# ISOLATION AND CHARACTERISATION OF THYROID HORMONE-RESPONSIVE GENES OF AMPHIBIAN TAIL

BY

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*"In memory of my Parents"*

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# DECLARATION

I hereby declare that all of the results presented within this thesis were obtained by myself under the supervision of Dr. Robert Old, with the exception of those instances where the contribution of others has been acknowledged. These results have not been submitted for a degree at any other institute

# ABBREVIATIONS

| | |
|---|---|
| **ATP** | Adenosine triphosphate |
| **BSA** | Bovine serum albumin |
| **CTP** | Cytidine triphosphate |
| **DTT** | Dithiothreitol |
| **dATP** | 2'-deoxyadenosine 5'-triphosphate |
| **dGTP** | 2'-deoxyguanosine 5'-triphosphate |
| **dTTP** | 2'-deoxythymidine 5'-triphosphate |
| **dCTP** | 2'-deoxycytidine 5'-triphosphate |
| **FCS** | Bovine foetal calf serum |
| **IPTG** | Isopropyl-thio-β-D-galactoside |
| **PBS** | Phosphate buffered saline |
| **Pfu** | Plaque forming units |
| **TEMED** | N,N,N',N'-tetra-methylethylenediamine |
| **X-Gal** | 5-Bromo-4-chloro-3-indolyl-β-D-galactoside |
| **SDS** | Sodium dodecyl sulphate |
| **TBE** | Tris-borate electrophoresis |
| **Tris** | Tris (hydroxymethyl) aminomethane |
| **µg** | Microgram |
| **µl** | Microlitre |
| **µM** | Micromolar |
| **nl** | Nanolitre |
| **DMSO** | Dimethyl sulfoxide |
| **DNA** | Deoxyribonucleic acid |
| **KDa** | Kilodalton |
| **Kb** | Kilobase |
| **Bp** | Base pair |
| **mg** | Milligram |

| | |
|---|---|
| **ml** | Millilitre |
| **PAGE** | Polyacrylamide gel electrophoresis |
| **Gln** | Glutamine |
| **LB** | Luria broth |
| **RNA** | Ribonucleic acid |
| **RNAase** | Ribonuclease |
| **cDNA** | Complementary DNA |
| **CIP** | Calf intestinal alkaline phosphatase |
| **EDTA** | Diaminoethanetetra-acetic acid, sodium salt |
| **Ef** | Elongation factor |
| **°C** | Degrees centigrade |
| **mRNA** | Messenger RNA |
| **O.D.** | Optical density |
| **RNAsin** | Human placental ribonuclease inhibitor |
| **tRNA** | Transfer RNA |
| **EtBr** | Ethidium bromide |
| **PCD** | Programmed cell death |
| **OLB** | Oligolabelling buffer |
| **ESTs** | Expressed sequenced tags |
| **MQW** | Milli-Q-water |

# SUMMARY

Amphibian metamorphosis is a post-embryonic process that systematically transforms different tissues in a tadpole. This transformation requires extensive remodelling of almost every tissue in the animal. Thyroid hormone plays a causative role in this complex process by inducing a cascade of gene regulation. One of the more dramatic effects of thyroid hormone (triiodothyronine $T_3$) is to induce a complete regression of tadpole tail in culture in a simple chemically defined medium.

The technique of differential display proposed by Liang and Pardee in 1992, has been applied in an attempt to isolate and then characterise responsive genes induced by thyroid hormone triiodothyronine ($T_3$).

Library screens using the PCR fragment xL52 as a probe allowed the isolation of ~2.5kb clone termed *xth-2*. Sequence analysis and database searches at the amino acid level revealed that this clone (*xth-2*) showed approximately 91% identity to some of the members of a recently discovered family of tissue-specific transmembrane proteins called Hem proteins. Temporal expression of *xth-2* using RT-PCR technique revealed that this gene is developmentally regulated.

Whole mount *in situ* hybridisation used for detecting the location of this mRNA in *Xenopus laevis* embryos at different developmental stages indicated that *xth-2* protein was highly expressed in the brain and the pattern of expression has been extended along the central nervous system (CNS) and the caudal region (tail bud).

Expression of *xth-2* protein in *Xenopus* embryos, did not show any significant effect on the phenotypic features of the embryos examined.

The *Nap1* protein, a member of Hem family proteins has recently been found to associate with the SH3 domain of *Nck* protein, and is thought to play an important role in signalling transduction. We could therefore, speculate that protein *xth-2* will have the same function as does the *Nap1* on the basis of their sequence similarity, tissue distribution and also the expression pattern.

# CHAPTER 1

## INTRODUCTION

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### 1.1. General

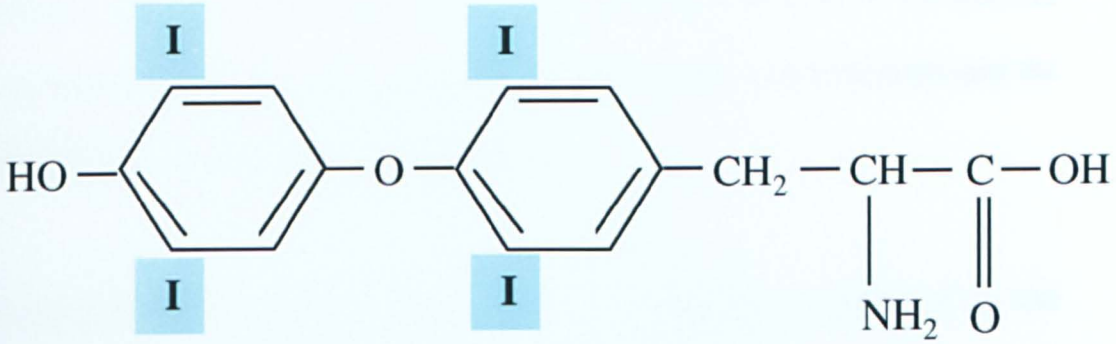
In 1912, J.F. Gudernatsch of Germany performed a landmark experiment. He fed young tadpoles with different horse tissues and observed their effect on tadpole morphology. Interestingly, he found that the thyroid gland could speed up the transformation of tadpoles to frogs. This unique observation sparked a series of studies that made amphibian metamorphosis one of the oldest and best studied hormone-regulated developmental processes (see Appendix A for the developmental series of *Xenopus* (Nieuwkoop and Faber, 1956)

It has long been shown that metamorphosis systematically transforms most, if not all, tissues and organs in a tadpole (Dodd and Dodd, 1976; Gilbert and Frieden, 1981). However, different organs undergo vastly different changes. Two of the extreme transformations include limb development and tail resorption. In the first example, cells predetermined to become a limb undergo rapid proliferation and subsequent differentiation to form the new structure. In contrast, the tail, a tadpole-specific organ comprising some of the same cell types (i.e., the connective tissue, muscle and epidermis) as the developing limb, completely degenerates during metamorphosis. Most other organs, however, are present in both the tadpole and the frog. To serve their function in a postlarvae frog, which has a different diet and

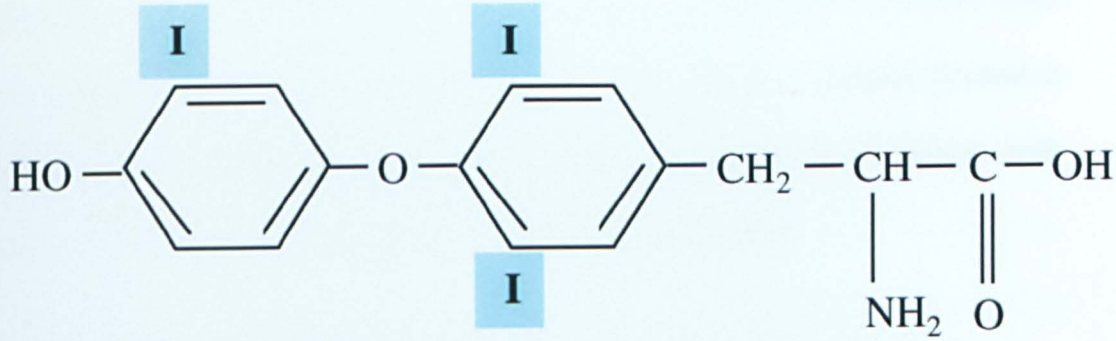
living habitat, several organs undergo drastic remodelling in a process that involves degeneration of specific larval cells, probably through apoptosis (programmed cell death) (Ishizuya-Oka and Shimozawa, 1992) and selective proliferation and differentiation of adult cells. For example, the liver is remodelled to produce an adult organ with a very different gene specific program. Thus, the activities of the urea-cycle enzymes in the liver are elevated in the frog compared to those in the tadpole as the animal changes from ammonotelism to ureotelism.

These early studies also provided considerable insights into the regulation of the developmental process by the thyroid gland. A few years after the first observation by Gudernatsch in 1912, Allen (1916) demonstrated that the thyroid gland is essential during natural frog metamorphosis because thyroidectomy prevented metamorphosis and consequently produced giant tadpoles. In addition, Kendal (1915) showed that the active ingredient in the thyroid gland is thyroid hormone TH (Kendall 1915). These studies led to the subsequent isolation and structural determination of the biologically active forms of the hormone, $T_3$ (3,5,3′-triiodothyronine) and $T_4$ (3,5,3′,5′-thyroxin) (see Figure 1).

FIG 1. Chemical structures of the thyroid hormones thyroxine and triiodothyronine. The two molecules differ by only one iodine atom, a difference noted in the abbreviations $T_3$ and $T_4$.




3,5,3',5'-Tetraiodothyronine (thyroxine $T_4$)




3,5,3'-Triiodothyronine ($T_3$)

Blocking the production of these hormones in the tadpoles by thyroidectomy or by using specific inhibitors of TH synthesis prevents metamorphosis, whereas adding pure TH to the rearing water of the tadpoles can initiate precocious transitions (Dodd and Dodd, 1976; Gilbert and Frieden, 1981). Although $T_4$ can induce metamorphosis, it is less active than $T_3$ and is generally believed to be converted to $T_3$ in order to function. Thus, these experiments unequivocally demonstrated the causative role of TH in frog metamorphosis.

Metamorphosis in amphibia and insects is a dramatic example of a late developmental switch, resulting in the reprogramming of morphological and biochemical characteristics of virtually every postembryonic and larval tissue (Gilbert and Frieden, 1981). Although all the major biochemical events also occur in mammalian embryonic or foetal development, little effort is currently devoted to studying the molecular mechanisms underlying metamorphosis, compared with work on gene expression during early embryonic development.

Two important features of metamorphosis render it an exceptionally attractive developmental process for understanding phenotypic switching. First, the process is under obligatory hormonal control, usually one hormone exerts juvenilising action by preventing or delaying development and the other initiates and sustains further development. Examples of these two hormone pairs are juvenile hormone and ecdysone in insects, and prolactin and thyroid hormone in amphibia (White and

Nicoll, 1981; Beckingham Smith and Tata, 1976; Tata, 1984). This dependence of metamorphosis on hormones offers a simple, effective tool to experimentally manipulate developmental changes in opposing directions. Second, the same hormonal signal initiates morphogenesis to generate new tissues, such as limbs or wings, restructuring some existing tissues to acquire new functions, such as the central nervous system and the hepatopancreas, while initiating cell death or apoptosis in others, such as in amphibian tail and gills or insect salivary glands.

Three groups of hormonal signals, released from specialised endocrine cells following environmental cues transmitted by the brain, determine the onset, rate and completion of metamorphosis. The hypothalmic peptides, thyroid releasing hormone (TRH) and corticotropin releasing factor (CRF) stimulate the pituitary to produce thyroid stimulating hormone (TSH) which, in turn, activates the thyroid gland of the tadpole. The activated thyroid gland secretes the thyroid hormones, L-thyroxine ($T_4$) and triiodo-L-thyronine ($T_3$), whose action is obligatory for the onset and completion of metamorphosis up to the adult stage. The pituitary also secretes the peptide hormone prolactin (PRL), which can prevent metamorphosis (White and Nicoll, 1981) an action essential for determining the timing of initiation of the programme for further development of the growing but not as yet fully differentiated tissues.

The simple exposure to $T_3$ can precociously induce metamorphosis in pre-metamorphic amphibian larvae, as well as in isolated larval tissues, and can induce the same adult gene products or processes as during normal development. The same hormone initiates a different developmental programme depending on the tissue, such as, for example, extensive morphogenesis, in limbs, the induction of hydrolases and other cell death determinants leading to the loss of whole organs, such as the tail and gills and functional reorganisation of the brain and gut, while producing more subtle changes as in the epidermis and eyes (see Table 1). All these thyroid hormone-dependent developmental changes can be blocked by raising the concentration of prolactin, thus extending the simple experimental manipulation of precociously inducing metamorphosis with $T_3$ to operationally ''freezing'' the process of postembryonic development of the early tadpole at any given stage.

**Table 1.** Diversity of biochemical responses during thyroid hormone-induced amphibian (anuran) metamorphosis (modified from Tata, 1996).

| Tissue | Biochemical response |
|---|---|
| Brain | Cell division, apoptosis and new protein synthesis |
| Liver | Induction of urea cycle enzymes and albumin,; Larval → adult haemoglobin gene switching |
| Eye | Visual pigment transformation (porphyropsin → rhodopsin); β–crystallin induction |
| Skin | Induction of collagen 63kDa (adult) keratin and magainin genes |
| Limb bud, lung | Cell proliferation and differentiation, chondrogenesis |
| Tail, gills | Programmed cell death; induction and activation of lytic enzymes (collagenase, nucleases, phosphatases); lysosome proliferation |
| Pancreas, intestine | Reprogramming of phenotype acquisition of new digestive functions |
| Immune system | Altered immune system and appearance of new immunocompetent components |

Hormonal induction of metamorphic processes and their inhibition by prolactin are direct and not systemic actions of the hormones, as they can be reproduced in organ culture. The ease with which many of the hormonal effects can be reproduced in tissue culture has considerably facilitated the study of developmentally programmed morphogenesis, cell death and specific gene expression, thus allowing a more detailed analysis of the mechanisms underlying postembryonic development (Tata, 1966; Yoshizato, 1989; Tata *et al.*, 1991). For example, it could be shown in organ cultures of *Xenopus* tails and limb buds that the addition of $T_3$ induces cell death and complete tissue regression and development, respectively (Tata *et al.*, 1991). In these experiments, prolactin prevented both regression of the tails and growth of limbs (i.e. the action of prolactin is to prevent both morphogenesis and cell death induced by thyroid hormones). Organ culture experiments have also made it possible to demonstrate that protein synthesis is required for programmed cell death.

Metamorphosis of amphibia is mainly considered. It is natural that many biologists have long been attracted by amphibian metamorphosis because it includes many basic biological processes, including cell differentiation, endocrinology, cell death, cell growth, and histolysis.

In the case of amphibia, all the tissues of tadpoles transform from the larval to the adult type during metamorphosis. The extent and the nature of the transformation

appear to be diverse and to depend on the particular organs; larva-specific, larva-to-adult, and adult-specific. The larva-specific organs exist and function only in a larva (e.g., gill and tail). The larva-to-adult organs exist and function throughout the larval phase to the adulhood; however, their structures and functions in the larva are different from those in the adult. This organ type includes the liver, gut, and the body skin. An example of the third type organ is the forelimbs, which do not exist in a larva but appear during metamorphosis and continue to function through adult life (Yoshizato, 1989).

### 1.2. Metamorphic transformation of larval organs and cell differentiation

In general, organs contain two types of tissues (i.e. epithelial and mesenchymal), the cells of which are called epithelial cells and mesenchymal cells, respectively. The cells of each type of tissues are thought to comprise two kinds of cell populations (1) undifferentiated and proliferative cells (germinative cells or stem cells), and (2) differentiated and nonproliferative cells (mature cells). Cells in an organ have their own life span and are engaged in a physiological turnover. When they come to the end of the span, they are lost from the tissue, the loss being compensated for by offspring of germinative cells. In this way the apparent cell number is kept relatively constant in a tissue (a state of dynamic equilibrium). In other words, a tissue, in general, contains specific populations of cells (germinative cells) that can enter the cycle of cell division to replace the mature cells that die (Yoshizato, 1989).

### 1.2.1 Larva-Specific-Organs

All the cells in this type of organ are subjected to cell death and destined to be removed from the body during spontaneous metamorphosis. Before metamorphosis, larva-type germinative cells proliferate and produce progeny that goes on to differentiate into larval type mature cells. At metamorphosis, it can be expected that both germinative and mature cells undergo cell death, because these larva-specific organs have no counterpart in the adult body. It should be emphasised that there seem to be two types of cell death (a) the physiological death of mature cells at the end of their life span (b) a metamorphosis related death of cells (metamorphic cell death). The latter type of death occurs in cells that are still proliferation-competent and may or may not be near physiological death. Even germinative cells could be subject to metamorphic cell death. Physiological cell death is considered to be thyroid hormone (TH)-independent, but metamorphic cell death appears to be thyroid hormone (TH)-dependent.

### 1.2.2. Removal of a larva-specific organ : Tail

Thyroid hormones play leading roles in these changes of larval cells. The larva-specific organ may be the simplest type to study in considering the metamorphic changes at the cellular level, because this organ type appears to be removed during metamorphosis and therefore does not require the presence of progenitor germinative cells for adult life.

### 1.2.3. Overview of the tadpole tail

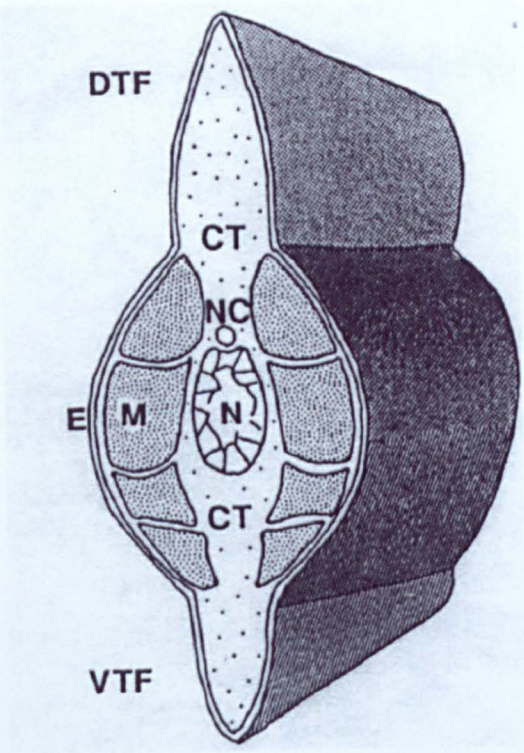
The tail constitutes a large proportion of the tadpole's body almost two-thirds of total body length in the case of bullfrog tadpoles. Tadpole tail has been one of the most frequently studied organs in experimental work on amphibian metamorphosis, for several reasons. The tail is easily recognisable, as an organ that is visible to the naked eye, without surgical intrusion, it shows dramatic changes during natural metamorphosis and also in response to exogenously administered TH (i.e., induced metamorphosis). It is known that the tail is one of the organs most sensitive to stimulation by TH. In addition, the tail can be cultured with relative ease for more than a week in a simple salt solution; furthermore, under these conditions the tail undergoes responsive changes in response to TH at physiological concentrations. Many studies on histolysis of the tail during spontaneous and TH-induced metamorphosis have accumulated since the last century (Frieden and Just, 1970).

The tail has a relatively simple architecture but does contain several tissues: nerve tissues, blood vessels, notochord, and muscle tissues (see Figure 2). Skeletal muscles are most voluminous. Highly hydrated gelatinous connective tissues surround the muscles and support the structure of the tailfin. The tail is covered by a thin epidermis. The cells proper to this organ therefore include epidermal cells, fibroblastic cells, macrophages, neurons, glial cells, endothelial cells and smooth muscle cells of blood vessels, chondrocytes, muscle cells. These are cells that

reside in the tail; the tail also contains cells that circulate through the entire body, red blood cells, leukocytes, and lymphocytes.

At the final stage of metamorphosis the whole structure of the tail disappears, as do various types of cells just mentioned as well as substances of the extracellular matrices (ECM) (Yoshizato, 1989).

FIG 2. A schematic representation of the tadpole tail. DTF, dorsal tailfin; CT, connective tissue; NC, nerve cord; E, epidermis; M, muscle, N, notochord, VTF, ventral tailfin (from Yoshizato, 1989).


DTF
CT
NC
E
M
N
CT
VTF

## 1.3. Morphological descriptions of breakdown of the tail

### 1.3.1. Overall features of tail regression

The tail regression is one of the most conspicuous morphological changes during anuran metamorphosis. All the materials of the tail tissues are subject to destruction. Regression of the tadpole tail seems to include two different processes: condensation and histolysis. Several workers have reported that the predominant initial response of the tissue to the metamorphic stimulus is water loss, resulting in an apparent tissue regression by the condensation of cells and extracellular matrix (ECM). The breakdown of tail requires cell-cell interactions and biochemical (enzymatic) reactions. The condensation might facilitate these cellular and biochemical processes. Another component of tail regression is a histolysis of the tissue. The condensation and the histolysis progress concomitantly from the beginning to the completion of the tail breakdown, which might be the basis of a well-organised process of degeneration. The condensation of tail tissues during metamorphosis might help to explain the mechanism of tissue breakdown, but this has received little attention. The mechanism of condensation or contraction of tissues has been largely unknown.

An experimental model in which fibroblasts are cultured three-dimensionally in a hydrated collagen lattice shows an extensive contraction of collagen fibrils resulting in a rearrangement of the fibrils. This cell-mediated condensation of the model is partly dependent on the presence of thyroid hormone TH (Yoshizato,

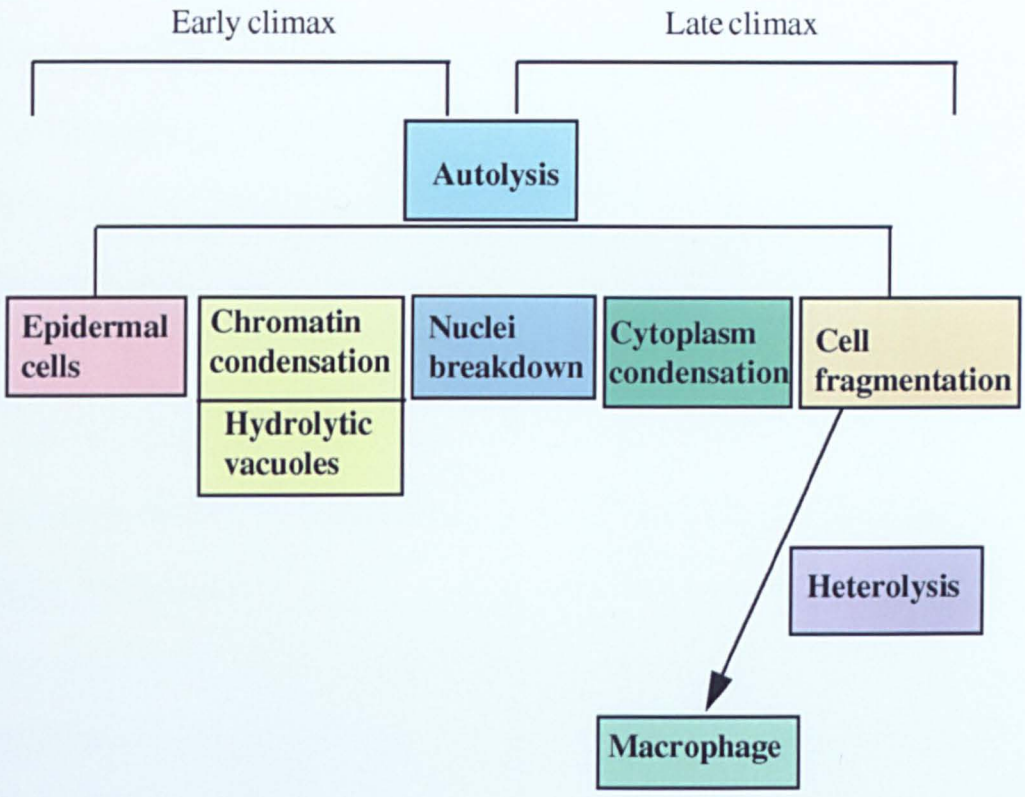
1989). These observations strongly suggest that tail dehydration during metamorphosis might also be induced by the action of TH. Thus condensation appears to be an important phenomenon in the mechanism of tissue regression.

There have been many terms used to refer to tail regression in the literature: breakdown, dissolution, autolysis, histolysis, phagocytosis, heterolysis, degeneration, regression, contraction, absorption, atrophy, and necrosis. It was suggested that the terms atrophy, necrosis, and degeneration are not suitable to describe the tail breakdown, because this process is composed of developmentally programmed cell death and organised removal of the dead cells and ECM by phagocytosis and enzymatic degradation. Apoptosis might be a suitable term for the tissue destruction of metamorphosing tadpoles (Yoshizato, 1989).

The metamorphic changes of tail epidermal cells were survyed electron-microscopically using tadpoles of *Rana japonica*. The earliest change is noticed in the outermost cells, where vacuoles with acid phosphatase activity appear and the cell membranes are destroyed. These changes spread toward the inner layers as metamorphosis progresses. The next changes observed are ruptures of structures of desmosomes, and the pyknosis of nuclei. Lymphocytes, neutrophils, and especially macrophages become conspicuous. Macrophages are often observed to phagocytose the dead cells. The destruction of tail skin cells proceeds in two sequential steps: autolysis and heterolysis. The autolysis is the death of epidermal

cells, which progresses inward the apical layer. The heterolysis is the phagocytosis of the autolysed cells by macrophages. The first change in the cells is a condensation of chromatin on nuclear membranes, followed by breakdown of nuclei and condensation of cytoplasm. As a result many fragmented cell bodies with cell membranes are produced. Organelles in the cell body such as mitochondria appear to be intact. These changes are not specific to metamorphosis but are generally observed in the process of programmed cell death in vertebrate embryogenesis and physiological turnover of cells. The fragmented cell bodies are named apoptotic bodies. Apoptotic bodies are then phagocytosed by macrophages (heterolysis) (Yoshizato, 1989). The process of removal of tail epidermal cells is schematically summarised in Figure 3.

FIG 3. Metamorphic changes in tail epidermal cells. (Adapted from Yoshizato, 1989).


