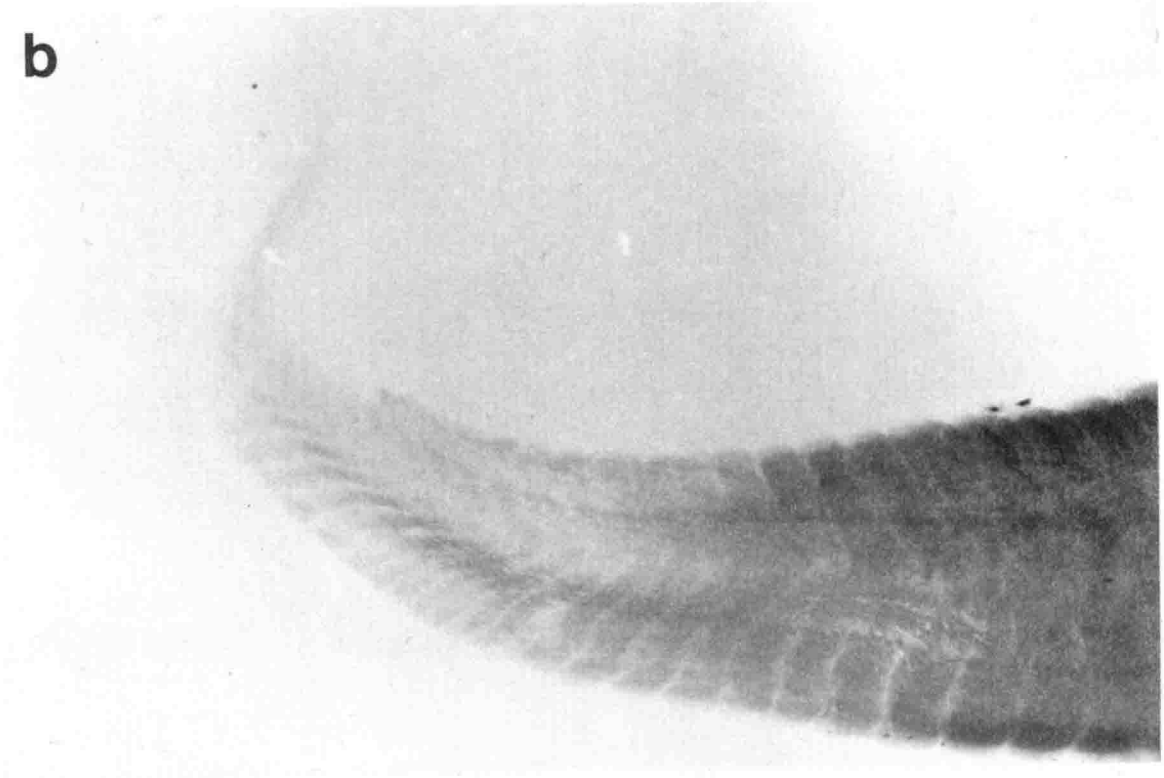

b

FIGURE 14 a, b. *In-situ* hybridization analysis of PTU-treated tail from whole tadpoles. Notice the hybridization of A7 around the borders of the somites (arrows) on the ventral and dorsal side of the tail at the base. There is no staining in the tip of the tail. This is similar expression seen in tails treated with T_3, and is unexpected based on the fact that PTU inhibits metamorphosis and T_3 production.


a


b

VITA

Bradley Thomas Butkovich was born on December 21, 1972, on Holloman AFB in Alamogordo, New Mexico. He graduated from Lake Braddock Secondary School, Burke, Virginia in 1990. He came to the University of Richmond on a football scholarship and pursued his interest in biology. He completed the requirements for the Bachelor of Arts in Biology in December 1993, and received it in May of 1994. In January, 1994, before receiving his B.A., he began work on his Master of Science Degree. He completed the requirements for the Master of Science in Biology in July, 1996. In the fall of 1996, he will be attending the Medical College of Virginia at Virginia Commonwealth University to pursue his M.D.