Early climax
Late climax
Autolysis
Epidermal cells
Chromatin condensation
Hydrolytic vacuoles
Nuclei breakdown
Cytoplasm condensation
Cell fragmentation
Heterolysis
Macrophage

### 1.4. Competence and stage-dependent metamorphosis of individual tissues

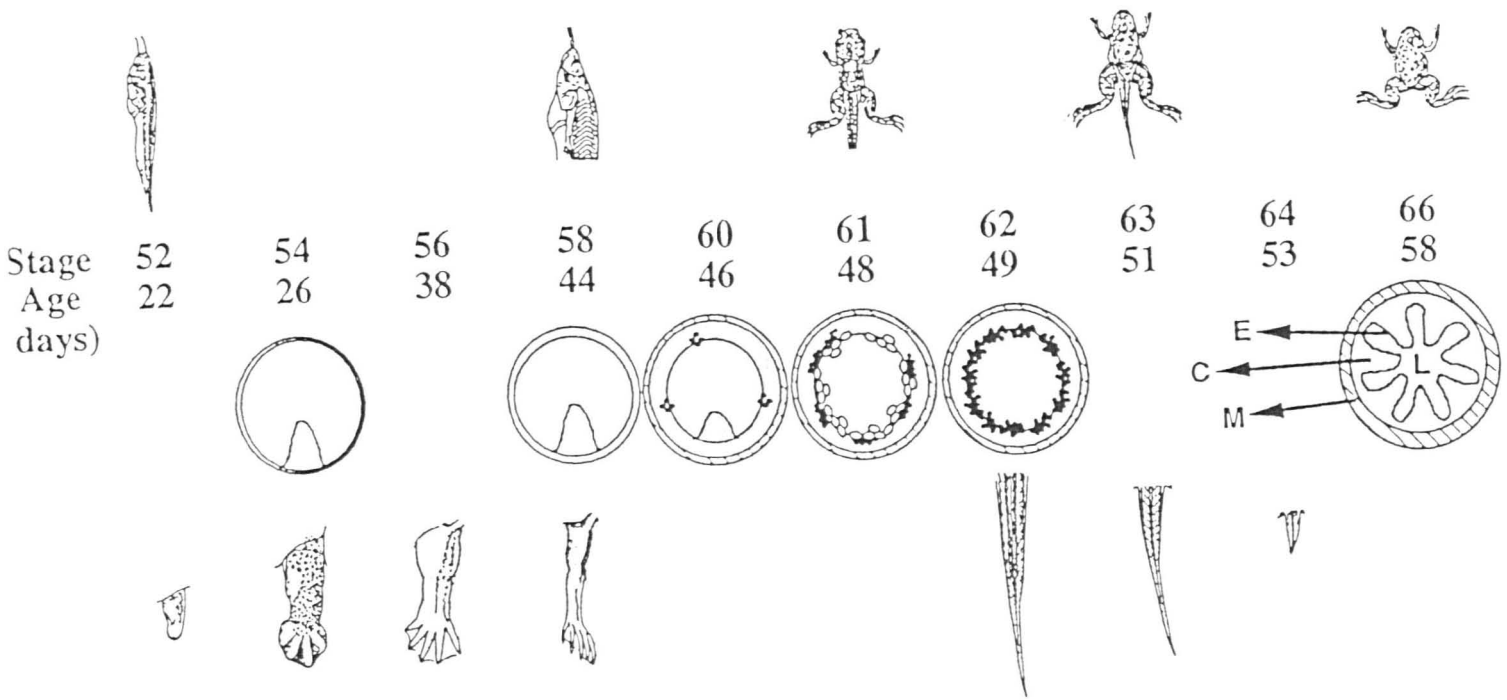
Natural metamorphosis takes place at precise developmental stages. This process however, can be precociously induced in premetamorphic tadpoles as early as stage 41 for *Xenopus laevis*, only 3 days after fertilisation, by treatment with exogenous TH (Dodd and Dodd, 1976; Gilbert and Frieden, 1981; Tata, 1968). Younger tadpoles or embryos were found to be refractory to this TH addition. This observation suggests that the whole animal is only competent to respond to TH at specific developmental stages.

This stage-dependent phenomenon is also observed to be true if one examines the metamorphic transitions of different tadpole organs during natural development; individual tissues undergo their unique metamorphic changes at distinct developmental stages. This is exemplified by the transformation of the hindlimb, tail and intestine in *Xenopus laevis* (see Figure 4). The earliest change to take place is hindlimb development (Nieuwkoop and Faber, 1956). The hindlimb bud begins to grow around stage 48 and the most dramatic morphological differentiation takes place around stage 53-56. Subsequently, the hindlimb undergoes mostly growth with few morphological changes. In contrast, tail resorption is one of the last changes to occur during metamorphosis (Nieuwkoop and Faber, 1956). While some changes in the tail, such as tail fin resorption, take place a little earlier (Dodd and Dodd, 1976), drastic tail resorption, as reflected by the reduction in tail length, begins around stage 62 and its completion marks the end of metamorphosis. The

intestine, on the other hand, begins its remodelling process around stage 58 when its connective tissue and muscle layers increase in thickness (McAvoy and Dixon, 1977). This is followed by degeneration of the larval epithelium through programmed cell death (apoptosis) around stages 60-62 (McAvoy and Dixon, 1977; Ishizuya-Oka and Shimozawa, 1992). Concurrently, secondary epithelial cells rapidly proliferate and differentiate toward the end of metamorphosis to form a much more complex frog organ (McAvoy and Dixon, 1977).

The molecular mechanisms underlying such developmental stage-dependent phenomena are still unclear. It is generally believed that the effect of TH is mediated through thyroid hormone receptors (TRs) by regulating gene expression. Such an assumption has been substantiated by molecular studies on TRs and genes that are regulated by thyroid hormone during metamorphosis (Tata, 1993; Shi, 1994).

FIG 4. Stage-dependent transformation of the hindlimb, intestine and tail of *Xenopus laevis* tadpoles. The developmental stages and ages of tadpoles are from Nieuwkoop and Faber (1956). Tadpole small intestine has a single epithelial fold, where connective tissue (C) is abundant, while a frog has a multiply folded intestinal epithelium (E), with elaborate connective tissue and muscle (M). Filled dots, proliferating adult intestinal epithelial cells, open circles, apoptotic primary intestinal epithelial cells, L, intestinal lumen (from Shi *et al.*, 1996).


Stage 52 54 56 58 60 61 62 63 64 66
Age 22 26 38 44 46 48 49 51 53 58
days)
E
C
M
L

## 1.5. Thyroid hormone receptors

In 1986 Evans and Vennstrom independently cloned the first TH receptors (Weinberger *et al.,* 1986; Sap *et al.,* 1986). These receptors were found to be transcription factors that can regulate gene transcription in response to TH (Tsai and O'Malley, 1994), supporting the earlier suggestion that TH controls metamorphosis by regulating gene expression in tadpoles (Dodd and Dodd, 1976; Gilbert and Frieden, 1981). Using the human TR cDNA as a probe, Brown's group cloned four TR genes in *Xenopus laevis* (Yaoita *et al.*, 1990), one of which is identical to the previously cloned *Xenopus* TRα gene (Brooks *et al.*, 1989). These genes are very similar to the TR genes in mammals and birds, and they belong to two families (α and β), as in other species. Similar observations have since been made in *Rana catesbeiana* (Schneider and Galton, 1991; Helbing *et al.,* 1992).

TRs belong to the rapidly expanding super-family of nuclear receptors, which has over 150 members including receptors for glucocorticoids, androgens, retinoic acids, etc. (Tsai and O'Malley, 1994; Mangelsdorf *et al.,* 1995). Members of this family share similar structural domains. Each of them has a DNA binding domain located in the amino terminal half and a hormone binding domain in the carboxyl terminal half. The DNA binding domains of different receptors share considerable similarity and are involved in the specific recognition of the corresponding hormone response elements, e.g. TRE (thyroid hormone response element) for

thyroid hormone receptors. All the other domains are, in contrast, unique to each receptor.

While TRs can bind TREs weakly as monomers and homodimers, they bind to TREs with much higher affinities as heterodimers with RXRs, the receptors for 9-cis retinoic acid (Tsai and O'Malley, 1994; Heyman *et al.,* 1992). More importantly, TR-RXR heterodimers can confer the specificity of gene regulation by TH in tissue culture cells, suggesting that they are *in vivo* partners of TRs (Tsai and O'Malley, 1994; Heyman *et al.*, 1992; Yen and Chin, 1994). Numerous studies, including *in vitro* DNA binding, cell culture transfection and functional studies in frog oocytes, have shown that TR-RXR heterodimers can bind to TREs even in the absence of TH (Tsai and O'Malley 1994; Zhang *et al.,* 1993; Yen *et al.*, 1994). However, such binding of a TRE present in a gene which is normally up-regulated by TH results in transcriptional repression. Upon TH binding to the heterodimers, the resulting liganded receptors become potent transcriptional activators (Tsai and O'Malley, 1994; Zhang *et al.* 1993; Yen, *et al.,* 1994; Ranjan 1994).

The exact mechanism of this activation by TH remains to be determined. However, TH binding has been shown to cause conformational changes in TRs (Bhat *et al.,* 1993 ; Toney *et al.,* 1993). More recently by using an oocyte transcription system, it was demonstrated that unliganded TR-RXR heterodimers can constitutively bind

to a TRE in a chromatinised template and that chromatin assembly and unliganded TR-RXR heterodimer binding can repress transcription synergistically. Addition of TH leads to over 200-fold transcriptional activation, accompanied by chromatin disruption. Such chromatin disruption is dependent upon TH but can occur even when transcription is blocked with α-amanitin. It was suggested so far that one mechanism of transcriptional activation by TH is through the disruption of the repressive chromatin structure.

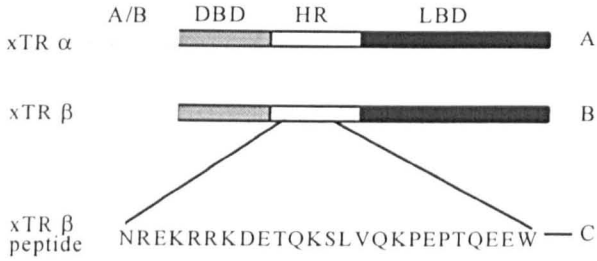
As the mediators of the effects of TH, TRs and RXR are expected to be present during amphibian metamorphosis. Indeed, the mRNAs of both TRα and TRβ are expressed during amphibian metamorphosis (Schneider *et al.*, 1991; Helbing *et al.*, 1992; Yaoita *et al.*, 1990; Kawahara *et al.*, 1991). In *Xenopus*, the TRα genes are activated shortly after tadpole hatching and their expression is maintained at high levels in tadpoles throughout metamorphosis. Although TRβ genes have little expression in premetamorphic tadpoles, they are dramatically activated during metamorphosis. Similarly, it has been found that both RXRα and RXRγ are also expressed in premetamorphic as well as metamorphosing tadpoles (Wong and Shi, 1995). It is expected that the third member of *Xenopus* RXR genes, the RXRβ gene which was cloned recently (Marklew *et al* 1994), is also expressed during metamorphosis.

More interestingly, when the expression of these receptor genes was analysed in individual organs during *Xenopus* metamorphosis, a strong correlation was found between their mRNA levels and organ-specific transformation (Wang and Brown, 1993; Yaoita *et al.* 1990; Wong and Shi, 1995; Shi *et al.* 1994). In general, TR and RXR genes are co-ordinately regulated during metamorphosis and their expression is high in a given organ when metamorphosis takes place and low before or after metamorphosis. Thus, the mRNA levels for these receptor genes (except for TRβ genes) are high in the hindlimb around stages 54-56 when hindlimb morphogenesis, e.g. digit formation, takes place. Similarly, in the tail, the genes are expressed at low levels until stage 62 when they are upregulated, coinciding with the rapid tail resorption. In the animal intestine, the mRNAs of both TRα and RXRα genes are present at intermediate levels and those of TRβ and RXRγ genes are upregulated during tissue remodelling (Wong and Shi, 1995; Shi *et al.* 1994). It should be pointed out, however that some differences do exist in the expression profiles of these receptor genes (Wong and Shi, 1995). For example, TRβ gene expression does not change as drastically as that of the TRα genes during hindlimb morphogenesis (stages 54-58), suggesting that TRα may play a more important role in this transition. In any case, these observations together suggest that TR-RXR heterodimers mediate the effects of TH during metamorphosis and that one mechanism for regulating stage-dependent tissue transformation is to modulate the levels of endogenous hormone receptors. The availability of these receptors may thus be required for the correct temporal regulation of changes in individual tissues.

So far there has been only one study reporting the distribution xTRα and β receptor proteins determined immunlogically with polyclonal antibodies in tissue extracts of *Xenopus* tadpoles (Eliceiri and Brown, 1994). According to these workers xTRα and β are maternally derived in early embryos before their transcripts are first detectable, and xTRα protein in the head and tail regions of the tadpole remains constant although its transcripts increase through metamorphosis. In contrast they found that both xTRβ mRNA and protein increased in parallel with endogenous TH during metamorphosis. However, it is worth noting that with the rat isoform TRβ2, there is no correspondence between TR mRNA and protein when the tissue distribution of mRNA and protein were analysed by *in situ* hybridisation and immunocytochemistry, respectively (Lechan *et al.*, 1993).

Fairclough and Tata (1997), made the first attempt to localise xTRα and β proteins in *Xenopus* tadpole tissues immunocytochemically with specific monoclonal antibodies (see Figure 5), during natural and $T_3$-induced metamorphosis.

FIG 5. Antigens used for raising and characterising antibodies against xTR α and β. A, full-length xTR α produced as a fusion protein (Machuca *et al.*, 1995; Ulisse *et al.*,1996). B, full-length xTR β produced as a fusion protein. C, synthetic 25-mer peptide representing a sequence in the hinge region that is unique to xTR β (Yoita *et al.*, 1990). A/B, N-terminal domain; DBD, DNA binding domain; HR, hinge region; LBD, ligand binding domain (from Fairclough and Tata, 1997).




As xTRα protein was present at relatively high levels and since its concentration was not significantly regulated during natural development and $T_3$-induced metamorphosis, the authors focused their attention largely on xTRβ protein. This receptor isoform is present at very low concentration in the liver and small intestinal epithelium at the limit of immunocytochemical detection in early tadpoles at stage 46 (see Table 2). The fact that *Xenopus* tadpoles at this stage exhibit a metamorphic response to exogenous $T_3$ (Tata, 1968; Tata, 1996) indicates the presence of functional TR. By metamorphic stage 52, that is, just prior to the activation of the larval thyroid gland, there is a low, but detectable level of TRβ, mostly localised in the nuclei, the liver, the intestine and hind limb-bud. This amount increased substantially in these tissues upon the onset of metamorphosis initiated by endogenous TH at stage 55. Exposure of stages 52 and 55 tadpoles to exogenous $T_3$ for 5 days caused xTRβ, but not xTRα, levels to increase substantially in all three tissues. Whereas this increase during onset of natural metamorphosis and artificial acceleration of the process in the liver and intestine

did not exhibit any preferential localisation within the tissue, it was topologically highly restricted to dense pockets of cells in the developing hind limb. This pattern resembled the localisation and migration of chondrocyte-rich cells in organ culture of hind limb-buds treated with $T_3$ (Tata *et al.,* 1991). Thus, these modulations of xTRβ protein in the liver, intestine and limb-bud are compatible with the developmental and hormonal regulation of accumulation of xTRβ transcripts (Baker and Tata, 1992; Yaoita and Brown, 1990).

The present results by Fairclough and Tata are in partial agreement with those of Eliceiri and Brown (1994), who quantified xTRα and β proteins by immunoprecipitation from extracts of *Xenopus* tadpole tissues. Like these authors, Fairclough and Tata found that xTRβ protein levels in non-hormonally treated tadpoles were generally lower than those of xTRα and that these increased more than xTRα during natural metamorphosis. They also found that in the cerebral cortex the levels were higher than in other tissues, but, unlike Eliceiri and Brown (1994), Fairclough and Tata found that neither isoform was substantially increased in the brain during natural and $T_3$-induced metamorphosis. Furthermore, their results show that xTRα levels are also enhanced, albeit to a lesser extent than those of the β isoform, during natural and $T_3$-induced onset metamorphosis (see Table 2).

**Table 2.** Summary of the data on the expression of xTRα and β proteins in the liver (Liv); small intestine (SI); and hind limb-bud (LB) of *Xenopus* tadpoles at different developmental stages during natural and thyroid hormone-induced metamorphosis. +/-, low (limit of detection); +, low (but easily detectable) ; ++, moderate ; +++, high ; ND, not determined (from Fairclough and Tata, 1997).

| | | xTR | Metamorphosis | |
|---|---|---|---|---|
| **Developmental stage** | **Tissue** | **Isoform** | **Natural** | **$T_3$-induced** |
| Early tadpole (46) | Liv | α | ND | + |
| | | β | + | ND |
| | SI | α | + | + |
| | | β | +/– | + |
| Premetamorphic (52) | Liv | α | ++ | ++ |
| | | β | + | ++ |
| | SI | α | + | ++ |
| | | β | +/– | ++ |
| | LB | α | + | ++ |
| | | β | +/– | |
| Onset of metamorphosis (55) | Liv | α | + | ++ |
| | | β | ++ | +++ |
| | SI | α | + | ++ |
| | | β | + | +++ |
| | LB | α | + | ++ |
| | | β | + | +++ |

## 1.6. Regulation of cellular TH levels

In addition to regulating the levels of hormone receptors another possible mechanism for determining the spatial and temporal aspects of metamorphosis would be the regulation of cellular TH levels in the animal. This could be achieved in several different ways. Firstly, is the actual production of TH in the thyroid gland. Secondly, the levels of cellular TH could be affected by cellular proteins that bind TH, called cytosolic thyroid hormone binding proteins (CTHBPs). The

metabolic conversion of $T_4$ to $T_3$ and or the inactivation of both hormones may be an important step in regulating the levels of functional TH.

### 1.7. Circulating thyroid hormone

Thyroid hormone is synthesised in the thyroid gland in two forms, 3,5,3′,5′-tetraiodothyronine ($T_4$-thyroxin) and its monodeiodinated form 3,5,3′-triiodothyronine ($T_3$). In *Xenopus laevis* the gland first appears in the embryo as a median thickening of the pharyngeal epithelium at the time of tadpole hatching (stages 35/36) (Dodd and Dodd, 1976). This rudiment then develops into a functional larval thyroid gland around stage 53. A similar developmental process has been observed in other anurans (Dodd and Dodd, 1976).

A systematic quantification of plasma TH levels during *Xenopus laevis* development was reported (Dodd and Dodd, 1976). They found detectable levels of $T_4$ in the larval plasma as early as stage 54, shortly after the formation of the functional thyroid gland. The levels of plasma $T_3$ appear to be lagging slightly behind $T_4$ during development. These low levels of endogenous TH could apparently trigger the initiation of metamorphosis with the morphogenesis of the hindlimb, as one of the first visible changes, to take place around stages 53-56. Subsequently, the plasma concentrations of both thyroid hormones increase, reaching peak levels around stage 60, the climax of metamorphosis. Currently, the mechanisms underlying the developmental regulation of the plasma TH levels are

unknown. However, many factors appear to be involved, including those that influence the growth and maturation of the thyroid gland, and hormonal clues that regulate thyroid hormone synthesis and release from the gland (Dodd and Dodd, 1976; Gilbert *et al.,* 1996). Regardless of the regulation mechanisms, the high levels of TH during development correspond exactly to the period of metamorphosis, consistent with the causative role of TH during this tadpole-to frog transition.

### 1.8. Cytosolic thyroid hormone binding proteins (CTHBPs)

In order to regulate cellular gene expression, the circulating TH in the plasma has to be transported through yet undefined mechanisms into target cells in different tissues. Upon entering the cytoplasm, it is likely to encounter cytosolic TH binding proteins (CTHBPs). Several such cellular proteins have been characterised (Shi *et al* 1994; Cheng, 1991; Yamauchi and Tata, 1994). In general, CTHBPs are multifunctional proteins that serve one or more other roles (e.g. pyruvate kinase, myosin light chain kinase and disulfide isomerase) and bind TH with 10-100 fold weaker affinity than the nuclear TRs.

The exact roles of these CTHBPs in TH signal transduction are still unclear. These proteins could participate in TH import from the extracellular medium, intracellular TH metabolism and transport to the nucleus, or serve as buffer to modulate intracellular free TH concentrations. This last function is strongly supported by

studies on the human pyruvate M2 kinase (Ashizawa and Cheng, 1992). As a monomer, it binds TH with affinity and specificity, while the homotetramer form functions as the M2 pyruvate kinase. Interestingly, over-expression of the monomer form of the protein leads to an inhibition of TH-dependent transcriptional activation by TR in a tissue culture cell line. Based on this observation, Cheng and co-workers suggest the monomer form of M2 pyruvate kinase functions as a chelator of cellular TH, thus reducing cellular free TH concentration and inhibiting the effect of TH (Ashizawa and Cheng, 1992).

To further understand the exact roles of cellular thyroid hormone binding proteins, two CTHBPs have been identified in *Xenopus laevis*. The first one was isolated from frog liver due to its ability to bind TH (Yamauchi and Tata, 1994). Partial peptide sequencing showed that it is likely to be the frog homolog of mammalian and avian cytosolic aldehyde dehydrogenase. Currently, it is unknown whether it is expressed during metamorphosis and how it is regulated. The second frog CTHBP gene was cloned on the basis of its homology to the human M2 pyruvate kinase gene (Shi, 1994). The deduced amino acid sequence of the frog CTHBP is about 90% identical to the human protein, with all known important regions completely conserved. Thus, while the biochemical function of the protein has not been studied, it is likely to function both as a pyruvate kinase in the tetramer form and a CTHBP in the monomer form.

The potential role of the frog M2 pyruvate kinase, referred to as xCTHBP, during metamorphosis is suggested by the tissue-dependent developmental expression of its mRNA. Of the three *Xenopus laevis* organs analysed, the intestine has very low but relatively constant levels of xCTHBP mRNA during development (Shi *et al.*, 1994). In contrast, the xCTHBP expression in the tail and hindlimb is drastically altered during metamorphosis. Low levels of xCTHBP mRNA are present in either the hindlimb during morphogenesis (stages 54-56) or tail during resorption (stages 62-64). Interestingly, the xCTHBP mRNA levels increase drastically in the growing hindlimb and decrease precipitously in the resorbing tail.

### 1.9. Deiodinases

TH is synthesised initially as $T_4$ or thyroxin in the thyroid gland (St. Germain, 1994a). A fraction of the $T_4$ in turn is converted in the thyroid gland into $T_3$ by 5'-deiodinases, the more potent form of TH (Jorgensen, 1978; Weber, 1967). Both $T_3$ and $T_4$ are then secreted into the serum and carried to the target tissues. The hormones are presumably transported into the cells either via carrier proteins or through energy-driven transport processes (Robbins, 1992). Within the target cells, the levels of the hormones can be further modulated due to local metabolism. This includes the conversion of $T_4$ to $T_3$ to enhance the effect of TH and the inactivation of both forms through 5'-deiodination, conjugation, deamination and oxidative decarboxylation (St. Germain, 1994a). Among them, the best studied is deiodination.

Two families of deiodinases have been discovered (St. Germain 1994a). These are the 5′-and 5-deiodinases. At least two different 5'-deiodinases have been identified that have different enzymatic characteristics. These enzymes appear to have different tissue distributions in mammals, with the type I form high in the liver, kidney and thyroid, and the type II form high in the brain, pituitary and brown adipose tissue of adult rat (St. Germain, 1994a). Thus, it seems likely that in developing animals, these activities are regulated in a tissue-specific manner. Unfortunately, the corresponding genes had not been cloned in amphibians, making it difficult to determine their expression during metamorphosis.

In contrast to 5′-deiodinases, which convert $T_4$ to the more active form $T_3$, 5-deiodinases convert $T_4$ and $T_3$ to $rT_3$ (reverse $T_3$) and $T_2$, respectively (St. Germain, 1994a). Since both $rT_3$ and $T_2$ have little affinity to TRs, the action of 5-deiodinases inactivates TH, thus inhibiting the effect of TH. A 5-deiodinase gene was recently cloned from *Xenopus laevis* due to its upregulation in the tail by the TH treatment of premetamorphic tadpoles (St. Germain *et al.*, 1994b). Its sequence shares considerable homology in several regions with the rat type I 5′-deiodinase. However, it functions primarily as a 5-deiodinase, with its 5′-deiodinase activity about 600-fold weaker (St. Germain *et al.*, 1994b). Interestingly, its expression during *Xenopus* metamorphosis is regulated in a tissue-specific manner (Wang and Brown, 1993; St. Germain *et al.*, 1994b). It has little expression in the intestine and in the hindlimb, it is not expressed until about stage 60, when its mRNA level

is upregulated, paralleling the increase in limb size. In contrast, relatively high levels of its mRNA are present in the tail during premetamorphic stages. Immediately prior to rapid tail resorption (stages 58-61), the expression of the 5-deiodinase gene is suddenly up-regulated several-fold. Its mRNA levels then return to the premetamorphic levels as tail resorption takes place after stage 61. Thus, as in the case of the xCTHBP, these expression profiles implicate a role for 5-deiodinase during the metamorphosis of the tail and the limb.

### 1.10. Molecular basis for competence and timing of tissue specific transformation

The development of amphibians proceeds by a complex genetic program, which produces a larval form in the absence of a functional thyroid gland (Dodd and Dodd, 1976; Nieuwkoop and Faber, 1956). This phase of development is followed by metamorphosis, a process involving a complicated co-ordination of the transformations of individual tissues. The development of the functional thyroid gland seems to play a critical role in initiating this metamorphic process. This is because tadpoles prior to the formation of a functional thyroid gland at stage 53 in *Xenopus* (Dodd and Dodd, 1976) can undergo precocious metamorphosis when exposed to exogenous TH (Dodd and Dodd, 1976; Gilbert and Frieden, 1981; Tata, 1968), suggesting that the only missing signal is the hormone itself. However, several levels of regulation are involved to ensure proper metamorphic transitions. Thus embryos and tadpoles younger than a critical stage (stage 41 for *Xenopus*)

(Tata, 1968) do not respond to exogenous TH. Furthermore, different tissues undergo transformations at very different developmental stages. Therefore, like the metamorphic process itself, the mechanisms underlying these regulations are expected to be very complex.

At least two factors are critical in the signal transduction by thyroid hormone during metamorphosis. These are the levels of the receptors and the concentrations of intracellular free TH. As summarised above, the expression of the receptor genes and those genes that can influence cellular free TH concentrations are regulated in a tissue-specific and developmental stage-dependent manner.

Assuming that in general the mRNA levels reflect the levels of the corresponding proteins, the observed regulation of these genes provides a molecular model for the competence of the tadpoles to respond to TH and tissue-specific developmental regulation of metamorphosis. Thus, in *Xenopus laevis* embryos and tadpoles before stage 40, neither the TRα nor the TRβ, the only known TR genes in all animal species, are expressed. This results in a lack of functional TR/RXR heterodimers and is probably responsible for the inability of embryos or young tadpoles to respond to the exogenous TH. After stage 40, the expression of TRα and RXRα genes is up-regulated. This up-regulation results in the formation of TR/RXR heterodimers, thus making the tadpoles competent now to respond to exogenous TH.

Interestingly, the exogenous TH synthesis is not detectable until around stage 54. It is unclear why the animal needs to have functional TR/RXR heterodimers before the formation of a functional thyroid gland. However, based on the fact that unliganded TR/RXR heterodimers can bind to TREs in chromatin and suppress basal transcription of the TRE containing the target genes, one possible function of the early expression of TR and RXR genes is to repress any potential leaky expression of genes that participate in metamorphosis, thus preventing premature tissue transformation. In fact, such a possibility has been suggested as an explanation for the bimodal expression of the matrix metalloproteinase gene stromelysin-3 in *Xenopus* (Patterton *et al.*, 1995). This gene is known to be regulated by TH directly at the trancriptional level (Wang and Brown, 1993; Shi and Brown, 1993). It has one period of expression during late embryogenesis and another during metamorphosis (Patterton *et al.*, 1995). Its repression after embryogenesis coincides with the up-regulation of TRα and RXRα expression in the absence of TH.

The regulation of receptor gene expression in individual tissues during metamorphosis will also probably contribute to the timing of tissue-specific metamorphosis. In addition, the tissue-specific regulation of at least two types of genes, the CTHBP and 5'-deiodinase genes, could influence the metamorphic timing as well. That is, the expression of these genes could lead to tissue-specific regulation of the cellular free TH levels, even though all organs/tissues are exposed

to the same levels of circulating TH synthesised in the thyroid gland. Thus, the combination of the levels of TR/RXR heterodimers and available cellular TH could act as a causative factors determining the timing of the transformations of individual tissues.

For example, in the hindlimb at stages 54-56, the xCTHBP and 5'-deiodinase genes are repressed. This should allow for the accumulation of relatively high levels of cellular free TH (see Table 3). The free TH could in turn interact with the very high levels of TR/RXR heterodimers to activate the limb morphogenic process. It should be pointed out that even though the plasma TH levels are low around stages 54-56, about 1-2nM $T_4$ and no detectable $T_3$, the dissociation constant of TR-TH complexes is even lower, about 0.1nM or less (Weinberger *et al.*, 1986).

**Table 3.** Comparison of the expression of TR/RXR and the levels of the factors affecting cellular free TH with organ transformation. The up and down arrows indicate that the mRNA levels of the corresponding genes change from low to high or from high to low, respectively, during the indicated stages. The cellular free TH concentrations are estimated on the basis of the plasma TH concentrations and the relative levels of CTHBP and 5'-deiodinase, assuming that the mRNA levels reflect the protein levels (modified from Shi *et al.*, 1996).

| | Hindlimb | | Intestine | Tail | |
|---|---|---|---|---|---|
| Stages | 54-56 | 58-66 | 58-66 | 54-60 | 62-66 |
| Plasma TH | Low | High | High | Low-High | High |
| CTHBP mRNA | Low | ↑ | Low-Moderate | High | ↓ |
| 5'-deiodinase mRNA | Low | ↑ | Low | ↑ | ↓ |
| Free cellular TH | Moderate | Low | High | Low | High |
| TR/RXR mRNA | Very High | Low | Moderate-High | Low | ↑ |
| Biological effect | Morphogenesis | Growth | Remodelling | Little change | Resorption |

Thus, it is possible to accumulate sufficient levels of cellular TH to bind to TR/RXR heterodimers, thus activating the limb morphogenic program. On the other hand, in the tail, the xCTHBP gene is expressed at very high levels up to approximately stage 62. Consequently, the cellular free TH levels could be limiting. Furthermore, when the plasma TH concentrations rises between stages 56 and 62, the 5-deiodinase expression also increases. This could counteract any resulting increase in cellular TH by converting the cellular TH into inactive forms. Thus, despite the high levels of plasma TH during this period, the intracellular free TH is likely to be very low (see Table 3). This, coupled with the relatively low levels of TR and RXR expression, will efficiently suppress tail resorption. After stage 60, the TR and RXR genes are up-regulated and the xCTHBP and 5'-deiodinase genes are down-regulated, thus allowing the activation of the tail resorption process.

Finally, in the intestine, the xCTHBP and 5'-deiodinase have very little expression throughout metamorphosis. The controlling factors appear to be the levels of the receptors and the plasma TH. While the TRα and RXRα genes are expressed at relatively constant levels, the expression of TRβ and RXRγ genes and the plasma TH levels are upregulated between stages 58-66 (Wong and Shi, 1995) (see Table 3). This period corresponds exactly to the period of intestinal remodelling. Thus, the tissue-specific temporal regulation of the receptor genes and intracellular free TH concentrations appear to be important molecular factors that help determine the

competence of premetamorphic tadpoles to respond to exogenous TH and the developmental regulation of tissue-specific metamorphosis.

While the mechanism of TH action proposed above for metamorphosis is based largely on the studies in one frog species, it is likely to be applicable for biological functions of other hormones of the nuclear receptor superfamily. However, compared to the regulation of amphibian metamorphosis by thyroid hormone, considerably less is known about the developmental and organ-specific regulation of these other hormones and their receptors. Interestingly, at least two other cases exist where the regulation of the levels of the hormones and their receptors appear to be important. The first one is insect metamorphosis. Like amphibians, insects undergo a larva-to-adult transition in a hormone (ecdysone) controlled process (Gilbert and Frieden, 1981). Recent molecular investigations have provided evidence that a complex regulation of ecdysone titers and its receptor gene expression is required to effect the proper tissue transformations (Thummel, 1995).

In the second case, the regulation of cellular retinoic acid concentrations by cellular retinoic acid binding proteins have been implicated to play a role during limb development an birds and mammals (Maden *et al.*, 1988). This is in part based on the fact that over-expression of a cellular retinoic acid binding protein decrease the retinoic acid-dependent gene activation in tissue culture cells (Boylan and Gudas, 1991). In addition, the expression of cellular retinoic acid binding protein gene is

regulated in a gradient fashion opposite to that of retinoic acid during limb development, suggesting that the function of these proteins may be to sequester retinoic acid in regions where it is not required, or required only at low levels (Maden *et al.*, 1988; Dolle' *et al.*, 1989).

### 1.11. Role of proteinases: Remodelling, Metastasis, and Apoptosis

For many years it has been proposed that proteolytic enzymes play a part in tail resorption and organ remodelling (Weber, 1969; Gross and Nagai, 1965). Collagenase enzymatic activity was demonstrated 30 years ago to be greatly increased during tail resorption (Gross and Nagai, 1965). The activation of lysosomal enzymes has been implicated as well (Weber, 1969). Four proteolytic enzymes have been identified as part of the tail program. Collagenase 3 and stromoelysin are members of a family of matrix metalloproteinases, while the third enzyme, FAPα, is an integral membrane protein that is a member of a family of serine proteinases (Ogata and Ikehara, 1989). The expression of stromoelysin 3 has also been correlated with remodelling and apoptosis. The invasion of collagen basement lamella by mesenchymal cells during tail resorption is reminiscent of metastases (Brown *et al.*, 1996).

The fourth proteinase (encoded by gene D) appears to be the first eukaryotic example of a class of proteinases related to a bacterial cytoplasmic peptidase (pepE). If pepE is cytoplasmic as its structure suggests, then it resembles the

interleukin converting enzyme (ICE) that has been shown to be involved in apoptosis and also cleaves aspartate residues. There are no other obvious similarities between the two proteins. The prominence of proteinases whose genes are up-regulated in the resorption program raises the question of whether the digestion of the extracellular matrix by these enzymes is responsible for the death of tail cells as well as their resorption (Brown *et al.*, 1996).

Isolated tails in organ culture (Weber, 1969) and tail cells in primary culture have been shown to respond to TH by resorption and growth arrest, respectively, but there is also evidence that epidermis is required for tail resorption (Niki *et al.*, 1982). Therefore, the extent that tail resorption is cell autonomous remains question. The large number of delayed genes that encode secreted or membrane-bound products emphasise the importance of events outside the cell or at its surface in the tail resorption program (Brown *et al.*, 1996).

### 1.12. Role of Prolactin in metamorphosis

Prolactin (PRL) is a peptide hormone secreted by the pituitary gland (White and Nicoll 1981). Exogenously administered PRL has been known to block $T_3$-induced metamorphosis in many species of amphibia in whole tadpoles; as well as in tail explants in culture (White and Nicoll, 1981; Ray and Dent, 1986). Its action can be compared with the antimetamorphic action of juvenile hormone in preventing ecdysteroid-dependent metamorphosis in insects (Gilbert and Frieden, 1981).

The addition of PRL to the $T_3$-induced, cultured *Xenopus* tadpole tails blocked tail regression. The regression of the cultured tails can be monitored simply by measuring the reduction in tail length. That this parameter is a good index of cell loss is shown by the parallel loss of DNA. Recently, widespread distribution of apoptotic cells has also been observed as judged by nuclear condensation and fragmentation, in *Xenopus* tadpole tails following $T_3$ treatment. How PRL acts to inhibit $T_3$-induced metamorphosis is not known. Only recently has the PRL receptor been characterised and it appears that the action of the receptor-ligand complex is transmitted intracellularly via the activation of JAK2 or src family of tyrosine kinases. As a consequence, the action of PRL would follow the classical pathway of signal transduction leading to the phosphorylation-dephosphorylation of key regulatory proteins, including transcription factors.

The interaction between PRL and $T_3$ can be considered as an example of cross talk between hormonal signals. It has recently been shown that the *Xenopus* TRβ gene promoter contains thyroid hormone responsive elements whose activities can be regulated by liganded TR (Ranjan *et al.*, 1994; Machuca *et al.*, 1995). Meanwhile, this hormone is a useful tool, for experimentally exploring the various facets of programmed cell death ( PCD) in a dual hormonally regulated system.

In addition, PRL would affect the autoinduction of *Xenopus* thyroid hormone receptors (xTR) while inhibiting the action of $T_3$ in whole *Xenopus* tadpoles and in

organ cultures of tails and limb buds (Tata *et al.*, 1991; Baker and Tata, 1992; Tata 1993; Rabelo *et al.*, 1994). There was a clear correlation between the prevention of $T_3$-induced metamorphic process by PRL with the inhibition of autoinduction of TRα and $\beta$ mRNA, as judged by biochemical analysis and *in situ* hybridisation (see Table 4)

**Table 4.** Relative accumulation of TRα and β in early stages of *Xenopus* tadpoles following treatment with $T_3$ and prolactin (from Tata, 1996).

| | Relative units | |
|---|---|---|
| Treatment | TRα | TRβ |
| None | 505 | 24 |
| $T_3$ | 1290 | 368 |
| $T_3$ + PRL | 799 | <10 |
| PRL | 405 | 43 |

It should be noted that PRL alone did not significantly affect the basal level of TR transcripts but that it prevented their up-regulation by $T_3$. This inhibition of TR autoinduction, coupled to loss of metamorphic response to TH, indirectly suggests that TR autoinduction is an essential requirement for the hormonal initiation and maintenance of amphibian metamorphosis (Tata, 1996)

## 1.13. Wider implications of thyroid hormonal regulation of amphibian metamorphosis

The interplay between thyroid hormone and PRL is relevant to the question of cross-talk, between signals acting via receptors located in the plasma membrane with those in the nucleus. There is now increasing evidence that such signal transduction processes, such as protein phosphorylation, $Ca^{2+}$ and $IP_3$ signalling G

protein-linked functions and *jun/fos* oncogene complexes modulate nuclear receptor function. Many recent investigations particularly highlight the modulation by peptide hormones and growth factors of phosphorylation of nuclear receptors, a process considered important for their nuclear transport, dimerisation and ligand binding. It is interesting to note that *Xenopus* TRβ promoter has several SP1 sites that are involved in regulation via the *fos/jun* pathway. There is also increasing evidence showing cross-regulation of nuclear receptors by their respective ligands Tata, 1994). An important consequence of interactions among nuclear receptors and between them and extranuclear factors whose activities are controlled by extracellular signals, would be to provide a combinatorial mechanism for hormone-specific positive and negative modulation of different gene that would not otherwise be effected via individual receptors. As cross-talk systems are better defined at the molecular level, it will become increasingly clear that complex intracellular networks of hormonal and non-hormonal signals facilitate a well-co-ordinated and homeostatically controlled regulation of growth and development (Tata, 1996).

How widely does the phenomenon of autoinduction of receptor occur? A survey of literature (Tata *et al.,* 1993; Tata, 1994) indicates its widespread manifestation during development regulated by ligands of various nuclear receptors (see Table 5). Besides the example of autoinduction of TR during metamorphosis, the up-regulation of the oestrogen receptor by its own ligand during amphibian

vitellogenesis has also been well documented (Perlman *et al.,* 1984). In embryonic mouse tissues, retinoic acid up-regulates all three isoforms of the retinoic acid receptor. More relevant to metamorphosis is the finding that ecdysteroid up-regulates the expression of its own receptor transcripts in *Drosophila* cells. The phenomenon of autoinduction of nuclear hormone, therefore, now appear to be a general feature of hormone-dependent postembryonic growth and development (Tata, 1996).

**Table 5.** Some examples of autoinduction of nuclear receptors by developmental signals (from Tata, 1996)

| Developmental signal | Receptor | Species | Function |
|---|---|---|---|
| Thyroid hormone | TRα and β | *Xenopus* | Metamorphosis |
| Ecdysone | EcR (E75) | *Drosophila* | Metamorphosis |
| Retinoic acid | RAR β | Mouse | Morphogenesis |
| Oestrogen | ER | *Xenopus* | Vitellogenesis |
| Androgen | AR | Rat | Morphogenesis |

## 1.14. Mechanisms of hormone action

Because they travel in the blood, hormones are able to reach virtually all tissues. Yet the body's response to a hormone is not all-inclusive but highly specific, involving only the target cells for that hormone. The ability to respond depends upon the presence on (or in) the target cells of specific receptors for those hormones.

The response of a target cell to a chemical messenger is only the final event in a sequence that begins when the messenger binds to specific receptors on the cell. Where in the target cells are the hormone receptors located? The receptors for steroid hormones and the thyroid hormones are proteins inside the target cells. Because these hormones are lipid-soluble, they readily cross the plasma membrane and combine with their specific receptors. In contrast, the receptors for the peptide hormones and catecholamines are proteins located on the plasma membranes of the target cells. However, many peptide hormones do in fact gain entry to the interior of cells by endocytosis of the plasma membrane hormone-receptor complex. It is likely that most of the hormone molecules internalised in this way simply undergo catabolism, but it is also possible that either they or the peptide fragments resulting from their catabolism exert additional effects upon the target cell.

Hormones can alter not only their own receptors but the receptors for other hormones as well. If one hormone induces a loss of a second hormone's receptors, the result will be a reduction of the second hormone's effectiveness. On the other hand, a hormone may induce an increase in the number of receptors for a second hormone. In this case the effectiveness of the second hormone is increased. This last phenomenon underlies the important hormone-hormone interaction known as permissiveness.

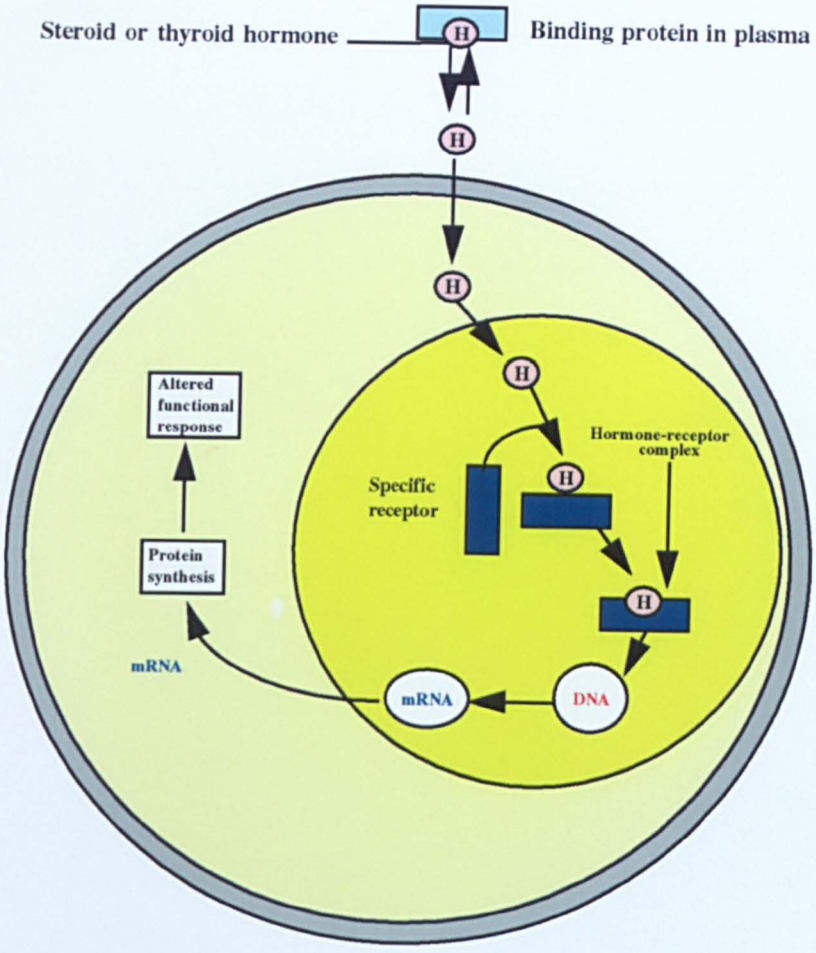
In general terms, permissiveness means that hormone A must be present for the full exertion of hormone B's effect. A low concentration of hormone A is usually all that is needed for this permissive effect, which is due to A's positive effect on B's receptors. For example, epinephrine cause marked release of fatty acids from adipose tissue, but only in the presence of permissive amounts of thyroid hormone, because thyroid hormone facilitates the synthesis of receptors for epinephrine in adipose tissue.

The ability of one hormone to influence the receptors of a second hormone also underlies certain situations in which a response requires actions of hormones in a sequence. For instance, ovulation requires the action of two different hormones secreted by the anterior pituitary; the first hormone paves the way for the second hormone by increasing the number of the latter's receptors in the relevant ovarian cells.

The common feature of the effects of steroid and thyroid hormones is an increased synthesis of specific proteins, enzymes, structural proteins and so on by their target cells. These hormones increase protein synthesis by stimulating the production of mRNA via the following sequence of events. First, a steroid or thyroid hormone enters a target cell and binds to specific receptors for it (Figure 6). The receptors for these hormones are synthesised in the cytoplasm but transported into the nucleus. In most cases, the hormone enters the nucleus and combines with the

receptor already there. However, in other cases, for example, cortisol, the hormone combines with the receptor still in the cytoplasm, and the entire complex is transported into the nucleus. In all cases, the receptor, activated by its binding of the hormone, interacts with segments of DNA specific for it to trigger transcription of those segments. That is, the activated receptor stimulates the synthesis of mRNA, which then enters the cytoplasm and serves as a template for synthesis of a specific proteins (Vander *et al.,* 1990).

FIG 6. Mechanisms of hormone action: Steroid and thyroid hormones enter the target cell and bind to their specific receptors, usually in the nucleus. The hormone-receptor complex is then transported into the nucleus. The activated receptor interacts with specific segments of DNA and stimulates transcription of new mRNA, which directs the synthesis of the proteins that mediate the cell's response. For generality, accessory proteins that associate with the nuclear receptors have been omitted.


Steroid or thyroid hormone
H
Binding protein in plasma
H
H
H
Altered functional response
Hormone-receptor complex
Specific receptor
H
Protein synthesis
H
mRNA
mRNA
DNA

## 1.15. Programmed cell death: an important feature of amphibian metamorphosis

Upon the onset of natural or induced metamorphosis, the amphibian tadpole shows rapid and substantial loss of cell numbers in many tissues until metamorphosis is completed. The substantial loss of mass and cell number is not restricted to tissues that undergo total regression, such as the tail and gills, but there is also extensive cell death in tissues that are restructured, such as the intestine, pancreas, skin and the central nervous system. With the establishment of the technique of TH-induced regression of isolated tadpole tails, it was possible to show that $T_3$ simultaneously augmented the amount of several lytic enzymes and the protein and RNA synthesising activity of *Xenopus* tadpole tails in organ culture. Several laboratories observed that actinomycin D, puromycin and cycloheximide prevent $T_3$ from inducing cultured *Xenopus* tails to regress (Tata, 1994). Actinomycin D produced a paradoxical situation in that an agent that would normally provoke cell death protected the tadpole tail programmed for total regression against the signal initiating cell loss. This phenomenon of the requirement of an active process of new RNA and protein synthesis to induce programmed cell death was also demonstrated in other postembryonic developmental systems (Saunders, 1966; Bowen and Lockshin, 1981; Tomei and Cope, 1991).

More recently, by exploiting the technique of subtractive hybridisation, it has been possible to demonstrate that $T_3$ rapidly and selectively activates or represses certain

genes in the *Xenopus* tadpole tail, although the nature of their products remains to be determined (Shi and Brown, 1993; Wang and Brown, 1993). However, the cloning of several cell death and anti-cell death genes, the identification of their products and their high degree of evolutionary conservation (Ellis *et al.*, 1991; Tomei and Cope, 1991; Dexter *et al.*, 1994) now offer possible candidates for ``early`` proteins involved in programmed cell death during metamorphosis. In a recent study, when the *Xenopus* homologue of mammalian *bcl*-2-like gene was cloned, its expression was found to be neither developmentally nor hormonally regulated before and during *Xenopus* metamorphosis (Cruz-Reyes and Tata, 1995). It should be pointed out that *bcl*-2-like genes are considered to be involved in cell survival or anti-cell death function (Ellis *et al.*, 1991; Dexter *et al.*, 1994).

## 1.16. Apoptosis

Amphibian metamorphosis is an ideal model for studying vertebrate postembryonic development (Tata, 1993). Among its several advantages are the following: (i) Many of the biochemical changes are similar to those seen during the acquisition of the adult phenotype at late embryonic or foetal stages of development in mammals, e.g., genetic switches in liver proteins and haemoglobin, skin keratinisation, and visual pigmentation. (ii) The process is under obligatory hormonal control. (iii) The same hormonal signal activates different cell-type-specific genetic programmes in different tissues. (iv) Metamorphic changes can be reproduced in culture. (v) Metamorphosis is an excellent model for studying the molecular mechanisms underlying programmed cell death (PCD) and apoptosis.

Apoptosis or programmed cell death (PCD) (Duvall and Wyllie, 1986) is a fundamental feature of development by which unwanted cells are eliminated from tissues during embryogenesis e.g. the disappearance of the interdigital cells during the formation of the digits from the solid limb paddle and in metamorphosis during the resorption of the tadpole tail. Similar phenomena are observed in the immune system during the negative selection of auto-reactive T-cell clones (Shi *et al.*, 1989; MacDonald and Lees, 1990) and attack by cytolytic T cells.

Recent reports also demonstrated that mature CD4+ T cells undergo apoptosis during an immune response *in vivo* (Kawabe and Ochi, 1991), and following

simultaneous stimulation by antibodies of both CD4+ and the T-cell receptor (Newell *et al.*, 1990).

### 1.16.1 Cell Morphology

The cellular changes in apoptosis are numerous, but it is still not clear which of them are directly associated with death, and which are of greatest physiological importance. The plasma membrane in apoptosis becomes ruffled and blebbed, in a way more pronounced than is seen in necrosis. The cell may break up into apoptotic bodies, but these are sealed and maintain their osmotic gradients. There is no spilling of intracellular contents, and no provocation of inflammation. It is possible that the apoptotic cell strengthens its membranes against the risk of lysis by the activation of cross-linked enzymes such as transglutaminase (Fesus, 1991).

The cell undergoing apoptosis shrinks remarkably, and shows an extremely condensed cytoplasm with normal appearing organelles (Wyllie, 1981). Biochemically, the cell quickly decreases its synthesis of RNA and proteins and degrades them (Cidlowski, 1982). The nucleus is the locus of much of the drama in apoptosis. In general the nucleus shrinks and its chromatin becomes very dense, collapsing into patches, then into crescents in tight apposition to the nuclear envelope, and finally (in many cells) into one or several spheres. This change is often accompanied by fragmentation of the DNA into a ladder of regular subunits,

the result of apparently random double stranded breaks in the linker regions between nucleosomal cores (Yuan and Horvitz, 1990).

### 1.16.2. Functions of apoptosis

Programmed cell death (PCD) serves many required functions during normal animal development (Bowen and Lockshin 1981). In many tissues, more cells are produced than are ultimately required by the organism, and the excess cells subsequently die by means of PCD. In some cases, the over-production of cells provide the organism with valuable developmental plasticity. For example, more spinal motor neurons are produced in the chicken embryo than are required to innervate potential targets. This excess ensures that adequate innervation is always available, independent of the size of the muscle encountered. In other situations, cells are removed because they present a threat to the developing organism. The best characterised example of this phenomenon is the death of immature T cells in the mouse thymus (Jenkinson *et al.*, 1989). The T-cell receptor on many immature T cells can recognise self-antigens present within the mouse. Should these cells be allowed to survive and proliferate, an auto-immune disorder could ensue. The immune system avoids this potentially lethal situation by removing self-reactive T cells by a process known as negative selection (MacDonald and Lees, 1990; Kappler *et al.*, 1987). Experimentally, the synchronised death of entire populations of immature T-cells can be induced with glucocorticoids (Wyllie, 1980). Much of

the data obtained during the study of apoptosis has been acquired with T-cells treated with the synthetic glucocorticoid dexamethasone.

A second developmental strategy is to produce cells that differentiate and assume some required, but transient, function and are then removed when the life style of the organism changes. One well-characterised example is the death of the inter-segmental muscles (ISM) during metamorphosis in moths. These embryonically derived muscles are used for various locomotory and defensive behaviours in the larva and pupa and in the eclosion (emergence) behaviour of the adult. The ISMs are not required for adult-specific behaviours and die during the 30hrs after eclosion. The trigger for this PCD is a fall in the steroid molting hormone 20-hydroxyecdysone late on the day preceding eclosion (Schwartz *et al.*, 1993).

### 1.16.3. Pathways of apoptosis

The programmed deaths of both mouse T cells and moth intersegmental muscles (ISMs) share two fundamental features. Both are initiated by specific physiological signals and both require *de novo* gene expression. However, once the cells begin to die, they do so in seemingly different ways. The T cells die by apoptosis, which is characterised by membrane blebbing, chromatin margination and the breakdown of chromosomal DNA into a ladder of regular subunits, the result of apparently random double stranded breaks in the linker regions between nucleosomal cores. None of these features are seen in the ISMs. Instead, these cells exhibit membrane

wrinkling, nuclear pyknosis, and the retention of high-MW, genomic DNA (Shwartz *et al*, 1993).

Two possible explanations for these results are (i) apoptosis includes a variety of different steps, and the T cells and ISMs represent different ends of this spectrum. (ii) the ISMs do not undergo apoptosis but, rather, die by a different molecular mechanism. Were the ISMs dying by a nonapoptotic cell death program, they might not be the only cells to do so. Data from literature suggests that a number of other cells die in a manner inconsistent with apoptosis. In addition to the ISMs, there are reports of several other dying cells that fail to generate chromosomal DNA ladders during the death process. These cells include glucocorticoid-treated hippocampal neurons, nerve growth factor-deprived neuronal *PC-12* cells, trophic factor-deprived oligodendrocytes and metamorphosing insect salivary glands.

There are some cases where cells might undergo apoptosis, but DNA fragments cannot be detected. The situation can arise when dying cells represent only a small percentage of total tissue mass. In such instances, an examination of the nuclear ultrastructure can facilitate characterisation of the cell death mechanism. As described above, DNA degradation correlates with the dispersion of chromatin along the nuclear envelop (Arends *et al.*, 1990). Many dying neurons display an apoptotic morphology, which has been referred to as type I degeneration by neuroanatomists (Clarke, 1990). However, other studies have documented that

certain dying neurons do not exhibit chromatin margination and possess a nuclear morphology comparable to that seen in ISMs. This pattern has been referred to as type II degeneration or autophagy (Clarke, 1990). Further evidence that type I and II degenerations do, in fact, reflect distinct cell death programs is provided on studies of the ciliary ganglion. During normal PCD, these cells undergo type II (nonapoptotic) cell death, whereas denervation-induced death proceeds via type I (apoptotic) cell death. Therefore, the same cell can apparently activate two distinct cell death programs, depending on the triggering stimulus (Shwartz *et al.*, 1993). The pathways to activation of apoptosis will be different in different cells, but the mechanism of death itself may always be the same, that is a final common pathway (Cohen, 1993).

In examining the data in the literature, apoptosis appears to be rare in invertebrates. In fact, the only demonstration of invertebrate cells generating DNA ladders are lepidopteran tissue culture cells infected with a specific baculovirus (Chem *et al.*, 1991).

The rapid breakdown of DNA seen in apoptosis ensures that even though a targeted cell does not immediately die, it is mitotically incapacitated. This feature would be invaluable for ensuring the efficient removal of dangerous mitotic cells, such as self-reactive T cells. In contrast, many other targeted cells appear to be postmitotic, and their inappropriate retention is relatively benign. Evidence for this comes from

nematodes, where mutations in the *ced* (cell death) genes can block the death of all the cells normally fated to die (Ellis *et al.*, 1991; Ellis *et al.*, 1986). Animals with these *ced* mutations are viable and appear to behave almost normally, despite a substantial increase in the number of neurons.

### 1.16.4. Genetic regulation of apoptosis

In several well-studied models of apoptosis, there is a requirement for new gene expression for both the morphological changes and death itself to occur (Cohen, 1991). Individual genes have been associated with apoptosis in two ways: either they are expressed in cells undergoing apoptosis, or their modulation affects the process. *Fas* for instance, is a gene whose product is a membrane-spanning protein homologous to tumor necrosis factor and nerve growth factor receptors (Itoh *et al.*, 1991; Oehm *et al.*, 1992). In a cell which expresses *Fas*, either naturally or by transfection, cross-linking by antibody to *Fas* induces apoptosis. *Fas* is identical to the human cell surface molecule identified as *APO-1*. Mice bearing the *lpr* mutation are defective in *Fas* (Watanabe *et al.*, 1992), and it is possible that their lymphoproliferation is more correctly lymphoaccumulation because cells die less readily than do their normal counterparts. The *Fas/APO-1* system provides an exciting model to study not only normal tissue turnover, but also the possibility of activating apoptosis as a therapeutic modality in many conditions including malignancies.

There are some genes whose expression increases in apoptotic cells, although their role in the process, if any, has yet to be determined. *TRPM-2*, whose protein product is known by many names including clusterin and *SGP-2*, is expressed in a number of tissues-primarily of the urogenital tract-during apoptosis (Buttyan *et al.*, 1989). It is not seen in tissues undergoing morphogenetic death in the embryo (Garden *et al.*, 1991). This gene product may play a role in secretion and lipid transport, and could be involved in the response to damage. *RP-2* and *RP-8* are two of a family of genes whose messages increase in abundance after the induction of apoptosis in thymocytes (Owens *et al.*, 1991).

One of the most important advances in understanding of the regulation of apoptotic cell death in vertebrates has come from studies of the oncogene *Bcl-2*. *Bcl-2* oncogene is unique among cellular genes in its ability to block apoptotic deaths in multiple contexts. Over-expression of *Bcl-2* in transgenic models leads to accumulation of cells due to evasion of normal cell death mechanisms (McDonnell *et al.*, 1989). Induction of apoptosis by diverse stimuli such as radiation, hyperthermia, growth factor withdrawal, glucocorticoids, and multiple classes of chemotherapeutic agents is inhibited by *Bcl-2* in *in vitro* models (Vaux *et al.*, 1988; Hockenbery *et al.*, 1990). In addition, the endogenous pattern of *Bcl-2* expression is highly suggestive of a role in the regulation of cell survival *in vivo* (Hockenbery *et al.*, 1991).

Despite the identification of genes necessary for cell death and the ability to regulate apoptosis by known genes, the essential biochemical events in apoptotic cell death remain largely unknown (Hockenbery *et al.*, 1993). The data presented support the hypothesis that more than one pathway for mediating PCD may exist. Clearly apoptosis has been well characterised and shown to be a major mediator of PCD (Shwartz *et al.*, 1993).

Due to the significance of programmed cell death and the genes that associate with this phenomenon, I was keen to use the differential display technique in an attempt to identify and then characterise early genes that are TH-induced and considered important for initiating cell death in *Xenopus laevis* tadpole tails. The analysis of changes in gene expression associated with apoptosis is an important one. Until recently, there were only two alternative approaches. Firstly, subtractive hybridisation (Zimmerman *et al.*, 1980) or differential hybridisation (St. John and Davis, 1979) used to identify genes which expressed in only one cell type of respective pairs of cells. These are mainly qualitative methods which do not allow quantitative measurements. In addition these methods are time consuming and not always satisfactory. Secondly nuclear run-on transcription is applied to analyse changes in the level of expression (Strauss *et al.*, 1990). However, this method can only be applied to the detection of changes in the expression of known genes. Thus, it would be important to have a method which detects all mRNA species expressed in a particular cell. By comparing the patterns of expressed mRNA from

two cell types one should be able to detect both qualitative and quantitative changes. This kind of method would allow not only to identify new genes but also the diagnosis of any changes in gene expression involved in a particular cellular process.

## 1.17. Comparison of Differential screening, Subtractive hybridisation and Differential display

### 1.17.1. Differential screening

The most commonly used technique to identify organ-specific messenger RNAs is the differential screening of cDNA libraries with cDNA probes of poly $(A)^+$ RNA from different tissues. Each tissue or environmental condition for which specifically expressed genes have to be isolated, requires the construction of an independent cDNA library. This particular problem can be circumvented by direct differential screening of a genomic library as has been shown for *Drosophila* and yeast (St. John and Davis, 1979; Kramer and Andersen, 1980).

To identify genes expressed in specific tissues, cDNA probes of different poly $(A)^+$ RNA preparations are used for hybridisation to the genomic sub-library. The intensity of the hybridisation signal reflects approximately the homologous mRNA in poly $(A)^+$ RNA used for cDNA probe synthesis.

The differential hybridisation of a genomic library is a useful and efficient approach to isolate genes expressed in specific tissues and under controlled conditions. The procedure has the advantage of avoiding the construction of independent cDNA libraries for each tissue or environmental condition for which specifically expressed genes are to be isolated. It is only applicable to the isolation of genes encoding abundant and moderately abundant mRNA. The remaining 95% of all structural genes are expressed too weakly to give reproducible signal.

### 1.17.2. Subtractive Hybridisation

Differential screening is applicable in many biological situations where it is desirable to isolate cDNAs derived from mRNAs that are induced by a particular treatment. In order to increase the efficiency of the procedure, it is beneficial to be able to create a cDNA library that is enriched in the desired sequences. This can be achieved by subtractive hybridisation, the essense of which is to remove those cDNA sequences that are ubiquitous or not induced.

Two early uses of subtractive library technology were the identification of genes that are activated at the gastrulation of *Xenopus laevis* (Sargent and Dawid, 1983) and cloning of the T-cell receptor (Hedrick *et al.*, 1984). Since those original studies, there have been many applications and modifications of subtractive library methodology, more recently incorporating PCR technology (Duguid *et al.*, 1988; Wieland *et al.*, 1990).

An example of this technique is the isolation of up-and down-regulated cDNA fragments induced by thyroid hormone during *Xenopus laevis* metamorphosis (Brown and Wang, 1991). Poly $(A)^+$ RNA was extracted from thyroid hormone treated (+) and untreated (–) stage 54 tadpoles. Oligo (dT) was used to prime the first strand of cDNA synthesis of poly $(A)^+$ RNA. Double-stranded cDNAs prepared from the two mRNA populations that are to be compared were cleaved with two four-base-pair-recognition restriction enzymes, and these cDNA fragments were ligated to linkers for PCR amplification. The amplified – and + cDNA fragments were the starting material for subtractive hybridisation.

The PCR-amplified cDNA was digested with restriction enzyme *EcoRI* to cleave the linker so that residual driver DNA could not be amplified later. The driver DNA was mixed with photoprobe biotin. The photobiotinylation reaction was repeated once to increase the density of biotin molecules so that the biotinylated driver DNA (BD) could be removed more efficiently.

Biotinylated driver and nonbiotinylated tracer DNAs were mixed. The DNA mixture was mixed with hybridisation buffer and boiled to ensure complete denaturation. The denatured cDNA samples were incubated in water bath for long hybridisation (LH). Streptavidin was mixed with the hybridised cDNA solution to form complexes with biotinylated DNA, and protein and protein-DNA complexes were removed by extraction with an equal volume of $CHCl_3$ / phenol (1:1, vol/vol).

The streptavidin binding and $CHCl_3$ / phenol extraction steps were repeated until there was no visible protein-DNA complex at the interface between the organic phase and aqueous phases. Usually it took four or five repeated extractions with streptavidin to remove >99% of the biotinylated DNA. The subtracted tracer cDNA (+1cDNA or −1cDNA) was mixed with biotinylated *EcoRI*-treated driver DNA as before. This was resuspended in hybridisation buffer and incubated for short hybridisation (SH). Biotinylated DNA was removed as before, and the enriched tracer DNA was ethanol precipitated. The DNA (+2 cDNA or – cDNA) was amplified by PCR as before.. The PCR product of 2 cDNA was purified and treated with *EcoRI* and biotinylated for use as a driver for the next cycle subtractive enrichment. Long hybridisation used biotinylated 2 cDNA driver and nonbiotinylated 2 cDNA tracer. This was followed by a short hybridisation with 1cDNA driver, producing 4 cDNA.

An essential aspect of this subtractive enrichment procedure is the use of both long and short subtractive hybridisation steps to remove common DNA fragments. Long hybridisation is needed to suppress the highly complex rare common cDNAs that comprise 50-60% of the total cDNA mixture. However, long hybridisation does not efficiently reduce the abundant common cDNAs and can actually suppress some differentially expressed cDNAs that have a baseline level in the driver cDNA. Thus, the short hybridisation is used to suppress the abundant common mRNAs. Up-regulated genes suppressed greatly in +1 cDNA, making these cDNAs better

drivers than the initial PCR-amplified cDNAs for short-term hybridisation steps. The enriched + cDNA hybridises only with itself and not detectably with enriched – cDNA vice versa. Further enrichment can be accomplished by driving out the most abundant enriched cDNA fragments , yielding 7 and 8 cDNA (see Figure 7).

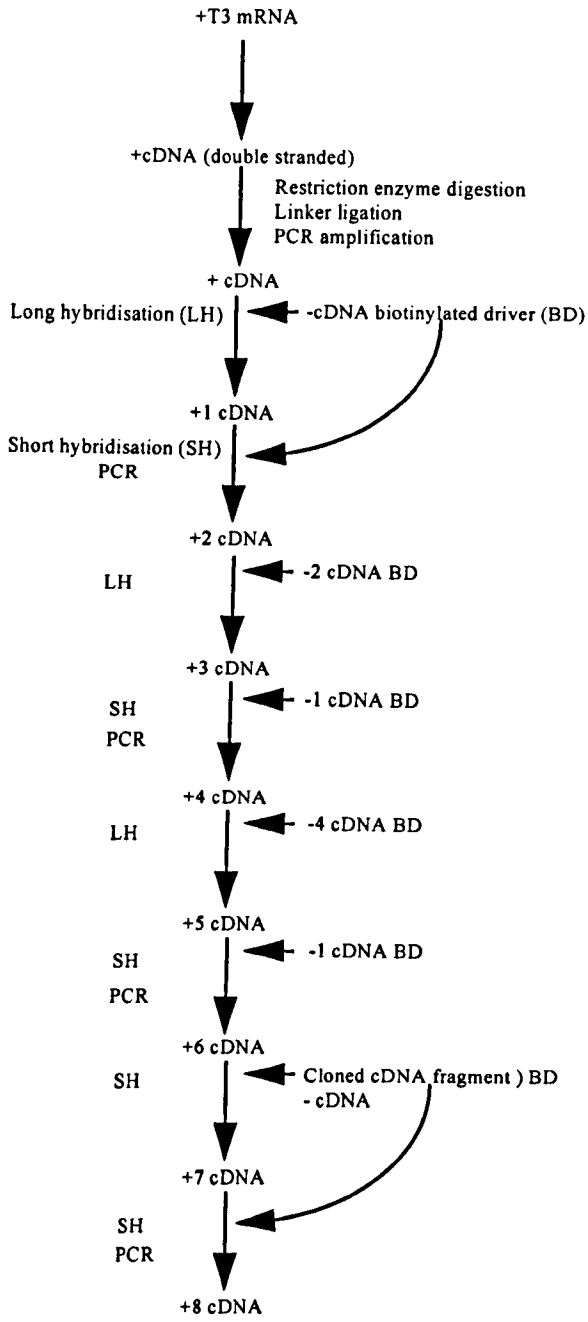



FIG.7. Flow diagram for isolation of up-regulated genes. A plus sign (+) refers to the mRNA isolated from tadpole tails treated with thyroid hormone (3,3',5-triiodo-L-thyronine, $T_3$) for 24 hr, as well as the cDNAs derived from this + mRNA; – refers to mRNA and cDNAs from untreated tadpoles, LH, long hybridisation; SH. short hybridisation; BD, biotinylated driver DNA (from Wang and Brown, 1991).

After three cycles of subtractive enrichment, + and – enriched cDNA fragments were amplified with PCR. The amplified cDNA was purified, and small portion of this purified product was subjected to an additional cycle of PCR amplification to ensure that all cDNA fragments were double-stranded. The products were cleaved with *EcoRI* and ligated to dephosphorylated pBluscript vector for transformation into competent *E.coli* DH5 cells. The clone fragments were then mapped to a high molecular weight cDNA library to find multiple fragments that are derived from the same mRNA.

This method finds differences between two samples of mRNA. Large genetic deltions can be isolated by gene amplification following subtractive hybridisation simply by driving mutant against control genomic DNA (Wieland *et al.,* 1990). Repeated rounds of enrichment and amplification of the remaining tracer genomic DNA, in principle could isolate ultimately a single-copy gene that was missing in the driver genomic DNA. A deleted 0.5kb genomic DNA fragment, within the size range that is amplified efficiently by PCR, would be enriched 12,000-fold to comprise 0.2% of total DNA and, therefore, be detected by this method. The highest enrichment in these experiments was about 2000-fold, so one or more additional rounds of subtractive hybridisation would be required. This method also identify a single gene responsible for a disease where the amount of mRNA accumulates in the abnormal cells differs from that in control cells. It should aid in the identification of multiple genes involved in a complex genetic disease where

the expression of more than one gene is altered. A gene expression screen can identify differentially expressed genes related to developmental, physiological, or pharmacological events in any organism.

The gene expression screen will not isolate all of the genes in the program. It will not identify genes that are critical to the program whose mRNA abundance is unaltered by TH. The screen will miss regulated genes whose cDNAs are not fragmented to the size range 200-800 bp by the two restriction enzymes used to cleave the cDNA. PCR does not preferentially amplify DNA by size in the range of 200-800 bp. About 15% of published *X.laevis* mRNAs lack the recognition sites for the two restriction enzymes. Any mRNA that has no poly (A) tail will be lost because oligo dT was used to prime cDNA synthesis.

A method recently published by (Liang and Pardee, 1992) which was used in our experiments is based on the assumption that every cell expresses some 15,000 genes and, in principle, every individual mRNA molecule can be reverse transcribed and amplified by the polymerase chain reaction (PCR). The idea was to use a set of arbitrary primers for PCR amplification of cDNA generated by reverse transcription from mRNA. This technique can be used for the following purposes:
1. To visualise mRNA composition of cells by displaying subsets of mRNAs as short cDNA bands; samples run in parallel reveal differences in their mRNA

patterns. A comparison will be made between normal and regressing tails in order to estimate populations of mRNAs that differ among them.

2. These cDNAs can be eluted from the gel, amplified further in a second PCR, for cloning and sequencing: thereby a tag for each mRNA can readily be obtained and compared with sequences in data banks.

3. Individual clones can be used as probes for northern or southern (DNA) blots and to isolate longer sequences either from cDNA or genomic libraries. This step may be necessary and important because the PCR display technique tends to produce sequences derived from 3' untranslated region of mRNA. These 3' untranslated sequences are typically A/T rich, and will not often be useful in data base searching especially because the untranslated regions are poorly conserved among species. The potential of this technique is to identify differentially expressed mRNAs and to clone their genes.

4. Homology may or may not be found in data banks. If however, recognition resulted in known genes in *Xenopus* OR *Mammals* (or more widely, in other organisms), then something about their function may be known. However, if a new cDNA is found then its function will not be apparent from the data banks.

### 1.17.3. Differential display reverse transcriptase PCR (DDRT-PCR)

A method recently described by (Liang and Pardee, 1992) termed DDRT-PCR was developed as a tool to detect and characterise altered gene expression in eukaryotic cells. Selection of 3′ primers takes advantage of the polyadenylate poly $A^+$ tail present on most eukaryotic mRNAs. The basic principle is to systematically amplify messenger RNAs and then distribute their 3′termini on a denaturing polyacrylamide gel. The essence of differential display method is to use for reverse transcription an anchored oligo-dT primer which anneals to the begining of a subpopulation of the poly $A^+$ tails of mRNAs. The anchor oligo-dT primers consist of 11or 12Ts plus two additional 3′bases which provide specificity, and have the general formula 5′-$T_{11}$MN-3′ where M may be dG, dA, or dC and N may be one of the four deoxynucleaotides G,A,C,or T. These are used in conjunction with a decamer oligonucleotide of arbitrary defined sequence for the subsequent PCR amplification. For example a primer such as 5′-$T_{11}$CA would allow anchored annealing to mRNAs containing TG located just upstream of their poly $A^+$ tails. This primer will recognise one-twelfth of the total mRNA population because there are 12 different combinations of the last 3 bases, omitting T as the ultimate base. The primer permits initiation of reverse transcription of only this subpopulation. Following reverse transcription the PCR reactions are performed using the cDNA and the corresponding 3′ primer in the presence of an arbitrary 10 mer 5′primer. These reactions are carried out in the presence of [$\alpha^{35}$S]-dATP and as a result amplified cDNA fragments of 3′ termini of mRNAs can thus be separated by size

on a denaturing polyacrylamide gel. Samples of RNA from different origins with different treatments can be put through this system and analysed side by side on the resultant gel. This allows qualitative and quantitative changes in expression pattern between the two samples to be identified. The differentially expressed bands identified by this system can then be isolated from the gel, reamplified and cloned.

Differential display has several technical advantages as compared to subtractive and differential hybridisations. It is much quicker, 2 months are required to isolate clones from cells by subtractive hybridisation, which includes mRNA isolations, cDNA library construction, subtraction, and screening by differential hybridisation. With differential display the patterns are obtained in 2 days and clones in 5 days. In addition, unlike subtractive hybridisation, differential display allows simultaneous detection s of both groups of differentially expressed genes (for example, candidate tumour suppresser genes and oncogenes). Most genes by statistics should be present in the patterns as single bands. Therefore, redundancy, underrepresentation of rare mRNAs, and false positive clones are minimised. In terms of sensitivity, because the method describe here is PCR-based, only 1μg of mRNA is required per 100 lanes, compared to 50 times as much or more for subtractive hybridisation. A direct comparison of the number of cDNA bands amplified by a given set of primers with either a cDNA library or mRNA of the same cell type indicates that the new method is much more sensitive. This suggests

possible underrepresentations of many genes during cDNA library constructions. Reproducibility from run to run of the method in displaying mRNA patterns with the same RNA sample is high (> 95% bands are always seen) in comparison with the great variations in the kinds and numbers of genes islated by subtractive hybridisation. The advantage of subtractive hybridisation is its enrichment and focus on only differentially expressed genes. Because of its simplicity based based on PCR and a DNA sequencing gel, two of the most widely used molecular biological techniques, the differential display should find wide and rapid applications in studying cancer, heart disease, cell differentiation, and aging, among others. These results demonstrate the potential of this technique to identify differentially expressed mRNAs and so to clone their genes. It should make possible the detection of the most mRNAs in a cell by use of multiple primer sets.

The procedure has several limitations: (a) Because the DNA fragments are separated on a DNA sequencing gel, the procedure is limited to fragments less than 600 bp. (b) The initially isolated PCR products have to be re-amplified once or twice, in order to generate adequate amounts for Northern blot assay, cloning, sequencing, etc. (c) The number of false positives obtained from differential display gels caused by co-migrating bands has been found to be a contributing factor to the validity of the technique.

## 1.18. Aims of the project

Application of the exogenous thyroid hormone (TH) exerts dramatic effects on amphibian metamorphosis. The diverse responses such as tail resorption, limb growth and differentiation and intestinal remodelling are mediated by TH.-regulated genes during amphibian metamorphosis. Each organ or tissue has been programmed (determined) to respond to the hormone in its own specificity with the TH-initiating the expression of each programme. We have chosen tail resorption as the simplest of these programmes because there is no concomitant growth and differentiation, merely death and resorption. These effects are of great interest in the study of developmental processes.

The project aims to isolate and characterise genes regulated by thyroid hormone in *Xenopus laevis*. The technique of differential display DDRT-PCR will be applied to RNA isolated from TH-treated and untreated *Xenopus laevis* tadpole tails, allowing genes differentially expressed as a result of the TH-treatment to be isolated.

Characterisation of clones isolated in this way will involve sequence analysis, identification of temporal and spatial expression patterns during embryogenesis by RT-PCR and *in situ* hybridisation and functional analysis including microinjection to identify any influences on developmental processes.

# CHAPTER 2
**Materials**

## 2.1. General reagents and Suppliers

Enzymes were obtained from the following suppliers:

Restriction endonucleases, *E.coli* DNA polymerase 1, T4 DNA ligase, T4 polynucleotide kinase, T7 and SP6 RNA polymerases, DNA polymerase Klenow fragment, MMLV reverse transcriptase were from Gibco (BRL); Taq DNA polymerase was from Promega; Calf intestinal alkaline phosphatase was from Boehringer Mannheim.

All radioisotpoes were supplied by Amersham international at the following specific activities: [$\alpha^{32}$P]-dGTP, 3000 Ci/mmol; [$\alpha^{32}$P]-CTP, 3000 Ci/mmol; [$\alpha^{35}$S]-dATP $\alpha$S, 1000 Ci/mmol; [$\alpha^{35}$S]-methionine, 1000 Ci/mmol.

Nitrocellulose filters (Hybond-C) were from Amersham.

Type II agarose, low melting point agarose and *E.coli* transfer RNA were supplied by Sigma.

Materials for bacteriological media were from Difco laboratories (Michigan, U.S.A.) and Oxford Limited (England).

Deionised formamide was from Fluka.

Acrylamide was from Fisons, and bisacrylamide from Kodak.

X-ray film was from Fuji Photo Company Limited (Japan).

Animals : *Xenopus laevis* tadpoles stages 49-54.

Materials : Triiodothyronine ( $T_3$ ) $5X10^{-9}$ M in DMSO kept at 4°C.

Methane sulfonate salt ( MS222 ).

Dimethyl sulfoxide ( DMSO ).

Potassium permanganate.

Ethanol.

Lebovitz medium with glutamin (L15) was from Gibco (BRL).

*Xenopus* Kidney cDNA library Uni-ZAP™XR vector from Stratagene.

Oligonucleotides were synthesised in the department using an Applied Biosystems automated synthesiser and also from Genosys.

All other chemicals and reagents were from BDH or from Sigma chemical company, unless otherwise stated.

**2.2. Stock solutions**

10×TBE: 0.9M Tris-borate pH 8.3, 20mM EDTA.

TE: 10mM Tris-HCL pH 8.0 1mM EDTA.

20×SSC: 3M Na Cl, 0.3M Sodium Citrate pH 7.0.

50×Denhardt's:1%(w/v)each of Ficoll, polyvinylpyrolidone, bovine serum albumin.

10×MOPS: 0.2M 3-(N-morpholino) propanesulphonic acid, 50mM Sodium Acetate, 10mM EDTA pH 7.0.

Phosphate Buffered Saline (PBS): 0.8% (w/v) Na Cl, 0.02% (w/v) KCl, 0.115% (w/v) $Na_2HPO_4$, 0.02% (w/v) $KH_2PO_4$.

1×Barth-X: 88mM NaCl, 1mM KCl, 2.5mM Na $HCO_3$, 15mM Tris-HCl pH 7.6, 0.3mM $CaNO_3$, 0.41mM $CaCl_2$, 0.82mM $MgSO_4$.

NAE: 0.3M Sodium Acetate pH 6.5, 1mM EDTA.

### 2.3. Bacteriological media

All media was autoclaved before use

Antibiotics: Ampicillin was used in plates and broth at a concentration of 100μg/ml. Also, it was used along with other antibiotics Streptomycin and Gentamycin in tissue culture.

LB-tetracyclin at 12.5μg/ml

LB-kanamycin at 50μg/ml

#### 2.3.1 Liquid media

LB Broth (per litre) 5g yeast extract, 10g bactotryptone, 10g NaCl.

NZY Broth (per litre) 5g NaCl, 2g $MgSO_4.7H_2O$, 5g yeast extract, 10g NZ amine (casein hydrolysate).

#### 2.3.2 Plates/Top agar/Top agarose

LB Plates (per litre) 10g bactotryptone, 5g yeast extract, 10g NaCl, 15g bactoagar.

For top agar, agar was added at 7g/litre.

For top agarose, agarose was added at 7g/litre.

## 2.4. Bacteria, Plasmids and Phage

### 2.4.1. Host strains and Genotypes

**MC1061**: -F-, *ara D139, Δ(ara,leu) 7696, Δlac Y74, gal U-, gal K-, hsr-, hsm+, strA.*

**XL1-Blue MRF'**: *Δ(mcrA) 183, Δ(mcrCB-hsdSMR-mrr) 173, endA1, supE44, thi-1, recA1, gyrA96, relA1, lac[F'proAB, lacI$^q$ZΔM15, Tn 10 (tet$^r$)].*

**SOLR™Strain:** e14$^-$*(mcrA), Δ (mcrCB-hsdSMR-mrr)171, sbcC, recB, recJ, umuC: Tn5 (kan$^r$), uvrC, lac, gyrA96, relA1, thi1, endA1, λ$^R$ [F'proAB, lacI$^q$ZΔM15]Su$^-$.*

### 2.4.2. Plasmid vectors

The Uni-ZAP™XR vector system (Stratagene) combines the high efficiency of lambda library construction and the convenience of a plasmid system with blue-white colour selection. The Uni-ZAP XR vector can be screened with either nucleic acid probes or antibody probes and allows rapid *in vivo* excision of the pBluescript SK(−) phagemid. The polylinker of pBluescript SK(−) has 21 unique cloning sites flanked by T3 and T7 promoters and a choice of 6 different primer sites for DNA sequencing. The phagemid has the bacteriophage f1 origin of replication, allowing rescue of single-stranded DNA, which can be used for DNA sequencing or site-directed mutagenesis. Transcripts made from the T3 and T7 promotors generate riboprobes useful in Southern and Northern blotting and the *lacZ* promoter may be used to drive expression of fusion proteins suitable for western blot analysis or protein purification.

### 2.4.3 f1 Helper Phage

VCSM13 (f1) for single-stranded rescue and ExAssist™ helper phage (M13) for the excision of the pBluescript phagemid from the ZAP vector.

# CHAPTER 3
## Methods

### 3.1. Gonadectomy (Testis removal)

Pentobarbitone 0.3ml to 0.5ml were injected into the lymph sac of a male frog as a vein is too small to find. It took about 20 minutes to take effect. The frog was left in water while the drug took its affect. The incision was made on either side of the groin. First to cut was the skin and then the muscle. The testes can be found on the end of the large yellow fat body which is usually easy to find.

### 3.2. Eggs and Embryos

Female *Xenopus laevis* were induced to ovulate by injection of 100 U of follicle stimulating hormone (FSH) 48 hours to one week prior to laying, and then injection of 600 U of human chorionic gonadotrophin (HCG) 16 hours prior to laying. Eggs were laid directly into and cultured in Barth X (BX) prior to fertilisation. So that the jelly coat remained unswollen and the eggs remained fertilisable *in vitro*. Testes were dissected from a male *X. laevis* and stored on ice in BX. Teased testis fragments were used to provide sperm to fertilise eggs in a small volume of BX. After approximately 2 minutes the eggs were flooded with distilled water. Fertilised eggs were identified as a result of rotation within the vitelline membane, which occurs approximately 20 minutes after fertilisation. Such eggs were dejellied in 2% cysteine (w/v) pH 8.0, washed with and subsequently cultured in 0.1x BX. At later stages, to avoid exogastrulation, embryos were transferred into fresh 0.1x BX containing 10µg/ml gentomycin sulphate (Sigma). The temperature

of development was preferentially kept low (14°C). Embryos were staged according to Nieuwkoop and Faber (1967).

### 3.3. Rearing of *Xenopus laevis* under laboratory conditions

#### 3.3.1. General information and precautions

No detergents or disinfectants to be used in frog room at all, apart from drains (to be used with care).

Frog room temperature was kept at 22°C. All water should stand for 24 hours to rid it of chlorine. The best system is to have an overhead tank to contain all water needed. Charcoal filters are incorporated to speed up the process. The water must also stand to allow it to be raised to the correct temperature. All frog water was changed daily.

### 3.4. Feeding

#### 3.4.1. Tadpoles:

Tadpoles were fed with nettle powder dissolved in water approximately 25 grams per 2 liters water, put on bubbler for 48 hours before feeding.

#### 3.4.2. Metamorphosis

Young frogs after metamorphosis were fed on Daphnia and then on Brineshrimp every day. When frogs reach 20mm, they were changed to blood worm. The

amount to use must be judged by user every other day. When they reach 40mm, they were changed on to *Xenopus* chips if they can be obtained.

When frogs start to grow, larger food can be used such as pond pellets with high a high protein content (60%), or they can have Beef heart on Bilice bottle maggots twice a week.

Recipe for Barth X :

Solution A. Composition per litre

| | |
|---|---|
| Na Cl | 128.17 gm |
| KCl | 1.85 gm |
| Na $HCO_3$ | 5.0 gm |
| Tris-base | 45.37 gm |

pH was adjusted to 7.8.

Solution B. Composition per litre

| | |
|---|---|
| Ca $(NO_3)_2$ $4H_2O$ | 1.925 gm |
| Ca $CL_2$. 6 $H_2O$ | 2.25 gm |
| Mg SO4. 7 $H_2O$ | 5.0 gm |

Solutions A and B are aliquoted into 40mls amounts in 100mls bottles. These bottles were kept separate upright when freezing. To make Barth X 40ml of each of A and B are mixed and made up to one litre with distilled water.

### 3.5. Microinjection of *Xenopus* embryos

Embryos were generally injected bilaterally with mRNA at the two cell stage. Prior to injection, embryos were transferred to 5% Ficoll (w/v) to reduce cytoplasm

leakage. After injection, the embryos were maintained in Ficoll until stage 6, after which they were cultured in 0.1x BX to avoid exogastrulation.

### 3.6. Tadpole treatments

To identify genes regulated by triiodothyronine ($T_3$) two treatment regimes for tadpole tails were performed, from which RNA was extracted and analysed by differential display reverse transcriptase polymerase chain reaction (DDRT-PCR).

(i) Controls-no treatment.

(ii) $T_3$ treated with $5\times10^{-9}$ M for 48 hrs.

Animals of the same developmental stages (52-56) and of similar sizes were selected. A total of six tadpoles were anaesthetised in 0.2% MS222, tails were then amputated using clean sharp scalpel or razor blades, washed thoroughly in potassium permanganate 10mg/ml, ethanol 70% and finally in sterile distilled water. They were then placed in a six well tissue culture cluster, each containing 5ml of Lebovitz medium. The medium was supplemented with the antibiotics ampicillin, streptomycin and gentamycin. It also contained glutamine. The tissues were left in the culture medium at 21°C ± 1°C for 24 hrs before the hormone was added. The day after the removal of tissues was counted as Day 0. Thereafter, 2.5µl of triiodothyronine (final concentration $5\times10^{-9}$ M) was added to three wells of culture medium, while the remaining ones were treated as controls. The culture medium with the additives was changed every 48 hrs, unless the regime of additives was altered after the commencement of culture. All manipulations until

this point were carried out under sterile conditions. The organ-cultured tails were then examined histologically by fixing them in a solution of 4% paraformaldehyde-PBS (Tata *et al*, 1991) for 24 hrs at room temperature and then dehydrated with a series of increasing concentrations of ethanol. These were processed for embedding into paraffin and then serially sectioned, 8μm in thickness. Transverse sections of the tails were stained with hematoxylin and eosin by the standard procedure.

### 3.7. Gels for resolving nucleic acids

#### 3.7.1. Non-denaturing agarose gels

DNA samples, to which 0.2 volumes of loading buffer (50% glycerol, 5×TBE, 0.1% bromophenol blue) had been added, were separated in 0.6 to 1.5% (w/v) agarose gels containing 0.2μg/ml ethidium bromide made in 1×TBE buffer. Gels were run in 1×TBE buffer containing 0.5μg/ml ethidium bromide at 80mA. The gels were examined and photographed on an ultraviolet light box.

#### 3.7.2. Low melting point agarose gels

These gels were used for isolation of DNA fragments generated by restriction enzyme digests. DNA samples to which 0.2 volumes of agarose gel loading buffer had been added were loaded on 0.8% (w/v) low melting point agarose gel made in 1×TBE buffer and containing 0.2μg/ml ethidium bromide. Gels were run in 1×TBE buffer containing 0.5μg/ml ethidium bromide at 40mA. The gel was examined under a UV light transilluminator and the desired bands excised as a

small gel slice. Exposure of the gel to the UV light was kept to a minimum to avoid UV induced DNA damage. The gel slice was then transferred to a 1.5ml eppendorf tube and weighed to calculate the volume of the gel slice (assuming 1g=1ml). 4 volumes of NAE (0.3M sodium acetate PH 6.5, 1mM EDTA) was added and the gel slice melted by heating to 65°C for 15 minutes. After cooling to 37°C an equal volume of neutral phenol was added, the solution was mixed vigorously and left on ice for 10 minutes. The tube was then centrifuged at 13,000 rpm for 5 minutes and the aqueous phase transferred to a fresh tube. The aqueous phase was ether extracted, and the volume reduced to less than 0.5ml by extraction with butan-1-ol. The DNA was then precipitated with two volumes of ethanol at –20°C.

### 3.7.3. Denaturing polyacrylamide gels

6% or 8% polyacrylamide (19:1 bis) gels containing 42% (w/v) urea in 1×TBE (to relieve certain compression in sequencing gels 25% formamide was included in the gel mix) were poured between 20×40cm gel plates with 0.4mm spacers. Nucleic acid samples in denaturing loading buffer (90% deionised formamide, 10mM EDTA pH 8, 0.01% bromophenol blue, 0.01% xylene cyanol) were heated at 100°C for 5 minutes, loaded onto the gel and electrophoresed at 38 Watts in 1×TBE. Gels were not fixed or dried prior to autoradiography. [$^{35}$S] samples (sequencing reactions) were autoradiographed at room temperature. However, [$^{32}$P] samples were autoradiographed at –70°C with intensifying screens. Gels for

differential display were not fixed or dried before autoradiography. The top gel plate was removed, the gel covered in cling film and exposed to X-ray film at –70°C.

### 3.8. Subcloning techniques

#### 3.8.1. Restriction enzyme digest

These were carried out according the manufacturers instructions. Plasmid DNAs were generally digested for one-two hours.

#### 3.8.2. Vector preparation for subcloning

Vectors that had been digested with two restriction enzymes generating incompatible ends were run on a low melting point agarose gel and the linear vector fragment recovered. Vectors cut with a single restriction enzyme were treated with calf intestinal alkaline phosphatase (CIAP, Boehringer) by adding 2 units of enzyme into the digestion reaction at the end of the digestion period and incubated for further 30 minutes at 37°C. The reaction product was then phenol extracted and ethanol precipitated.

#### 3.8.3. Preparation of target DNA for subcloning

Target DNA was generally a restriction fragment isolated from a low melting agarose gel. In some cases it was necessary to ligate target DNA to vector with incompatible sticky ends. In these cases 5′ overhangs (on both vector and target DNA) were made blunt by the addition of all 4 dNTPs (final concentration 1mM

each) and 10 units of Klenow fragment of DNA polymerase into the digestion reaction (following completion of digestion) and incubated for 15 minutes at room temperature.

### 3.8.4. Ligations

Ligation reactions were carried out in 1× ligation buffer (Gibco), vector DNA, target DNA and T4 DNA ligase. Standardly 20ng of vector and a concentration range of target DNA was used. A control ligation containing no target DNA was always included. For sticky end ligations 0.2 units of T4 DNA ligase was used, while one unit was used for blunt end ligations. These reactions were performed at 14°C overnight.

## 3.9. Transformations

### 3.9.1 Preparation of competent cells

L-broth (10ml) was inoculated with the appropriate *E.coli* strain (MC1061). The culture was placed overnight in a rotary shaker (200rpm) at 37°C. A 1ml aliquot of this culture was removed and used to inoculate 20ml of fresh L-broth. The incubation was resumed for approximately 2-2.5 hours. The growth of the culture was monitored by measuring its optical density at 550nm against an L-broth blank. When this value reached 0.45-0.5 O.D. units, the cells were harvested by centrifugation of the culture at 3000rpm for 10 minutes at 4°C. The supernatant was discarded and the bacterial cell pellet gently resuspended in 10ml ice cold

$MgCl_2$ (0.1M). The cells were pelleted as mentioned before and resuspended in 10ml ice cold $CaCl_2$ (0.1M). The cells were immediately pelleted as previously mentioned and resuspended in 1ml ice cold $CaCl_2$ and kept on for 90minutes until required for transformation.

### 3.9.2. Transformation with plasmid DNA by heat-shock

An aliquot of cells (100-200µl), rendered competent by the $CaCl_2$ washing method, were used for each transformation. Plasmid DNA (1-10ng) was added to the cells, which were subsequently incubated on ice for 90 minutes to enable adsorption of the DNA to the cell surface. In order to determine the transformation efficiency of the prepared batch of cells, a range of known concentrations of plasmid DNA was incubated with the cells. The cells were transferred to 42°C for 2 minutes and then quenched on ice for 15 minutes. L-broth (500µl) was added to each Eppendorf and the cells were incubated for 60 minutes at 37°C to enable expression of the ampicillin resistance gene born on the plasmid. A range of dilutions of the cell suspension was spread onto agar plates containing the antibiotic at 100µg/ml. The plates were incubated overnight at 37°C. A control aliquot of cells was cultured on Lamp plates, in order to confirm that the strain was not inherently ampicillin resistant. The viability of the cells, following $CaCl_2$ treatment, was assessed on agar plates without antibiotic.

### 3.10. Preparation of Plating Bacteriophage Cultures

A single colony of the appropriate bacterial host (XL1-Blue) was inoculated into a 50ml LB broth supplemented with 0.2% (v/v) maltose and 10mM $MgSO_4$ in a sterile flask. The culture was grown overnight with shaking at 30°C. This temperature ensures that the cells will not overgrow. The culture was monitored by measuring O.D. at 600nm until it reached 0.5. The cells were pelleted by centrifugation in a conical tube for 10 minutes at 2000rpm. Carefully the media was decanted and the cell pellet was gently resuspended without vortexing in 15ml of 10mM $MgSO_4$. The cells were diluted to $OD_{600}$ = 0.5 with 10mM $MgSO_4$. Approximately 600 µl of $OD_{600}$ = 0.5 cells were needed for each 150-mm plate and 200 µl of $OD_{600}$ = 0.5 cells for each 100-mm plate. 1µl of lambda phage in SM buffer (50mM Tris-HCl pH 7.5, 0.58% (w/v) NaCl, 0.2% (w/v) $MgSO_4.7H_2O$, 0.01% (w/v) gelatin) was added to 200 µl of host cells, well mixed and incubated for 15 minutes at 37°C to allow the phage to attach to the cells. Up to $1x10^5$ phage were absorbed with 600µl of host cells. 6.5ml of melted top agarose was aliquoted in to sterile tubes in a heating block at 48°C and allowed to equilibrate to 48°C. 0.6 ml of infected cells was added to the top agarose and this was then poured onto dried 15cm x 15cm NZY plates and allowed to set evenly. The plates were then incubated inverted at 37°C until plaques of suitable size formed.

### 3.11. Bacteriophage Lambda Plaque Lifts

This procedure was performed to transfer bacteriophage DNA from plates to nitrocellulose filters as described by the manufacturer. The host strain XL1-Blue was used. Bacteriophage lambda were plated on a large NZY plates to 600,000 pfu/plate with 600µl of host cells using 15ml of top agarose and then incubated at 37°C for 8 hours until suitably sized plaques formed. The plates were then refrigerated for 2 hours at 4°C to chill. Pieces of nitrocellulose filters cut slightly smaller than the plates, were carefully lowered onto the surface of the top agarose ensuring that no air bubbles formed. These were left for 2 minutes. With the help of a syringe needle the filter was pricked through the membrane into the agar for orientation. Using a pair of forceps, the filters were carefully removed with the DNA side up onto a tray containing several sheets of filter paper (Whatman 3mm) moistened with denaturing solution (1.5M NaCl and 0.5M NaOH) and left for 2 minutes during which time a second filter was applied to each plate. This replica filter was allowed to transfer for 4 minutes oriented in the same position of the first filter. Following denaturation, the filters were submerged in another tray containing neutralising buffer (1.5M NaCl 0.5M Tris-HCl pH 8 for 5 minutes followed by a 30 seconds rinse in 0.2M Tris-HCl pH 7.5 and 2xSSC buffer. The filters were then transferred onto 3MM Whatman paper and allowed to air dry. They were then baked at 80°C under vacuum for 2 hours.

### 3.12. Prehybridisation, Hybridisation, Autoradiography of Filters

Filters were prehybridised in 5xDenhardt's, 6xSSC, 0.1% SDS, 100µg/ml *E.coli* tRNA, 50% formamide and was performed in a heat-resistant glass tubes at 42°C for 2-20 hours. The prehybridisation solution was replaced by a fresh aliquot of the same buffer containing radiolabelled nucleic acid probe and hybridisation was carried out overnight at 42°C.

Filters were washed twice in 2xSSC, 0.1% SDS at room temperature for 10 minutes each and then twice for 30 minutes each in the final wash conditions at 65°C (varied according to the hybridisation stringency required). The filters were then allowed to air dry on a filter paper and wrapped in a clingfilm and exposed to X-ray film with intensifying screen at −70°C. For orienting the filters, "Putative" clones with the strongest signal on film was determined by lining up the film and mark numbers and dots where the needle poked through. A square centimetre where the putative clone lined up with the film spot was cut with the help of an inverted yellow tip and transferred to fresh sterile tube containing 1ml of SM buffer and 20µl of chloroform, well vortexed and kept stored at 4°C. This phage stock was replated at a lower density and plaque lifts performed. Positive plaques were identified and the process repeated until the agar plug taken contained a single positive plaque.

### 3.13. *In vivo* Excision Using The EXASSIST/SOLAR System

This system was designed to allow efficient excision of the pBluescript phagemid from the Uni-ZAP vector.

The plaque of interest from the agar plate was transferred to a sterile microfuge tube containing 500µl SM buffer and 20µl of chloroform. This was well vortexed to release the phage particles into the SM buffer and incubated for 1-2 hours at room temperature or overnight at 4°C. This was kept at 4°C as phage stock. An overnight culture of XL1-Blue MRF' and SOLR cells was grown in LB broth at 30°C. To a 50ml of LB broth, 0.5ml of the overnight culture was added and grown at 37°C for 2-3 hours until the $OD_{600} = 0.5$. The XL1-Blue MRF' cells were gently pelleted by centrifugation at (1500 x g) and resuspended in 10mM $MgSO_4$. The SOLR cells was allowed to grow to OD600 = 0.5-1.0 for excision and then removed from the incubator and left at room temp.

In a 50ml conical tube, 200µl of XL1-Blue MRF' cells, 250µl of phage stock (> $1x10^5$ phage particles), 1µl of ExAssist helper phage (>$1x10^6$ pfu/ml) were combined and the mixture was incubated at 37°C for 15 minutes. 3ml of LB broth was added and a further incubation with shaking at 37°C for 2-2.5 hours to overnight was performed. The cells were pelleted by centrifugation at 2000 x g and the supernatant was transferred to a fresh tube and the tube was heated at 70°C for 15 minutes. This was again centrifuged at 4000 x g for 15 minutes. Carefully, the

supernatant was transferred into fresh sterile tube. This stock contained the excised pBluescript phagemid packaged as filamentous phage particles. To plate the rescued phagemids, 200μl of freshly grown SOLR cells were added to 10-100μl of the phage stock. The tubes were incubated at 37°C for 15 minutes and 10-50μl were plated on LB-ampicillin plates (100μg/ml). The plates were incubated inverted overnight at 37°C.

### 3.14. Isolation and recovery of DNA fragments

#### 3.14.1. The GENECLEAN II method

DNA fragments were resolved by agarose gel electrophoresis and visualised under long wave UV light. The desired gel slice was excised and subjected to the GENECLEAN II procedure. This, briefly, involves dissolution of the agarose in sodium iodide and subsequent incubation of the mixture with a specially formulated silica matrix (GLASSMILK), which binds single and double stranded DNA in favour of agarose, proteins, or small RNA. The matrix is extensively washed in a Tris-ethanol buffer to remove DNA contaminants before elution of the DNA in a small volume of water or TE. A modification of this protocol, known as the "DOUBLE GENECLEAN" method, was employed in the case of subcloning single PCR products. The PCR mixture was treated as above to remove excess primers, enzymes and salts and the resulting elute (50μl) was incubated with the appropriate restriction enzyme (s) and recommended buffer for 1-4 hours. The reaction was stopped with 0.5M EDTA pH 8.0 (1μl per 100μl reaction volume) and

the restricted PCR fragment purified by a second round of the GENECLEAN procedure. No further purification was necessary and the elute could be used directly in ligation reactions.

### 3.14.2. Wizard ™Miniprep DNA purification system (Promega)

This system was used to rapidly isolate small quantities of plasmid DNA suitable for direct sequencing. It was used exactly as described in the product guide. The cells from 1.5ml of an overnight were collected by micrcentrifugation for 2 minutes, and resuspended in 200µl of resuspension buffer (50mM Tris-HCl pH 7.5, 10mM EDTA, 100µg/ml RNase A). 200µl cell lysis solution (0.2M NaOH, 0.1% SDS) was added and the contents mixed by inverting the tube. 200µl of neutralisation solution (1.32M potassium acetate) was added, the contents mixed. The contents were microfuged for 5 minutes and the cleared supernatant was transferred to a tube containing 1ml of wizard purification resin. This mixture was passed through a minicolumn using a syringe. 2ml of column wash solution (100mM NaCl, 10mM Tris-HCl pH 7.5, 2.5mM EDTA, 50% ethanol) was passed over the minicolumn. The washed minicolumn was transferred to a 1.5ml eppendorf tube and the tube was microfuged for 30 seconds, to dry the resin. The minicolumn was transferred to a new eppendorf, and 50µl of water was applied to the minicolumn to elute the DNA from the resin. Plasmid DNA was collected from the minicolumn by microfugation for 1 minute.

### 3.15. Estimation of DNA concentration

An estimation of the concentration of DNA in aqueous solution can be obtained spectrophotometrically by measuring its optical density at 260nm. At this wavelength an optical density of 1 absorbance unit signifies a solution containing 50μg/ml double stranded DNA or 40μg/ml single stranded DNA or approximately 20μg/ml single stranded oligonucleotides. These figures only apply for homogeneous or pure DNA solutions. The ratio between the readings at 260nm and 280nm gives an indication of the purity of the solution concerned. Thus, if the $OD_{260}$:$OD_{280}$ ratio falls between 1.8 and 2.0 this is deemed satisfactory, if it is less than 1.8 accurate quantification is not possible by simple OD measurements.

Fragments generated by digestion of plasmid DNA were visualised on ethidium bromide stained agarose gels and their concentration estimated by direct comparison of the intensity of the corresponding bands, with that of known DNA standards included on the gel. The fluorescent yield of DNA bands, as viewed under UV light is not unconnected with fragment size as larger fragments will intercalate more ethidium bromide. Standards were prepared by linearising known concentrations (100ng/μl and 50ng/μl of commercially available pBR322 DNA with the restriction enzyme *EcoRI*).

### 3.16. Large scale preparation of plasmid DNA and purification by caesium chloride/ethidium bromide centrifugation

The method used was the alkaline lysis method described by Maniatis *et al* (1982). A single colony was inoculated into 10ml LB containing the appropriate antibiotic, and grown overnight at 37°C. The next day 0.5ml of this culture was used to inoculate 50ml of LB, and this culture was grown on a shaker at 37°C for 2-3 hours. This 50ml culture was then used to inoculate 500ml of LB in a 2litre flask which was shaken (200rpm) overnight at 37°C. The next day the cells were pelleted by centrifugation at 5000rpm for 10 minutes. The following quantities are those used for cells from a single 500ml bacterial culture. The pellets were resuspended in a total of 10ml of ice cold solution I (50mM glucose, 25mM Tris-HCl pH 8.0, 10mM EDTA pH 8.0, 5mg/ml lysozyme). The slurry was transferred to an oakridge tube. After incubating at room temperature for 5 minutes. 10ml of freshly prepared solution II (0.2M NaOH, 1% SDS) was added, and the tube was inverted several times to mix the contents. The tube was left on ice for 10 minutes after which 7.5ml of ice cold 5M potassium acetate pH 6.0 was added. The contents of the tube were mixed by inverting several times and then left on ice for 10 minutes. The pH of the solution was then tested with pH paper to ensure that the sodium hydroxide had been completely neutralised, and more potassium acetate added if necessary. Bacterial debris and chromosomal DNA were pelleted by centrifugation at 13,000 rpm for 30 minutes. 18ml of the resultant supernatant was transferred to a 30ml corex tube and nucleic acids were precipitated by adding

12ml of isopropanol. after 15 minutes at room temperature the tubes were centrifuged at 10,000 rpm for 30 minutes at 20°C, and the pellets resuspended in a total of 20ml TE. Exactly 20g of caesium chloride was dissolved in this and 0.6ml of ethidium bromide (10mg/ml) was added. Using a syringe this solution was transferred to a Beckman heat-sealable centrifuge tube. The tubes were topped up with liquid paraffin, balanced and heat sealed. The tubes were then centrifuged at 45,000 rpm in a vertical rotor for 20 hours at 20°C. The tubes were viewed under UV light and the lower band (which is supercoiled plasmid DNA) was removed from the gradient using a syringe. The solution was extracted five times with water-saturated butan-1-ol to remove the ethidium bromide, and then dialysed against two litres of TE overnight. The DNA was precipitated in a corex tube at –20°C by the addition of sodium acetate to a final concentration of 0.3M and two volumes of ethanol. The DNA was recovered by centrifugation at 10,000 rpm for 30 minutes at 4°C and resuspended in 0.5ml of TE. The solution was then extracted with neutral phenol and reprecipitated with ethanol. The DNA was pelleted by microcentrifugation for 10 minutes and then resuspended in 0.5ml of TE. The concentration of the DNA was then determined by measuring the $A_{260}$.

### 3.17. Sequencing of double stranded DNA

Approximately 5μg of plasmid DNA was denatured in 0.2M NaOH and 2mM EDTA (5 minutes at room temperature). The mixture was neutralised with 0.1volumes of 3M Sodium Acetate pH 4.8 and the DNA was ethanol precipitated.

The dried pellet was resuspended in 7µl of sterile distilled water and mixed with 2µl of 5x annealing buffer and 1µl sequencing primer (10µg/ml). Annealing was promoted by incubation at 37°C for 15-45 minutes. Sequencing reactions were performed using the USB sequenase version 2.0 system according to the manufacturer's instructions. The protocol employed is based on the dideoxynucleotide chain-termination method described by Sanger *et al.,* (1977). Following denaturation at 80°C for 3 minutes, 3µl of the samples were loaded onto a denaturing urea/polyacrylamide gel and electrophoresed as detailed in section 3.18.

### 3.18. Polyacrylamide gel electrophoresis for products of sequencing reaction

The DNA ladder generated by the chain-termination method of DNA sequencing can be resolved by vertical electrophoresis through a 38cm polyacrylamide/urea gel, consisting of 7.5ml 38% (w/v) acrylamide 2% (w/v) bisacrylamide, 5ml 10x TBE, 21g urea and made up to 50ml with distilled water. Polymerisation was initiated with the addition of 450µl 10% (w/v) APS and 70µl TEMED. The solution was mixed and poured into the gel casting apparatus. A shark-tooth comb was inserted with its straight edge into the gel, which was then allowed to polymerise in a horizontal position for at least one hour.

The polymerised gel was placed in a vertical electrophoresis tank and 1×TBE was added to upper and lower reservoirs. The comb was removed and replaced in the

opposite orientation, with the teeth inserted slightly into the gel so as to form the wells. The gel was pre-run for 30 minutes. Immediately prior to sample loading, the wells were washed out with buffer in order to remove traces of urea and acrylamide. Samples were heated to 80°C for 3 minutes, loaded onto the gel and electrophoresed under constant power (38 watt) for 1-4 hours. After this time the gel plate was prised a part and the gel immersed in a fixing solution of 10% (v/v) acetic acid and 10% (v/v) methanol for 15 minutes. The gel was dried onto Whatman paper under the combined action of heat and vacuum. The dried gel was exposed overnight to X-ray film at room temperature.

### 3.19. Preparation of total RNA from *Xenopus laevis* tadpole tails

Tadpole tails after treatment regimes were washed thoroughly in sterile distilled water, frozen in liquid nitrogen for few seconds and then placed in a glass homogeniser or eppendorf tubes depending on volume containing the denaturing solution (25g guanidine thiocyanate 4M and 33ml CSB buffer (42mM sodium citrate, 0.83% N-lauryl sarcosine and 0.2mM β-mercaptoethanol). For 1g of homogenate, 1.2ml of 2M sodium acetate pH 4.0 was added and the tube contents were mixed thoroughly by inversion.12ml of phenol/chloroform/isoamyl alcohol mix (25.24.1) were also added mixed by inversion, shaken vigorously for 10 seconds and chilled on ice for 15 minutes. This mixture was then transferred to a 50ml thick-walled polypropylene tube (DEPC-treated) and centrifuged at 10,000 x g for 20 minutes at 4°C. Carefully, the top aqueous phase which contained the

RNA was removed and transferred into a fresh DEPC treated tube. DNA and proteins were retained in the organic phase and at the interphase. RNA was precipitated by adding an equal volume of isopropanol and incubated the sample at –20°C for at least 30 minutes. To obtain the maximum RNA yield from tissue samples that contain relatively low amounts of RNA, longer precipitation (up to overnight) is recommended. RNA was pelleted by centrifugation at 10000x g for 15 minutes at 4°C, and resuspended in 5ml of denaturing solution. In some instances, heating to 65°C may be required. An equal volume of isopropanol was added and the RNA was precipitated as described above. The pelleted RNA was then washed with ice-cold 75% (10ml) ethanol and centrifuged as above. The pellet was then dried in a vacuum desiccator for 15-20 minutes and resuspended in RNAse-free deionised $H_2O$ and stored at –20°C.

### 3.20. Differential Display Reverse transcription-PCR

This method was first described by Liang and Pardee (1992). It is used to identify messages that are differentially expressed among RNA samples and involve reverse transcription as well as PCR steps.

### 3.21. Reverse transcription reactions

Reverse transcription reactions were carried out on 2 μg of total RNA extracted from *Xenopus laevis* tadpole tails using superscript M-MLV reverse transcriptase (BRL) with reaction buffer and DTT added as described by the manufacturer. The

reaction was carried out in the presence of 2.5μM of 3'primer (one of set of twelve) and 20μM dNTPs. In addition, 200 units of reverse transcriptase and 10 units of human placental ribonuclease inhibitor (BRL) were used in each 20μl reaction. Reactions were incubated at 37°C for one hour and then the enzyme was heat inactivated at 70°C for 10 minutes.

### 3.22. PCR reactions

One-tenth of the product cDNA from the reverse transcription reaction was introduced into each 20 μl PCR reaction. PCRs were then carried out in the presence of 2.5 μM of 3' primer same as the one used in reverse transcription step, 0.5μM of 5' primer arbitrary 10mer and 2μM dNTPs. The reaction mix included 1μl [$\alpha^{35}$S]-dATPαS, manufacturers reaction buffer (BRL) and magnesium chloride to 1.25mM. The reaction mixture was overlaid with 20μl of mineral oil and 2.5units of Taq DNA polymerase (BRL). Following the denaturation of the reaction mixture at 94°C for 30 sec, annealing at 42°C for 1min, and an extension at 72°C for 30sec. 6.5μl of the PCR reaction products was analysed on a 6% denaturing polyacrylamide gel. Samples utilising the same primer but originating from different treatment regimes were run side by side to allow identification of differentially expressed products. Gels were not fixed or dried prior to autoradiography. The gel was left on the gel back plate, wrapped in cling film, radioactive ink was used to mark the gel to subsequently allow the gel to be lined up with the autoradiograph and autoradiography was carried out at –70°C.

### 3.23. Quantitative RT-PCR

Reverse transcription reactions were carried out using 0.5μg (2μl) of total *Xenopus* RNA from thyroid hormone treated and untreated samples made up to a total volume of 20μl sterile distilled water, and heated to 75° for 5 minutes in a heating block or PCR machine. Reactions were then stored on ice. The following components were added . Random hexamers (100μM stock) (1μl, at 3.3μM final concentration), 3μl (10x PCR buffer), 1.8μl (50mM $MgCl_2$), 1.5μl (10mM dNTPs), 0.6μl (50u/μl RNase inhibitor. The reaction mixture was allowed to incubate at 42°C for 5 minutes before adding 2μl of MMLV reverse transcriptase (Gibco BRL, 200u/μl), and a further incubation at 42°C for 60 minutes was performed. The reaction was stopped by heating to 95°C for 5 minutes and stored at –20°C. 2μl of the synthesized cDNA was used in a 50μl PCR reaction. The cDNA can be aliquotted into 0.5ml eppendorfs and stored on ice while the PCR reaction stock is made. 2.5μl of 10x PCR buffer was added to the aliquotted cDNAs, followed by the addition of 0.75μl of $MgCl_2$ (50mM), 0.5μl dNTPs (10mM), 0.05μl [$\alpha^{32}$P]-dGTP (10μCi/μl), 2μl L+R primer mix (12.5μM), 0.2μl Taq DNA polymerase (5u/μl), and the volume was adjusted with 14.1μl $H_2O$. 25μl of light paraffin oil was topped on tubes to prevent evaporation. PCR cycles were performed as follows: (denaturation at 94°C for 3 minutes), (annealing at 55°C for 1minute), (extension at 72°C for 1minute). The number of cycles varies, for elongation factor Ef-1α, 18 cycles were sufficient, however the others are less abundant and need 25 cycles. The important thing is to stay in the exponential phase of the amplification.

### 3.24. Oligolabelling / Random Priming

This is the fast and easy way to make a probe from an isolated DNA fragment. Briefly, the fragment of interest from LMP agarose gel (low melting point) was isolated and purified by melting the agarose at 65°C. The DNA was then precipitated by adding 3M sodium acetate to 0.3M final concentration followed by extraction with phenol/0.3M sodium acetate then phenol / chloroform/ isoamyl alcohol. The DNA was then checked on a minigel for estimating quantity. 50-100ng of the DNA fragment was made up to 32.5µl distilled water, heated to 95°C for 3 minutes and immediately cooled on ice. 10µl OLB solution (i.e., oligolabelling buffer) A:B:C at 100:250:150 ratio was added (Soln O: 2.5ml/2M Tris-HCl pH 8, 0.5ml/1M $MgCl_2$, 1ml $H_2O$; Soln A: 1ml soln O,18µl 2-mercaptoethanol, 5µl dATP, dCTP, dTTP each 0.1M all that except used for labelling ; Soln B: 2M HEPES pH 6.6; Soln C: 6-mer random hexamer oligos at 90 OD units/ml in TE). 2µl BSA/10mg/ml, 5µl 50µCi [$\alpha^{32}$P]- dGTP and 2µl Klenow DNA polymerase were added in order. They were then allowed to incubate well shielded at room temperature for 2 hours to overnight. The probe was then precipitated by adding 50µl $H_2O$, 1µl 5mg/ml tRNA, 100µl chloropane (i.e. phenol: chloroform: isoamylalcohol) and well vortexed followed by 5 minutes spin. The aqueous layer was then taken and an equal volume (100µl) of 5M ammonium acetate, 2.5 volume (500µl) of ethyl alcohol was added and left at -20°C for 30 minutes, dried and resuspended in 100µl $H_2O$. Another way of precipitating the probe was by adding 10µg tRNA, 12.5µl 3M Sodium acetate, 50µl isopropanol and

mixing well followed by a 5 minutes incubation on ice and a 15 minutes spin. A radioactive counts comparison was made between supernatant and pellet. These ought to be equal. Resuspended pellet was then stored shielded at –20°C for later use. Alternatively, a spin column (from Pharmacia) was used to elute and purify the probe from the unincorporated nucleotides.

### 3.25. Paraffin wax sections of tadpole tails

#### 3.25.1. Fixation

Tadpole tails isolated from *Xenopus laevis* stage 52 after their treatment regime, were fixed in a solution of paraformaldehyde-PBS (Tata, *et al.*, 1991) for 24 hrs at room temperature. They were then placed in 70% ethanol for 15 minutes at room temperature. The liquid was drawn off and replaced by 100% ethanol for 15 minutes, this was then replaced by xylene and let to stand for 15 minutes. Xylene was then decanted and replaced by warm xylene at 60°C for 15 minutes, this was replaced by xylene-wax warmed at 60°C (saturated or near saturated solution) followed by an incubation for 30 minutes at 60°C. This was again replaced with 100% wax at 60°C. and a further incubation for 30 minutes. The last step was repeated and finally the wax was changed and left at 60°C. for one hour to overnight.

### 3.25.2. Embedding

In a watch-glass smeared with glycerol, a small amount of wax was poured in, and the tails was carefully and quickly oriented with the help of warmed forceps, paper label and more wax were added to fill the watch-glass, this was allowed to set somewhat and the whole assembly was immersed in cold (preferably chilled) water and left to set completely, preferably 2 hours after which the wax block was floated out of the watch-glass.

### 3.25.3. Sectioning

#### 3.25.3.1. Preparation of subbed slides for tissue sections

Subbed slides were prepared in advance by washing them in strong detergent, and then rinsed in distilled water, dipped into a solution of gelatin (0.5%, Sigma) plus 0.5% chrome alum. This solution was prepared by dissolving gelatin by boiling without the chrom alum until cooled to room temperature. The slides were then air dried and baked at 80°C for 8 hours to overnight.

The wax block was the trimmed by cutting away from the specimen and note was taken for the required orientation. The wax was then mounted onto a wooden block by melting the wax (i.e., using a heated scalpel), and the wax-embedded specimen was then placed on top. The edges were well sealed by using a heated blade. The assembly was allowed to cool thoroughly so that the wax was fully set in place. The wax block was finally trimmed. In order to produce a good ribbon, the sides of the wax block were placed so as to present a rectangular face to the

knife. The blade was cleaned by wiping with tissue soaked in xylene. The block was then mounted in the microtome with the blade adjusted in position. The angle of the blade was set to 16 degrees, and sections of 8 microns were cut to form a ribbon. As the ribbon formed, suitable lengths were taken by lifting them with a soft paintbrush and placed on a subbed slides. The sections were placed in a logical order of which a note was kept, and the slides were labelled using a diamond pencil. Carefully, some water was pipetted onto the slide to float the sections. The slides were then placed on a hotplate at 40°C to extend the sections. When the sections were fully extended, the water was sucked off and the slide left to dry on the hotplate.

### 3.25.4. Dewaxing

It is essential that sections are completely dewaxed. The slides were placed in xylene for 15 minutes. They were then transferred into clean xylene for few minutes.

### 3.25.5. Staining

The sections were placed in 25% ethanol, stained in Hematoxylin for 3-8 minutes,washed in alkaline tap water with many changes for 5 minutes.

| | | | |
|---|---|---|---|
| **Scott's Hematoxylin:** | Hematoxylin | 1.25 | g |
| | Glycerol | 100 | ml |
| | Dist.water | 100 | ml |
| | Ethanol | 100 | ml |
| | Glacial acetic acid | 10 | ml |
| | Potash alum | 7 | g |

The sections were also stained 1% aqueous eosin for one minute, taken up through ethanol series (30, 60, 80, 95, 100%) , air dried and mounted in DePex (i.e., commercial mountant).

## 3.26. Preparation of synthetic RNA for microinjection into oocytes / embryos

### 3.26.1. *In vitro* transcribed capped RNA

This was essentially performed according to the manufacturer instructions (Ambion's message machine T3 RNA polymerase). Briefly, for synthesis of run-off transcripts of defined size, the double-stranded DNA template was digested to completion with suitable restriction enzyme that cleaves distal to the promoter (*Xho1*). After restriction enzyme digestion, the template was checked on 1% agarose gel, isolated and purified using GENECLEAN II kit and the DNA concentration was estimated. 1µg of linearised template DNA (2µl of 0.5µg/µl) was used in a 20µl reaction volume. In a 1.5ml microfuge tube at room temperature, 4µl RNase-free water, 2µl 10×Transcription buffer, 10µl Ribonucleotide Mix (15mM ATP, CTP, UTP; 3mM GTP and 12mM cap analogue), 2µl 10×Enzyme Mix (a combination of placental ribonuclease inhibitor, RNA polymerase and other components required for optimal RNA synthesis) were mixed and microfuged briefly to collect all the reaction mixture at the bottom of the tube. The reaction was incubated at 37°C incubator for 90 minutes. 1µl of RNase-free DNase I (2U/µl) was added to the reaction, mixed thoroughly and a further incubation at 37°C for 15 minutes was carried out. The reaction was terminated

and the RNA was recovered by adding 30µl of RNase-free $dH_2O$ and 25µl of Lithium Chloride Precipitation solution (7.5M Lithium Chloride, 75mM EDTA), mixed thoroughly and chilled for at least 30 minutes at –20°C. The RNA was pelleted by centrifugation at 4°C for 15 minutes at maximum speed. Carefully the supernatant solution was removed and the pelleted RNA was washed once with 70% ethanol and re-centrifuged to maximise removal of unincorporated nucleotides. The supernatant solution was carefully removed and the RNA was resuspended in RNase-free $dH_2O$.

### 3.27. *In vitro* translations

*In vitro* transcribed RNA was used to synthesise proteins *in vitro* using rabbit reticulocyte lysate (Promega). The reaction involved 1µl (0.1-0.5µg) of synthetic capped transcript with 10µl of rabbit reticulocyte lysate and 20µCi [$^{35}$S]-methionine. The reaction was incubated at 30°C for 1hour and stopped by placing the reaction on ice. Samples of the translation products were analysed by SDS-PAGE. Prior to loading on the gel the sample was mixed with an equal volume of 2x SDS-loading buffer (112mM Tris-HCl pH 6.8, 3.6% SDS, 18% glycerol, 0.01% bromophenol blue, 1.4M 2-mercaptoethanol) and boiled for 3 minutes.

### 3.28. SDS-protein polyacrylamide gel electrophoresis

A separating gel consisting of 10-15% acrylamide (37.5:1 bis), 0.3M Tris-HCl pH 8.8, 0.08% SDS was poured into the assembled gel plates (minigels), leaving sufficient space at the top for the stacking gel to be added later. After polymerisation (10-15 minutes), the surface of the separating gel was first rinsed with water to remove any unpolymerised acrylamide and then with a small volume of stacking gel mix (3% acrylamide (20:1 bis), 0.125M Tris-HCl, 0.1% SDS). The remaining space was filled with stacking gel and the comb was immediately inserted. After the stacking gel had polymerised (30-45 minutes), the comb was removed and the wells were rinsed with water to remove the unpolymerised acrylamide. A small aliquot of protein sample was mixed with an equal volume of loading buffer (112mM Tris-HCl pH 6.8, 3.6% SDS, 18% glycerol, 0.01% bromophenol blue, 1.4M 2-mercaptoethanol), heated to 95°C for 5 minutes and then loaded on the gel. Typically, electrophoresis was carried out at a constant current (15mA in the stacking gel and 30mA in the separating gel) in 1x running buffer (50mM Tris, 400mM glycine, 0.1% SDS). Electrophoresis was usually performed until the bromophenol blue dye front had run off the bottom of the gel. Gels were fixed in 45% methanol, 10% acetic acid for 30 minutes, vacuum dried and autoradiographed.

### 3.29. Whole-mount *in situ hybridisation* analysis

*In situ* analysis was performed as described in Harland (1991).

#### 3.29.1. Embryo preparation

Various stages of embryos and tadpole tails at stage 52 were fixed in MEMFA (0.1M MOPS pH 7.4, 2mM EGTA, 1mM $MgSO_4$, 3.7 formaldehyde). The vitelline membranes were removed from the pre-hatched stages. Embryos were then fixed overnight at 4°C. MEMFA was rinsed from the embryos washed thoroughly in distilled water and replaced by methanol. Such embryos can be kept at –20°C.

#### 3.29.2. Prehybridisation

Embryos and were rehydrated through a methanol series (75%, 50%, 25%, methanol (v/v) for 5 minutes in each). They were then transferred to PTw (PBS+0.1% (v/v) Tween-20). Digestion with Proteinase K to 5μg/ml was carried out at room temperature for 10 minutes. They were then washed in PTw (2×5 minutes), and refixed in 3.7% formaldehyde in PTw for 20 minutes. After fixation, they were washed in PTw (5×5 minutes) to remove any fixative before the hybridisation step. They were then transferred to hybridisation mix [(50% formamide, 5×SSC, 1mg/ml torula RNA (Sigma), 100μg/ml Heparin (Sigma), 2% Blocker powder (Boehringer), 0.1% Tween-20 (BDH), 0.1% CHAPS (Sigma)] and prehybridised at 60°C for at least 6 hours.

### 3.29.3. Probe synthesis

This is a standard transcription reaction with DIG labelled UTP (DIG-UTP) being incorporated in the place of UTP using the DIG RNA labeling kit (Boehringer). Subclones were introduced into transcription vectors in an orientation that allowed synthesis of the antisense strand. Then a 20µl labelling reaction contained 1× transcription buffer, 10mM ATP, 10mM CTP, 10mM GTP, 6.5mM UTP, 3.5mM DIG UTP, 1µg linearised template DNA, 20units human placental RNase inhibitor and 20 units RNA polymerase. The reaction was incubated at 37°C for 1-2 hours. The product was ethanol precipitated at –20°C for at least 30 minutes and resuspended in 10µl water.

### 3.29.4. Hybridisation

Embryos and tails were transferred to fresh hybridisation mix containing probe (1µg/ml). Hybridisation was carried out at 52°C overnight. Both embryos and tails were then washed in 2×SSC / 0.3%CHAPS at 60°C (3×20 minutes). RNase A digestion (20µg/ml) was then carried out at 37°C for 30 minutes. Additional washes comprising 2×SSC / 0.3% CHAPS at room temperature for 10 minutes, 0.2×SSC / 0.3% CHAPS at 60°C for 2×30 minutes, PTw / 0.3% CHAPS at 60°C for 2×10 minutes, PTw at room temperature for 10 minutes were carried out.

### 3.29.5. Detection

PTw was replaced with PTw / 0.5% Blocker powder and incubated at room temperature for 15 minutes. This was then replaced with a fresh aliquot of the same solution containing anti-digoxygenin antibody (1/2000 dilution) (Boehringer) and incubated overnight at 4°C. Embryos and tails were then washed in Ptw/5mM Levamisol (Sigma) at 4°C (4×1 hour) and transferred to chromogenic buffer (100mM Tris-HCI pH 9.5, 50mM $MgSO_4$, 100mM NaCl, 0.1% Tween-20, 5mM Levamisol) and incubated at room temperature for 10 minutes. The chromogenic buffer was replaced by chromogenic buffer+20µl/ml NBTand BCIP mix (Boehringer). Samples were covered in foil and maintained at room temperature to allow the colour reaction to develop. Colour reactions were stopped by rinsing in TE and fixed in MEMFA. Samples were then dehydrated in methanol and cleared in 2:1 benzyl benzoate : benzyl alcohol (BDH) prior to photography.

# CHAPTER 4

## Isolation of thyroid hormone (Triiodothyronine) $T_3$-induced genes by DDRT-PCR

### 4.1. Introduction

Until recently analysis of changes in gene expression relied mainly on subtractive hybridisation or differential hybridisation used to identify genes that are expressed in only one cell type of respective pairs of cells. These are mainly qualitative methods which do not allow quantitative comparisons. In addition, these methods are time consuming and not always satisfactory.

A method recently published by Liang and Pardee (1992) allows identification and isolation of genes that are differentially expressed. This technique was used to attempt to identify genes regulated by thyroid hormone (triiodothyronine $T_3$) in *Xenopus laevis* tadpole tails.

### 4.2. Treatment of *Xenopus laevis* tadpole tails stage 52 with $5\times10^{-9}$ M $T_3$

#### 4..2.1. Introduction

Amphibian metamorphosis is a post-embryonic process that systematically transforms different tissues in a tadpole. Thyroid hormone plays a causative role in this complex process by inducing a cascade of gene regulation. While natural metamorphosis does not occur until endogenous thyroid hormone has been synthesised, tadpoles are competent to respond to exogenous thyroid hormone

shortly after hatching. In addition, even though the metamorphic transitions of individual organs are all controlled by thyroid hormone, each occurs at distinct developmental stages. Recent molecular studies suggest that this competence of premetamorphic tadpoles to respond to the hormone and the developmental stage-dependent regulation of tissue-specific transformations are determined in part by the levels of thyroid hormone receptors and the concentrations of cellular free thyroid hormone. In addition, at least two genes, encoding a cytosolic thyroid hormone binding protein and a 5'-deiodinase, respectively, are likely to be critical players in regulating cellular free thyroid hormone concentrations.

One of the more dramatic effects of $T_3$ and $T_4$ in metamorphosis is to induce complete regression of the tadpole tail. The dependence of this resorption upon the local action of the thyroid hormone has been clearly established since isolated tadpole tails maintained *in vitro* in a simple chemically defined medium will undergo complete regression in the presence of very low doses of $T_3$ and $T_4$ (Tata, 1994).

The rate of tail regression was determined by daily measurements of its length as a result of $T_3$ stimulation (see Figure 8). In addition, Figure 9 depicts the morphology of stage 52 tail explants, representative of six samples cultured in multiwell dishes. In culture, wells 1, 2 and 5, showed a highly conserved dorsal and ventral fins on each side of the central body composed of muscle and

connective tissue, skin and muscle were observed after 5 days of the commencement of the experiment. By this time $5x10^{-9}$ M $T_3$ caused the total loss of both fins as well as considerable loss of connective tissue, skin and muscle (wells 3,4 and 6).

In transverse sections of the tails cultured, both the more substantial ventral and the smaller dorsal fins are easily seen in the untreated tails. The mesenchyme and the closely packed muscle fibres surrounding the central notochord, spinal chord and connective tissue are also clearly seen. In parallel with the shortening of the tail length, transverse sections revealed the total elimination of dorsal and ventral fins as well as connective tissue accompanied by considerable compaction and loss of muscle tissue upon addition of $5\times10^{-9}$ M $T_3$ (Figure 10).

FIG 8. Cultured tail regression induced by triiodothyronine ($T_3$). The rate of tail regression was determined by daily measurements in cultures of stage 52 *Xenopus laevis*. Symbols for the controls and $T_3$ treated samples are indicated. Day 0 indicates tail explants without any additives; Day 1 to 5 represent tail explants treated with $5 \times 10^{-9}$ M $T_3$.

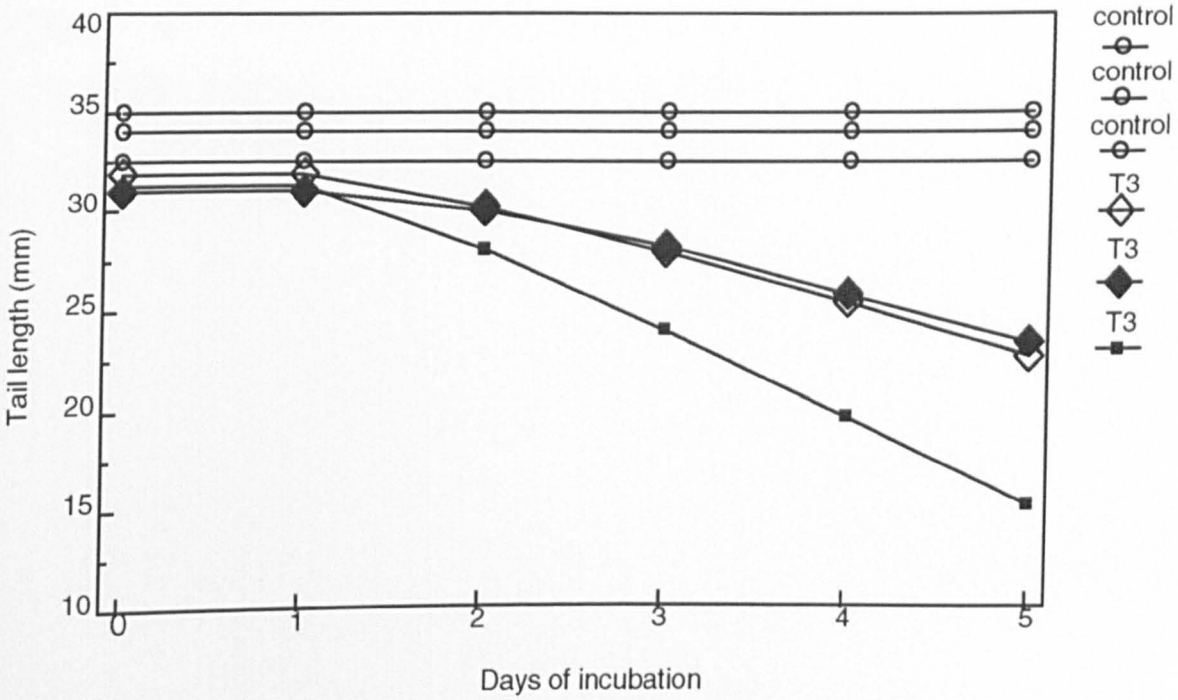
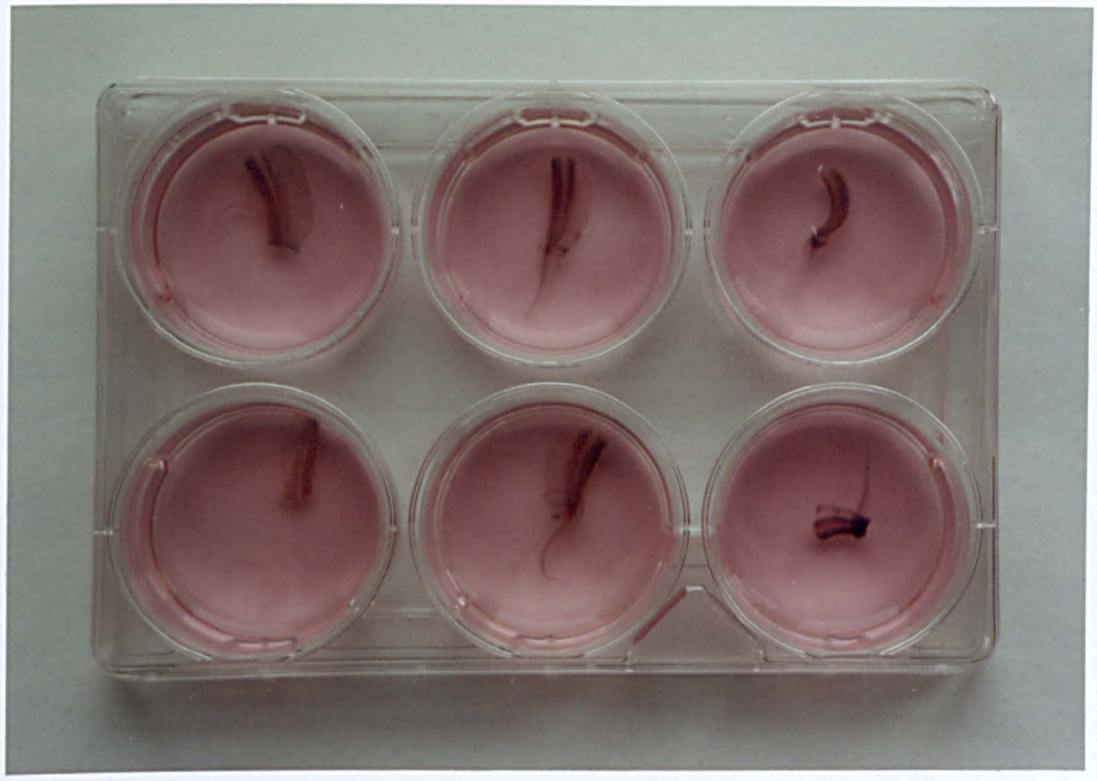

40
35
30
25
20
15
10
Tail length (mm)
0
1
2
3
4
5
Days of incubation
control
control
control
T3
T3
T3

FIG 9. Effect of triiodothyronine ($T_3$) on *Xenopus laevis* tadpole tails stage 52 in organ culture. Tadpole tails were cultured in multi-well dishes for 24 hrs before the hormone was added. Additions: well 1, 2,5; control; well 3,4,6; $5x10^{-9}$M $T_3$. Note in culture well 1,2,5 the highly conserved dorsal and ventral tail fins on each side of the central body, composed of muscle and connective tissue, 5 days after the commencement of the experiment. By this time $5x10^{-9}$M $T_3$ caused a considerable loss of connective tissue, skin and muscle (wells 3,4 and 6).

1 2 3



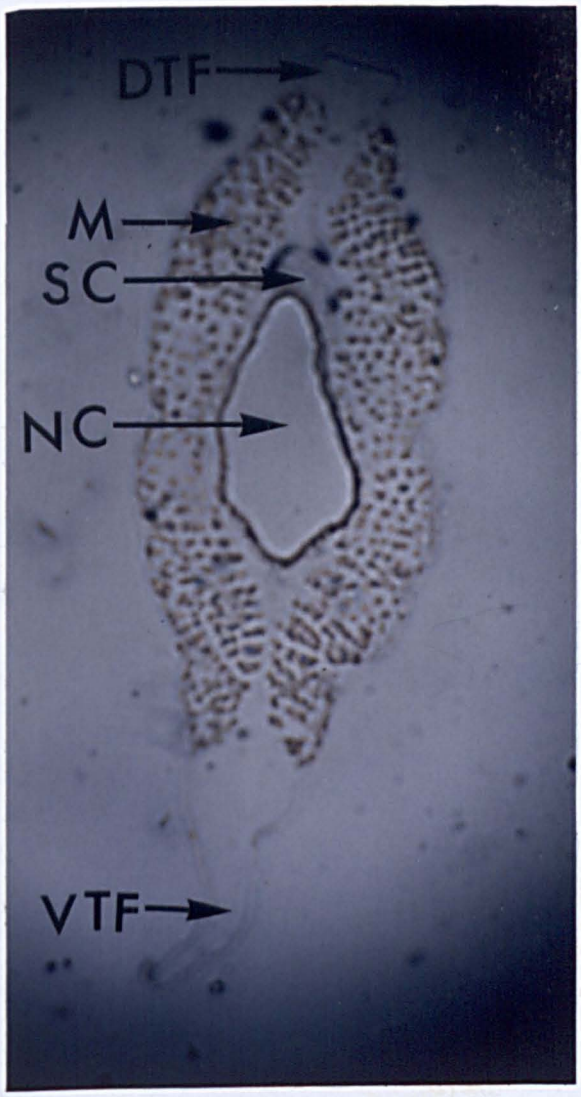
4 5 6

FIG 10. Histological sections of stage 52 *Xenopus laevis* tadpole tails after 5 days in culture and following treatment with $5x10^{-9}M$ $T_3$ stained with eosin and hematoxylin.

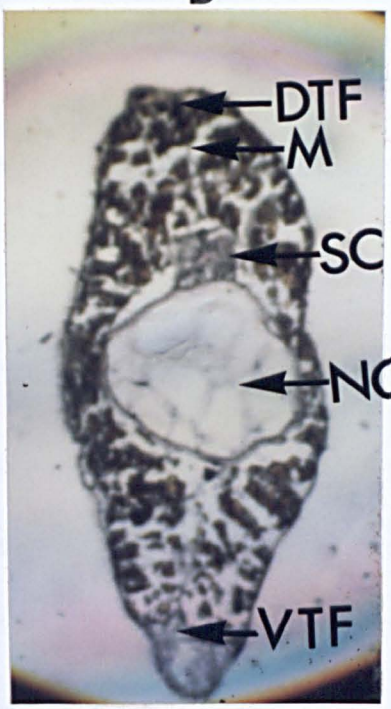
Panel A. Shows the more substantial ventral and the smaller dorsal fins in the untreated tails. The mesenchyme and the closely packed muscle fibers surrounding the central notochord, spinal chord and connective tissue are also clearly seen.

Panel B. Reveals total elimination of dorsal and ventral fins as well as connective tissues accompanied by considerable compaction and muscle loss upon the addition of $5x10^{-9}M$ $T_3$. Abbreviations: DTF and VTF; dorsal and ventral tail fins; SC, spinal cord; NC; notochord; M, muscle.

A




B



## 4.3. Differential display reverse transcriptase polymerase chain reaction

## DDRT-PCR

RNA extracted from stage 52 *Xenopus laevis* tadpole tails following their treatment regimes was subjected to DDRT-PCR. Each of the set of twelve 3′ primers were used to prime reverse transcription reactions. Following RT the subpopulation of cDNAs generated were introduced to a PCR reaction primed by the same 3′ primer that was present for the cDNA synthesis and a 10 mer 5′primer arbitrary in sequence. The 5′primer used was the same in all differential PCR reactions performed, this primer was termed 504 and had the sequence AGACTTCGAG. The resulting PCR products were compared across the treatment regimes, side by side, following electrophoresis on a 6% denaturing acrylamide gel and autoradiography.

In order to limit the number of false positives that are isolated, it is important to obtain reproducible RT-PCR band patterns with the system under investigation. This has been achieved with the method outlined in the methods section by optimising the annealing temperature.

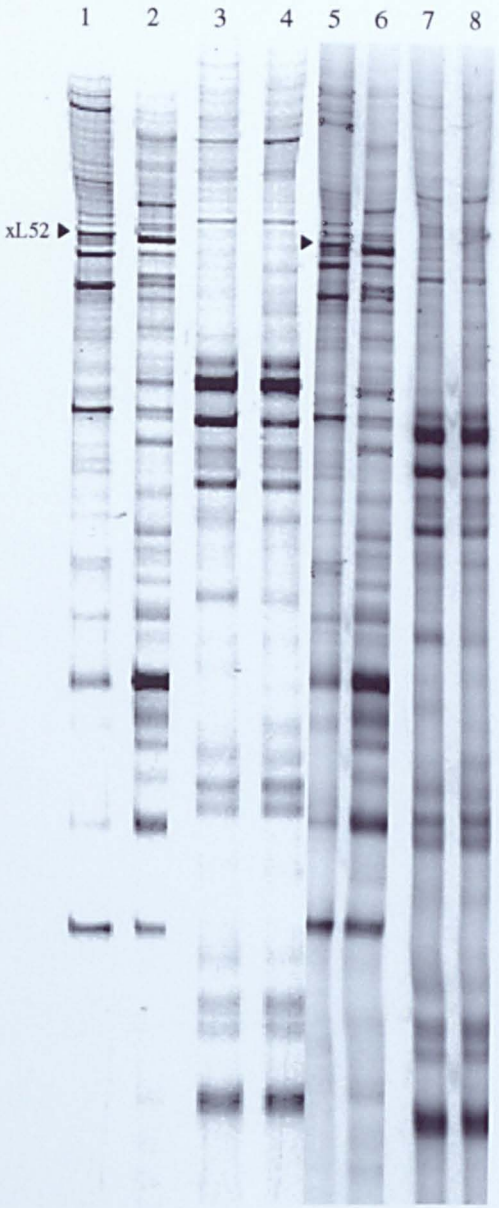
Duplicate reactions ensure that bands are reproducible and therefore likely to represent a defined priming site. This reproducibility enhances the possibility that differences seen in the band pattern are more likely to indicate differentially expressed genes. With this in mind, only bands indicating differential gene

expression in duplicate were isolated (figure 11). These gels were repeated twice to ensure that the band pattern obtained in the first gel is similar to the one in the second gel when repeated.

The primers that generated clear banding patterns varied in number of distinct bands present in a track. The number of bands varied from 50-100 from a single gel run, the size of these products was in the range between 100-350 bases. The majority of the bands were present at similar intensity across the treatments.

I was keen to isolate genes that altered in response to thyroid hormone and so I was looking for bands either stronger or weaker (i.e. up-regulated or down-regulated) by thyroid hormone. From the four sets of reactions that gave good banding patterns it was possible to select ten bands that appeared to be obviously differentially expressed, these ten bands represent 1% of the total number of bands that were looked at. Of these ten bands eight were up-regulated by thyroid hormone and two were down-regulated. Obtaining more differential bands by varying the run length of the acrylamide gel and by substituting in different 5′ primers in the PCR remains possible. The ten differential bands were excised from the gel and the DNA eluted, ready for further characterisation.

FIG 11. An example of differential display technique (DDRT-PCR) on total RNA extracted from thyroid hormone treated ($5x10^{-9}$M $T_3$, lanes 1,3,5,7) and untreated, (lanes 2,4,6,8) *Xenopus laevis* tadpole tails. The autoradiograph shows the banding pattern obtained from two sets of primers. (i) 5′Primer-504 (ii) 3′ Primer- $T_{12}CA$ on lanes (1,2,5,6) (iii) 5′ Primer-504 (iv) 3′ Primer- $T_{12}GC$ on lanes (3,4,7,8). Duplicate gels ensure the reproducibility of the DDRT-PCR. Only bands in duplicate were isolated. xL52 band is indicated by arrow.


1
2
3
4
5
6
7
8
xL52

### 4.4. Excision, reamplification and cloning of differential bands

Differentially expressed bands were identified on the autoradiograph oriented on the gel by lining up the radioactive ink mark. After developing the film, cDNA bands of interest were located by cutting through the film. A scalpel blade was used to cut the gel slice. The gel slice was then transferred to an eppendorf tube containing 50µl of NAE (300mM sodium acetate, 1mM EDTA). The tube was left at room temperature overnight to allow DNA to elute from the gel slice. 200ng of carrier glycogen was added and phenol extraction was carried out, after removing the acrylamide slice. Nucleic acids were precipitated from the aqueous phase at –20°C for one hour following the addition of two volumes of ethanol. Nucleic acids were then pelleted by centrifugation and the pellet was dried in a vacuum drier and resuspended in 20µl of distilled water. 7µl of this eluted DNA was reamplified in a 40µl PCR reaction volume using the same primer set and PCR conditions as used in the mRNA display except the dNTP concentrations were 20µM and no [$\alpha^{35}$S]-dATP αS was added. 10µl of PCR samples were run on a 1% agarose gel and stained with ethidium bromide which showed the majority of amplifications to have worked (see figure 12.). These PCR products were phosphorylated using T4 polynucleotide kinase and electrophoresed on a 1% agarose gel, the correct PCR product was cut from the gel and purified using GENECLEAN II (see Methods 3.14.1.). The PCR products were then ligated into the dephosphorylated *HincII* site pBluescript KS vector. Five of these ten differential cDNAs were selected, reamplified, and cloned (see Figure 13). Because of the time constraints, only one

clone was proceeded with for further characterisation at any extent. The remaining samples were stored at −20°C for future use.

### 4.5. Sequencing differential products

The identified clones (Figure 13) for each differential band, were then sequenced by both the dideoxy chain termination method using a sequenase version 2.0 kit (Stratagene) and the automated sequencing utilising the pBluescript T3 primer. Depending on the orientation of the insert this was sequenced either from the polyadenylate tail or the 5' end. The sequence obtained in each case was very A/T-rich, a characteristic of 3'untranslated sequence, with no significant open reading frames. These sequences (see Appendix B), were in turn used to search the GenEMBL nucleotide sequence databases, using the non-redundant BLAST search. Due to the small size of the inserts, subcloning steps to obtain complete sequence were not needed.

FIG12. Differential display PCR products eluted from 6% polyacrylamide gel and amplified further in a second PCR. After amplification, these products were analysed on 1% agarose gel and visualised by ethidum bromide under the UV illuminator. The products were run side by side along with DNA ladder. The size of each PCR product was determined. xL52 band is indicated; other bands: 1, 3, 4, 5 correspond to DDTA, DDTC3, DDTD3, DDTE respectively.

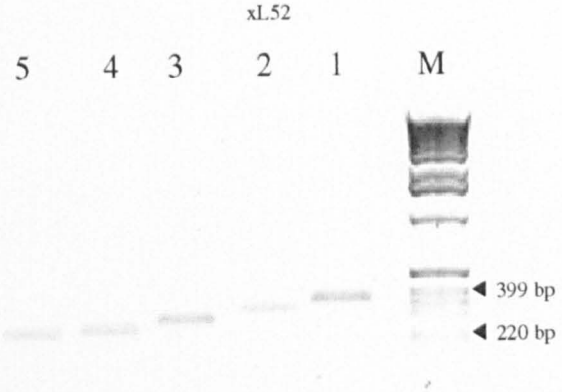
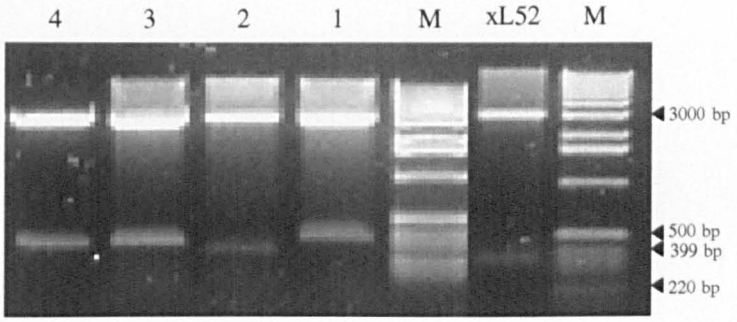

xL52
5
4
3
2
1
M
399 bp
220 bp

FIG 13. PCR products cloned into *HincII* site cut pBluescript (KS). These products represent cDNAs of interest eluted from 1% low melting point (LMP) agarose gel, after being purified with DNA purification kit (Promega). The cloned cDNAs were released from the pBluescript (KS) using restriction enzymes *KpnI* and *SmaI*. The size of each clone was determined by using DNA ladder marker. (M, marker; xL52 clone is shown along with the other cloned products 1,2,3,4. These cloned products (i.e., 1,2,3,4), refer to the sequenced products in Appendix B as: 1, DDTA; 2, DDTE; 3, DDTC3; 4, DDTD3.


4
3
2
1
M
xL52
M
3000 bp
500 bp
399 bp
220 bp

## 4.6. Confirmation of the inducibility of cloned products by RT-PCR

### 4.6.1. Introduction

Levels of individual mRNAs have been analysed by procedures such Northern blots, RNase protection. These common methods for detection and analysis of gene transcripts, require amounts of total RNA in excess of several micrograms, even when examining gene transcripts expressed at high levels. Typically, RNA analysed by these methods must be further enriched for mRNA by oligo (dT) cellulose chromatography. RT-PCR not only provides a more sensitive method requiring smaller amounts of RNA, but in some cases is the only method that can be used. For example, the dystrophin gene, defective in patients with muscular dystrophy, is expressed at very low levels (representing only 0.01-0.001% of total muscle mRNA), making it difficult to study by conventional methods. RT-PCR was successfully used by (Chelly *et al.*, 1988), to study levels of this mRNA in clinical samples. The poorly expressed multidrug resistance gene *mdr*-1 has also been studied by RT-PCR, whereas conventional methods were unsuccessful at finding transcripts (Fuqua *et al.*, 1990; Murphy *et al.*, 1990). In some experimental models, genes may be expressed at moderate to high levels, but only in tissues of minute size, such as early mouse embryos (Gaudette and Crain, 1991).

Reverse transcription (RT) followed by polymerase chain reaction (PCR) amplification is a widely used, highly sensitive method for the expression analysis of low abudance messenger RNAs (Kawaski and Wang, 1989). Oligo (dT),

random (hexamer) primers and the PCR antisense primer are used as primers for the reverse transcription reaction. Oligo (dT) primers are often inefficient for transcripts with long 3'untranslated regions, since the efficiency of reverse transcription decreases as distance from the primer increases. Both oligo (dT) and random hexamer primers are not sequence-specific and, therefore, an alternative lies in the use of sequence-specific primers, such as the PCR antisense primer itself.

### 4.6.2. RT-PCR assays to confirm thyroid hormone inducibility of xL52 clone

#### 4.6.2.1. Determination of the number of rounds of amplification used in PCR with primers designed in this study

A number of primer sets were designed for use in this study. All PCR primer pairs were designed using Primer Designer software (Scientific & Educational Software) to optimise annealing temperatures and avoid the risk of primer dimer and secondary structure formation, which might prevent proper priming. In order to establish the conditions at which these primers could amplify the target cDNA. RNA from *Xenopus* tadpole tails isolated at the stage desired for this analysis, was reverse transcribed and amplified for various numbers of cycles. The elongation factor Ef-1$\alpha$ primers were tested. This primer set was chosen to act as a control, since it is non-inducible and varies little in post-neurula stages. The data obtained were analysed and the minimum number of cycles required for amplification was established. The reason behind this is that because PCR amplification is an

exponential process, small variations in amplification efficiency can drastically affect the yield of products. In addition, the efficiency at the later stages of amplification due to depletion of reaction components, diminished enzymatic activity, and accumulation of products. Therefore, any attempt to quantitate mRNA levels by PCR must be limited to the analysis of products generated only during the exponential phase of the amplification. Under these conditions, RT-PCR can yield reasonably precise information about relative changes in mRNA levels.

The PCR product concentration is proportional to the starting target DNA as long as product accumulation remains exponential. The point at which exponential accumulation plateau can be roughly estimated by noting the point at which continued cycles do not produce significantly increased product yields.

Preparation of total RNA from tadpole tail explants was described in the Methods 3.19. Care was taken to avoid contamination of the reactions with either RNase or any DNA which could be amplified with the PCR primers; Gilson barrels, plungers and seals were routinely cleaned with ethanol and sterilised in a UV plate drier. All incubations were carried out using a Hybaid thermal cycler to ensure consistency. For reverse transcription (RT) reactions 0.5μg of total RNA was made up to a total volume of 20.1μl using freshly thawed milli-Q water (MQW). The diluted RNA

was then heated to 75°C for 5 minutes and cooled on ice. Reverse transcription mix was made as in Table 6.

**Table 6.** Reverse transcriptions for RNA analysis

| Component | Amount per 30µl | Final concentration |
|---|---|---|
| 100 µM Random hexamers | 1µl | 3.3 µM |
| 10x PCR buffer | 3µl | 1x |
| 50mM $MgCl_2$ | 1.8µl | 3mM |
| 10mMdNTPs | 1.5µl | 500µM |
| 50 u / µl RNase inhibitor | 0.6µl | 1u / µl |

To each RNA sample 7.9µl of RT mix was added and the reaction was incubated for 5 minutes before adding 2µl (400 U) of MMLV reverse transcriptase (RT ase) enzyme and incubating at 42°C for a further hour. Control tadpole tail RNA incubated as respective positive and negative controls for each set of reactions. RT reactions were immediately heated to 95°C for 5 minutes on completion as apart of the thermal cycler program. cDNAs were stored at – 20°C until use.

Since only 1µl was sufficient for each PCR reaction, the cDNA was first diluted 5x with MQW and divided into 5µl aliquots after thorough mixing. Each aliquot was used in PCR with a primer pair specific to one of the marker gene. PCR reactions were set up on ice in a 25µl volume by adding 20µl of PCR reaction mix, as in Table 7.

**Table 7.** Components of the PCR reaction

| Component | Amount per 25µl | Final concentration |
|---|---|---|
| 10x PCR | 2.5µl | 1x |
| 50mM $MgCl_2$ | 0.75µl | 1.5mM |
| 10mM dNTPs | 0.5µl | 200µM |
| 10µCi / µl [$\alpha^{32}$P]- dGTP | 0.05µl | 0.5µCi |
| 12.5µM each Left/Right Primer Mix | 2µl | 1µM each |
| MQW | 14.1µl | |
| 5 u / µl Taq DNA Polymerase | 0.1µl | 0.5 U |

After an initial denaturing step (94°C for 3 minutes) PCR reactions were cycled as below for the appropriate number of rounds of amplification.

Annealing temperature (x°C) for most primer sets was 55°C.

| | | |
|---|---|---|
| **Denaturing** | 94°C | 30 seconds |
| **Annealing** | x°C | 1 minute |
| **Extension** | 72°C | 1 minute |

9µl of each PCR reaction was mixed with 6µl of formamide loading buffer (95% deionised formamide, 10mM EDTA pH 8.0, 0.1% xylene cyanol, 0.1% bromophenol blue), boiled for 5 minutes and loaded onto a 0.4mm thick 6% sequencing gel. Gels were run at 20 watts for 1 hour, fixed, and dried.

RNA isolated from *Xenopus laevis* tadpole tails after a treatment regime in exactly the same way as was described for the differential display system was used as a template for reverse transcription to complementary DNA (cDNA), using random hexamers. The cDNA in turn is used as a template for PCR, using Ef-1α upstream and downstream primers to act as controls and gene specific primers termed

GSP1and GSP2 designed to amplify a selected cDNA region (see Table 8) Subsequently, the PCR product was analysed on a 6% polyacrlamide gel.

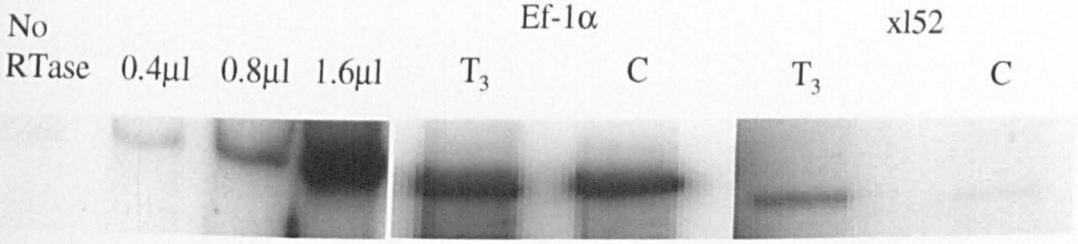
**Table 8.** Primer sequences used in RT-PCR assay

| PRIMER | SEQUENCE<br>5'→3' |
|---|---|
| Gene-specific primer 1 GSP1 | TGTGGCTGTTTCTATGCCAACG |
| Gene-specific primer 2 GSP2 | CAGTCTCTTGGCCAATCTT |
| Ef-1α Upstream primer | CAGATTGGTGCTGGATATGC |
| Ef-1α Downstream primer | CACTGCCTTGATGAC |

The amplified cDNA was identified by the size of the PCR product which is predicted from the knowledge of the cDNA nucleotide sequence of clone xL52. An example of such induction by the $T_3$ is shown in Figure 14.

The induction seen in this assay is presumed to be real because the signal for the control primers of the elongation factor (Ef-1α) is constant across the two samples. The untreated track shows a low level of expression compared to the level of expression seen in $T_3$-treated track.

FIG 14. RT-PCR to confirm differential expression of cloned PCR products. Two gene specific primers were designed from xL52 clone and used for amplification procedure. Ef-1α was used as a control on cDNA prepared from both $T_3$-treated $5x10^{-9}$M for 48hrs and C-untreated control tadpole tails. To ensure linear amplifications, range of cDNA concentrations (0.4μl, 0.8μl, 1.6μl) were included: (No reverse transcriptase as a negative control is also included); this range of cDNA concentrations was used as a standard, and incorporated in all RT-PCR figures: 15 and 23. The products were analysed on 6% polyacrylamide gel.


No
RTase
0.4µl
0.8µl
1.6µl
Ef-1α
$T_3$
C
xl52
$T_3$
C

## 4.7. Summary Discussion

Our experiments were designed to isolate and then characterise the responsive genes in amphibian tail, that occur during $T_3$-induced regression, the final change in amphibian metamorphosis. The entire process is cell autonomous (Brown *et al.*, 1996). Even though the target organ consists of a variety of cell types, they have a single uniform fate, namely resorption. The tail can be cultured in a salt solution for many days, and it responds to TH in a fashion that cannot be distinguished from the normal process of tail resorption that occurs at the climax of metamorphosis when endogenous TH is at its highest level (Brown *et al.*, 1996).

Differential display performed on RNA samples isolated from cultured triiodothyronine $T_3$ treated and untreated *Xenopus laevis* tadpole tails, produced ten differentially expressed bands. Eight were up-regulated and two were down-regulated in response to the thyroid hormone $T_3$. These cDNA fragments isolated from a displaying sequencing gel, were reamplified in a second PCR and used for RT-PCR analysis to confirm the up-regulation of these genes in response to $T_3$ stimulation.

The next step is to determine the identity of these cDNA fragments by sequencing analysis. However, because a decamer (at upstream) and $T_{12}MA$ (at downstream) were used for the differential display PCR, neither of these primers is long enough to specifically prime the sequencing reactions using a normal sequencing protocol.

Therefore, the isolated cDNA fragments from the differential display was subcloned into a cloning vector. The differentially gel isolated bands of interest were cloned into pBluescript (KS) vector cut at *HincII* site. These cloned products were completely sequenced using both sequenase version 2.0 kit (Stratagene) and automated sequencing.

Sequence analysis and database searches showed that none of the clones revealed significant matches to any of the known genes, since the PCR technique tends to produce sequences derived from 3'untranslated region of mRNA. These 3'untranslated sequences are typically A/T rich, and will not often be useful in database searching especially because the untranslated regions are poorly conserved among species.

I was then keen to look at the identity of these sequenced differentially PCR products in more details. The first step towards identifying these bands of interest was to confirm that they were in fact, products generated as a result of thyroid hormone induction $T_3$. Therefore, it was decided to use the RT-PCR technique as a tool for this purpose.

RT-PCR assays showed that of these thyroid hormone treated products, only one clone appeared to be up-regulated by the hormone which was termed xL52.

Having confirmed the inducibility of this clone, as an initial step towards gene identification, isolation of longer sequences from the coding region of clone xL52 became priority, since the original cDNA fragments isolated by the differential display which were obtained by priming mRNA with oilgo (dT) were therefore more likely to be located within or near the 3′ end of their respective mRNA. The choice of cDNA library was based on tissue distribution of the clone xL52 among various *Xenopus laevis* adult tissues.

# CHAPTER 5

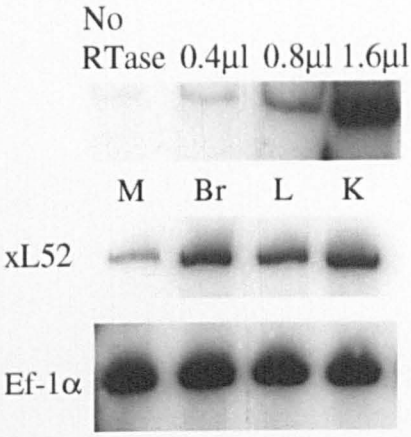
## Isolation of cDNA clones from adult *Xenopus laevis* Kidney library

### 5.1. Distribution of xL52 clone among *Xenopus laevis* adult tissues

Having confirmed the thyroid hormone inducibility, it was decided to look at the distribution of xL52 clone mRNA among the various *Xenopus laevis* adult tissues as an initial step towards the isolation of longer sequences from the coding region of xL52 clone using cDNA libraries.

The same technique ,RT-PCR, was applied on total RNA extracted from brain, kidney, liver and muscle to see how xL52 clone was distributed through the various *Xenopus laevis* adult tissues. Total RNA isolated from the above mentioned tissues was reverse transcribed and cDNA was synthesised. The cDNAs were then subjected to amplification procedure. using the same gene specific oligonucleotide primers for the confirmation of thyroid hormone inducibility. Assays were performed on comparable samples using Ef-1α primers to confirm integrity and quality of RNA samples. The xL52 clone was observed to be most abundant in brain, kidney, liver and less abudant in muscle as shown in the autoradiograph Figure 15.

FIG 15. The distribution of clone xL52 in *Xenopus laevis* adult tissues. RT-PCR analysis was performed on total RNA extracted from liver, kidney, muscle and brain adult frog. Gene specific primers termed GSP1 and GSP2 were designed from clone xL52 and used for the amplification procedure. To ensure cDNA quality, range of cDNA concentrations were included (0.4μl, 0.8μl, 1.6μl). A negative control (No RTase) is also included to show the lack of contamination from genomic DNA. The PCR products were analysed on 6% polyacrylamide gel. The clone xL52 was represented in all the tissues examined as seen in the Figure below. The elongation factor-1α is also included as control with these preparations.. Abbreviations: M , muscle; Br, brain ; K, kidney ; L, liver.


No
RTase 0.4μl 0.8μl 1.6μl
M Br L K
xL52
Ef-1α

## 5.2. *Xenopus laevis* Kidney cDNA library

### Uni-ZAP™XR Vector

The cDNA library (*Xenopus*, outbred adult) was purchased from (Stratagene), and was performed as essentially described by the manufacturer.

**Description:**

**Primer:** Oligo dT

**Insert Size:** > 0.5 kb

**Average Insert Size:** 1.0 kb

**Cloning Site:** *EcoRI* and *XhoI*

**Primary Plaques:** $2.0\times10^6$ pfu

**Estimated Background:** 2% nonrecombinants

**Estimated Titer:** 2.5×106 pfu / ml

**Actin Screen:** 0.26%

**Host Strains**: The XL1-Blue MRF' strain is the recommended host strain for amplification and screening of Uni-ZAP™ XR cDNA libraries. The XL1-Blue MRF' strain is a restriction minus strain and allows blue / white color selection of recombinant clones when grown on plates containing IPTG and X-gal.

**f1 Helper Phage:**

**VCSM13 (f1)**: $1\times10^{11}$ pfu/ml, supercoiled single-stranded DNA migrates at ~6 kb on an agarose gel (for single-stranded rescue).

**ExAssist™ helper phage (M13)**: $1\times10^{10}$ pfu/ml, supercoiled single-stranded DNA migrates at ~5 kb on an agarose gel (for excision). Exassist helper phage has α-

complementing β-galactosidase sequences, which may interfere with sequencing or site-directed mutagenesis where oligonucleotide primers hybridise to β-galactosidase sequences (e.g., M13-20 primer). It was therefore, recommended VCSM13 helper phage for single-stranded rescue and ExAssist for excision of the Bluescript phagemid from the ZAP vector.

### 5.2.1. General Vector Description

The Uni-ZAP XR vector system combines the high efficiency of lambda library construction and the convenience of a plasmid system with blue-white color selection. The Uni-ZAP XR vector is double digested with *EcoRI* and *XhoI* and will accommodate DNA inserts from 0 to 10 Kb in length. The Uni-ZAP XR vector can be screened with either nucleic acid probes or antibody probes and allows rapid in vivo excision of the pBluescript SK(−) phagemid, allowing the insert to be characterised in a plasmid system. The polylinker of pBluescript SK(−) has 21 unique cloning sites flanked by T3 and T7 promotors and a choise of 6 different primer sites for DNA sequencing. The phagemid has the bacteriophage f1 origin of replication, allowing rescue of single-stranded DNA, which can be used for DNA sequencing or site-directed mutagenesis. Unidirectional deletions can be made with exonuclease III and mung bean nuclease by taking advantage of the unique positioning of 5′ and 3′ restriction sites. Transcripts made from the T3 and T7 promotors generate riboprobes useful in Southern and Northern blotting, and

the *lac-Z* promoter may be used to drive expression of fusion proteins suitable for Western blot analysis or protein purification.

The library was synthesised using the ZAP-cDNA synthesis method. The linker-primer was designed with a GAGA sequence to protect the *XhoI* restriction enzyme recognition site and an 18-base poly(dT) sequence. The restriction site allows the finished cDNA to be inserted into the vector unidirectionally in the sense orientation with respect to the *lac-Z* promotor.

The linker-primer is a 50-base oligonucleotide with the following sequence:

**5′** GAGAGAGAGAGAGAGAGAGAACTAGT**CTCGAG**$(T)_{18}$ **3′**

GAGA sequence *XhoI*

The adaptors are comprised of 9- and 13-mer oligonucleotides, which are complementary to each other and have an *EcoRI* cohesive end. The adaptors have the following sequence:

**5′** AATTCGGCACGAG **3′**

**3′** GCCGTGCTC **5′**

The pBluescript SK(−) plasmid in the Uni-ZAP XR vector contains the N-terminus of the *lac-Z* gene, which can be α-complemented by the specific host strain used. There are 36 amino acids from the MET sequence to the *EcoRI* site. A total of 131 amino acids are coded for, but this is interrupted by the large polylinker.

## 5.3. Isolation of full length cDNA clones from adult *Xenopus laevis* Kidney library screened with xL52 clone

### 5.3.1. Introduction

A cDNA library is different from a genomic library in that it represents only a small subset of all genes in a genome. cDNA libraries use mRNA as a starting point, and thus represent only the expressed sequences in a given cell type, tissue or stage of embryonic development. The decision whether to construct a genomic or a cDNA library depends on the question at hand. If you are interested in a particular gene, it may be easier to prepare a cDNA library from a tissue that expresses that gene. For example, red blood cells make large amounts of hemoglobin, and most of the mRNA in these cells is β-globin mRNA.

A cDNA library prepared using mRNA isolated from red blood cells is a direct way to isolate a globin cDNA. If the regulatory sequences adjacent to the globin gene are of interest, then a genomic library would need to be constructed, since these regulatory sequences are not present in the globin mRNA and would not be represented in the cDNA library.

## 5.4. Screening of the *Xenopus* kidney cDNA library

To analyse clone xL52, the full length cDNA was isolated by screening adult *Xenopus laevis* kidney cDNA library. Each screen was carried out as described in the Methods 3.11. Duplicate lifts were made to allow for the detection of false

positives. Nitro-cellulose membranes were oriented by pushing a sterile needle, in three different places through into to the agar plate, the holes were then marked in a marker-pen on the bottom of the petri-dish. Autoradiography were aligned with the filters using radioactive ink and A and B lifts were exposed to X-ray films, so the detection of positive signals could made by simply lying A and B autoradiographs together on a lightbox. Clone xL52 was labelled by digesting the insert from the pBluescript using restriction enzymes *Kpn I* and *Sma I*. A 100ng cDNA insert was labelled with [$\alpha^{32}$P]-dGTP by random priming hexamers (Pharmacia), and the unincorporated nucleotides were removed using Sephadex G-50 Columns (Pharmacia). Probe was added after prehybridising the filters and hybridisation was carried out for 16 hours at 42°C. Wash stringencies were carried out as follows (twice in 2x SSC, 0.1% SDS at room temperature for 10 minutes each; twice in 0.2x SSC, 0.1% SDS at 65°C for 30 minutes each). Filters were then dried before exposing to X-ray film, at –70°C with an intensifying screen.

### 5.4.1. Primary screen using clone xL52 probe

Screening was performed as described in the Methods, using the following wash conditions (twice for 10 minutes each in 2xSSC, 0.1% SDS at room temperature; twice for 30 minutes each in 0.2x SSC, 0.1% SDS at 65°C). The probe screened for cDNAs containing the untranslated region present in clone xL52. The use of a *Xenopus laevis* kidney cDNA library was decided because of the signals detected in kidney, brain, muscle and liver of *Xenopus* adult tissues by RT-PCR assay

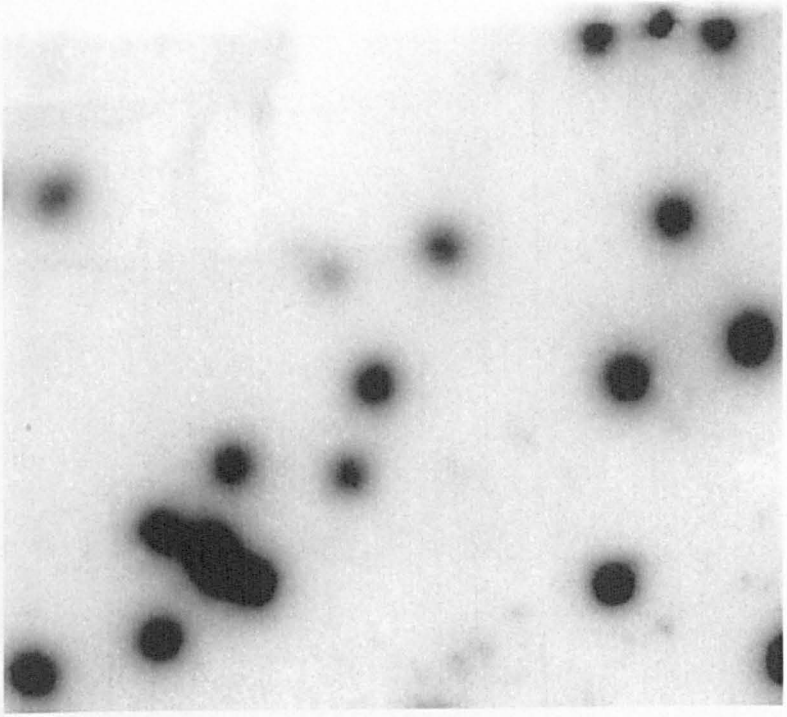
suggesting that this clone would be represented at some level. Approximately $6\times10^5$ phage were screened with the clone xL52 probe described in the Methods section. Replica filter lifts were taken from each plate. Seven positive plugs were obtained from the first round of screening, these were picked using the large end of sterile blue tips. The phage plug was stored in 500µl SM buffer and 10µl chloroform at 4°C.

### 5.4.2. Secondary and tertiary screen

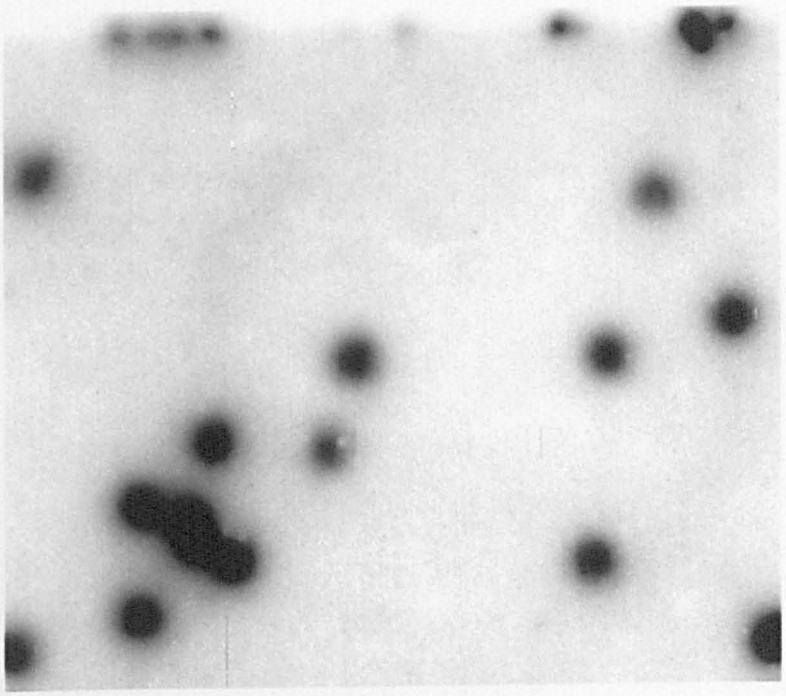
One phage plug, termed R4 from the originally picked 7 positives was replated at a lower density of approximately 500 pfu/plate. The screening procedure was then repeated and the X-ray film exposed for two days, positive plaques were detected on the filters. 6 positives were picked using sterile inverted yellow tips and stored as before. One phage plug was replated at a lower density of ~50 pfu/plate. The screening procedure was then repeated and exposed to X-ray film for 24 hours, positive plaques were seen on the filter. Two positives were picked this time using sterile inverted yellow tips and stored as above. The results showed all plaques to be positive and plaque pure. An example of the third round screen is shown in Figure 16.

FIG 16. Third round screen of positive cDNA clone from *Xenopus laevis* kidney library. An autoradiograph showing the replica filters.

First lift



Second lift



### 5.4.3. *In vivio* Excision of pBluescript from the lambda Uni-ZAP XR vector

The insert-containing pBluescript vector had to be excised from the plaque pure isolates. This was done using the ExAssist SOLAR system (Stratagene see methods 3.13.) which allows efficient excision of the pBluescript phagemid from the Uni-ZAP-XR vector. The ExAssist helper phage contains an amber mutation that prevents replication of the phage genome in a non-suppressing *E.coli* strain such as SOLAR cells. This allows only the excised phagemid to replicate in the host, removing the possibility of coinfection from the ExAssist helper phage. The excision was performed according to the manufacturer's instructions. This plaque pure isolate was taken for further analysis below.

## 5.5. Restriction map of clone *xth-2* cDNA

A restriction map is a compilation of the number, order, and distance between restriction enzyme cutting sites along a cloned segment of DNA. Restriction maps provide information that can be used for subcloning fragments of a gene, or for comparing the organisation of a gene and its cDNA so as to identify exons and introns in the genomic copy of the gene.

Fragments generated by cutting DNA with restriction enzymes can be separated by gel electrophoresis and visualised by staining the DNA with ethidium bromide and viewing under ultraviolet illumination. The size of the individual fragments can be determined by running a set of marker fragments of known size on the same gel.

Restriction maps provide an important way of characterising a DNA segment, and it can be constructed in the absence of any information about the coding capacity or function of the mapped DNA. In conjunction with other techniques, restriction mapping can be used to define the boundaries of a gene, and it provides a way of dissecting the molecular organisation within a gene and its flanking regions. Mapping can also serve as a starting point for the isolation of an intact gene from cloned segments of DNA, and it provides a means for locating mutational sites within genes.

Restriction enzyme cutting sites can be used as genetic markers, thus reducing the distance between sites on a map, increasing the accuracy of maps, and providing reference points for the correlation of genetic and physical maps (Klug and Cummings, 1997).

Figure 17 shows standard single and double restriction enzyme digests used to determine the restriction enzyme map from clone *xth-2* that contains cutting sites for restriction enzymes. For the construction of this map, a number of samples of clone *xth-2* were digested with restriction enzymes, one with *EcoRI* and one with *XhoI* and one with both *EcoRI* and *XhoI*. The fragments generated by digestion with the restriction enzymes were separated by electrophoresis. The sizes of the separated fragments were estimated by comparison to a set of standard markers run in adjacent lanes. To construct the map, the fragments generated by the restriction

enzymes were analysed (see Figure 18 for the restriction enzyme sites of clone *xth-2*)

When the DNA was cut with the restriction enzyme *XhoI*, no fragments were produced except that the cloning vector pBluescript (SK) was linearised with this enzyme.

When the cloned DNA (*xth-2*) was cut with *EcoRI*, two fragments were produced, one 3.3 Kb which represented the cloning vector plus an additional 300 bases from the insert *xth-2*, and one ~2.2 kb represented the clone *xth-2* on its own. This was confirmed by the double restriction enzyme digests. Using both restriction enzymes, *EcoRI* and *XhoI*, three fragments were generated: 3 kb for the cloning vector, ~2.2 kb and ~0.3 kb for the insert *xth-2* respectively.

Taken together, the results show that there was one restriction site for *EcoRI* as indicted by the single and double restriction digestion, but there was no site for the restriction enzyme *XhoI* as indicated by the single restriction digestion. The same analysis was carried out on the other restriction enzymes.

*KpnI*, *SmaI*, *XbaI*, *SacII*, *EcoRV*, *BamHI*, *BglI*, *ClaI*, *DraI*, *NotI*, *SalI*, *PstI*, *DraIII*, or *BglI* were found not to cut the molecule *xth-2*. However, *HindIII* and *StuI* restriction digestion each released two fragments, indicating the presence of one

site for each enzyme. In addition, *HincII*, digestion released four fragments, indicating the presence of three sites for the enzyme.

FIG 17. illustrates examples of the construction of restriction map of clone *xth-2*. The cloned cDNA was subjected to standard single and double restriction digests: (A): M, marker; 1, the uncut plasmid containing the insert; 2, linearised DNA with restriction enzyme *XhoI*; 3, single restriction digest with *EcoRI*; 4, double restriction digest with *EcoRI* and *XhoI*. (B): represents single restriction digest with *HincII*. (C): shows single restriction digest with *PstI*, and *HindIII*.

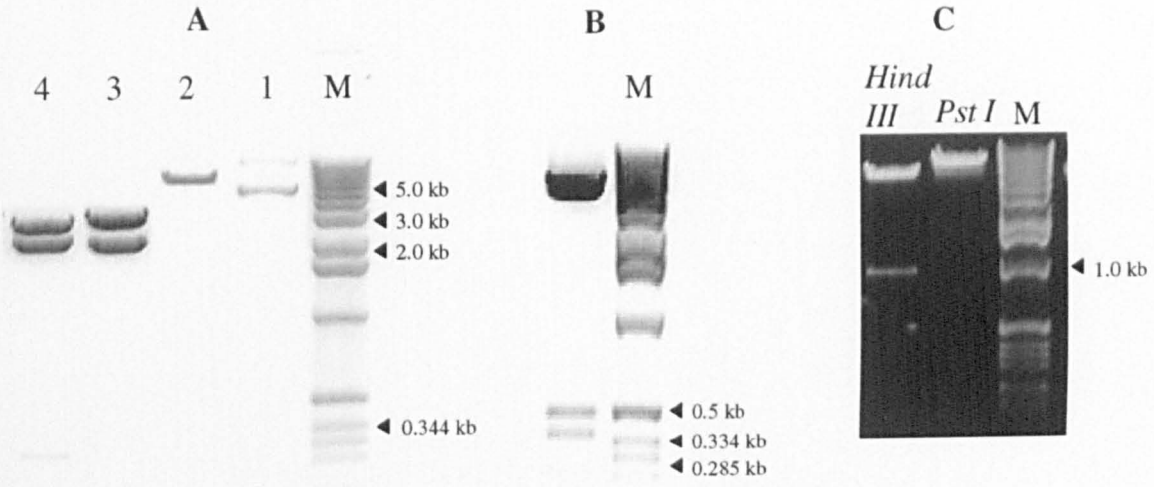
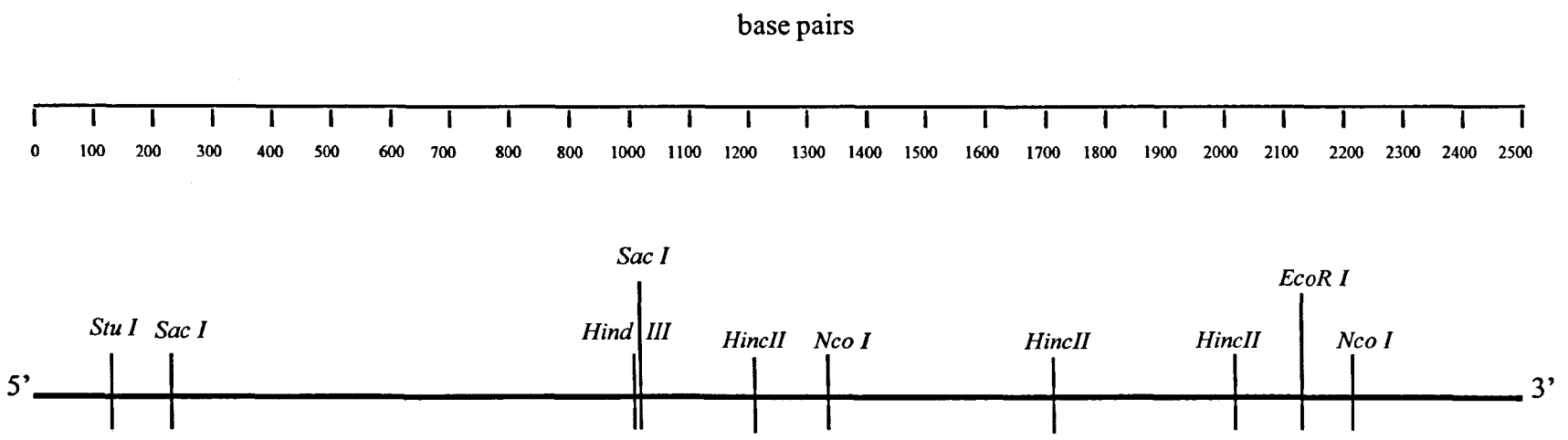

A
4
3
2
1
M
5.0 kb
3.0 kb
2.0 kb
0.344 kb
B
M
0.5 kb
0.334 kb
0.285 kb
C
Hind III
Pst I
M
1.0 kb

FIG 18. Restriction enzyme sites of clone *xth-2* cDNA isolated using the differential display clone xL52 probe.


base pairs
0 100 200 300 400 500 600 700 800 800 1000 1100 1200 1300 1400 1500 1600 1700 1800 1900 2000 2100 2200 2300 2400 2500
Sac I
EcoR I
Stu I
Sac I
Hind III
HincII
Nco I
HincII
HincII
Nco I
5'
3'

## 5.6. Sequence analysis of *xth-2*

In a sense, the ultimate characterisation of a cloned DNA segment is the determination of its nucleotide sequence. The ability to sequence cloned DNA has added immensely to our understanding of gene structure and the mechanisms of gene regulation.

The DNA sequencing method provides information about the organisation of genes and the nature of mutational events that alter both genes and gene products, confirming the conclusion that genes and proteins are collinear molecules. Sequencing has also been used to study the organisation of regulatory regions that flank prokaryotic and eukaryotic genes, and to derive the amino acid sequence of proteins.

In addition, to identify DNA defects that cause mutant phenotypes, DNA sequencing is used to study the organisation of a gene (the number of introns and exons and their boundaries), to provide information about the nature and function of proteins encoded by genes, including the size, number and type of domains (membrane spanning, DNA binding) and relationship to similar proteins and proteins from other organisms (Klug and Cummings, 1997).

The sequencing strategy adopted for clone *xth-2* is outlined in Figure 19. Sequencing (by the dideoxy chain termination method and automated sequencing)

was from double stranded plasmid templates, derived from pBluescript subclones, utilising T3 and T7 primers. Oligonucleotides were derived internally from both ends of the clone (see Table 9) and sequencing was carried out until overlapping sequences were obtained, indicating that the entire clone was completely sequenced. Presumptive 5′ and 3′ ends of the clone were allocated following identification of the probe sequence. The cDNA length of *xth-2* clone is ~2.5kb of an open reading frame starting from the AUG, in reading frame 1, at the 166nt position and ending at a TAA stop site at the 2377nt position. There are no stop codons upstream of the AUG site at the 166nt position. Therefore, it can be assumed that the *xth-2* cDNA does not appear to be a full length The nucleotide sequence obtained for the clone *xth-2* in the presumptive coding region is shown in Figure 20. The longest open reading frame from the in frame methionine is indicated. The ~2.5kb cDNA (*xth-2*) includes a 2.2 kb open reading frame capable of encoding a protein of 737 amino acids with a calculated molecular mass of ~84.0 kD. An alignment of the amino acid sequences of all known Hem family proteins in database, showing regions of similarity with *xth-2* are shown in Figure 21. Also, a comparison of members of Hem family as a matrix of amino acid residues is shown in Figure 22.

FIG 19. Sequencing strategy of clone *xth-2*. The *xth-2* cDNA was first sequenced at both ends using pBluescript T3 and T7 primers. Arrows indicate length read in each sequencing reaction and asterixes indicate automated sequencing reactions using internal primers. SP1, SP2, SP3 primers were used for sequencing from the 5'end of the clone; R1, R2, R3 primers were used for sequencing from the 3'end of the clone. SP3 and R3 primers indicate the overlapping region.

**Table 9.** Sequencing Primers Used For Clone *xth-2*

| PRIMER | SEQUENCE<br>5'→3' |
|---|---|
| pBS SK (T3) | ATTAACCCTCACTAAAG |
| pBS SK (T7) | AATACGACTCACTATAG |
| SP1 | CCGTCATGCAGACAACATTC |
| SP2 | TAGTGGAGATGTTGGTGGAGAC |
| SP3 | CAATCAGTCAAGCCGTT |
| R1 | ATAGGCTGGAGGAAGCAAG |
| R2 | CATCTCTCAGTGCTTCCTGTG |
| R3 | CATTGCTTACCTGCCTCAGT |

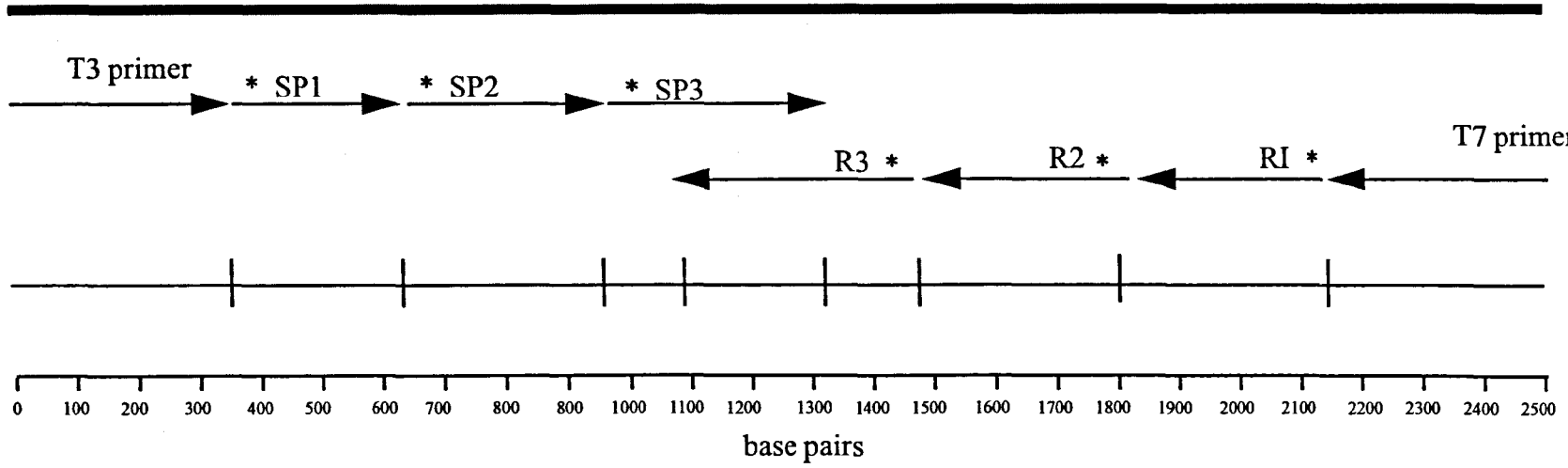

T3 primer
* SP1
* SP2
* SP3
R3 *
R2 *
RI *
T7 primer
0 100 200 300 400 500 600 700 800 800 1000 1100 1200 1300 1400 1500 1600 1700 1800 1900 2000 2100 2200 2300 2400 2500
base pairs

FIG 20. Nucleotide sequence of *xth-2* cDNA excluding linkers. The first in frame methionine is underlined at 166nt position (but translation *in vivo* probably runs through the region upstream, as this is an incomplete cDNA, see text). Stop codon (TAA) is underlined at 2377nt position.

```
1     AACAAAAGGA  TAAATGACAT  CAGGGAATGT  AAAGAGAATG  CAGTATCACA

51    TGCAGGAAGC  ACACACAGAG  AAAGGCGCAA  ATTTTTAAGG  TCTGCATTGA

101   AAGAACTTGC  TACCGTACTT  GCCGATCAGC  CAGGCCTTCT  GGGTCCCAAA

151   GCACTTTTTG  TATTCATGGC  ATTATCTTTT  GCCCGTGATG  AAATAATATG
                        M     A  L  S  F    A  R  D    E  I  I

201   GCTACTCCGT  CATGCAGACA  ACATTCCCAA  GAAATTTGCA  GATGACTTCA
      W  L  L  R    H  A  D    N  I  P    K  K  F  A    D  D  F

251   TGGATAAGCA  CATTGCTGAG  CTCATATTTT  ATATGGAAGA  ACTTCGAGCG
        M  D  K    H  I  A  E    L  I  F    Y  M  E    E  L  R  A

301   CATGTACGGA  AGTATGGACC  AGTGATGCAG  CGATACTATG  TGCAGTACTT
        H  V  R    K  Y  G    P  V  M  Q    R  Y  Y    V  Q  Y

351   GTCTGGCTTT  GATGCAGTTG  TATTAAATGA  GCTTGTTCAG  AATCTTTCTG
      L  S  G  F    D  A  V    V  L  N    E  L  V  Q    N  L  S

401   TGTGCCCTGA  GGATGAATCC  ATTATAATGT  CGTCATTTGT  AAACACTATG
        V  C  P    E  D  E  S    I  I  M    S  S  F    V  N  T  M

451   ACCTCCCTGT  GTGTGAAACA  AGTTGAAGAT  GGAGAGGTTT  TTGACTTCAG
        T  S  L    C  V  K    Q  E  V  D    G  E  V    F  D  F

501   AGGAATGAGA  CTGGATTGGT  TTAGATTGCA  GGCATATACC  AGTGTTTCCA
      R  G  M  R    L  D  W    F  R  L    Q  A  Y  T    S  V  S

551   AAGCATCGTT  AAGTCTCGCT  GACCACAGAG  AACTTGGAAA  AATGATGAAC
        K  A  S    L  S  L  A    D  H  R    E  L  G    K  M  M  N

601   ACTATAATAT  TCCATACCAA  AATGGTGGAT  TCCTTAGTGG  AGATGTTGGT
        T  I  I    F  H  T    K  M  V  D    S  L  V    E  M  L

651   GGAGACATCA  GACCTTTCAA  TATTTTGCTT  TTACGGCCGA  GCTTTTGAAA
      V  E  T  S    D  L  S    I  F  C    F  Y  G  R    A  F  E

701   AAATGTTTCA  GCAGTGTTTG  GAGCTGCCTT  CCCAGTCAAG  ATATTCCATC
        K  M  F    Q  Q  C  L    E  L  P    S  Q  S    R  Y  S  I

751   CCCTTCCCAC  TTTTATGCAC  TCACTTTATG  AGTTGCACTC  ATGAACTTTG
        P  F  P    L  L  C    T  H  F  M    S  C  T    H  E  L

801   CCCTGAAGAG  CGGCATCACA  TTGGAGACCG  CAGTTTGTCA  CTATGTTACA
```

```
      C  P  E  E    R  H  H    I  G  D    R  S  L  S    L  C  Y

851   TGTTCTTGGA   TGAAATGGCA   AAGCAAGCTC   GAAATCTAAT   TACAGACATC
        M  F  L      D  E  M      K  Q  A      R  N  L      I  T  D  I

901   TGCACAGAAC   AATGTACTCT   TTGTGATCAG   TTGCTGCCAA   AGCACTGTGC
        C  T  E      Q  C  T      L  C  D  Q      L  L  P      K  H  C

951   CAAAACAATC   AGTCAAGCCG   TCAATAAAAA   GCAGACTGGA   AAGAAAGGAG
        A  K  T  I      S  Q  A      V  N  K      K  Q  T  G      K  K  G

1001  AACCAGAAAG   GGAAAAACCT   GGAGTTGAAA   GCTTGAGAAA   GAATAGACTT
        E  P  E      R  E  K  P      G  V  E      S  M  R      K  N  R  L

1051  GTGGTAACAA   ACTTGGATAA   ATTGCACACT   GCACTTTCAG   AGCTCTGTTT
        V  V  T      N  L  D      K  L  H  T      A  L  S      E  L  C

1101  CTCTATCAAC   TATGCACCAA   ATATGGTTGT   ATGGGAACAC   ACATTTACCC
        F  S  I  N      Y  A  P      N  M  V      V  W  E  H      T  F  T

1151  CAAGAGAGTA   TTTGACGTCG   AACTTGGAAA   TCCGCTTTAC   CAAGTCTATT
        P  R  E      Y  L  T  S      N  L  E      I  R  F      T  K  S  I

1201  GTTGACATGA   CCATGTACAA   TCAAGTCACC   CAAGAGATTG   CTAAACCTTC
        V  D  M      T  M  Y      N  Q  V  T      Q  E  I      A  K  P

1251  TGAGTTGTTT   ACAGTGTTAG   AAGCCTACAT   GACTGTACTC   CAGTCAATAG
        S  E  L  F      T  V  L      E  A  Y      M  T  V  L      Q  S  I

1301  AAAACTATGT   GCAGATTGAC   ATCACAAGGG   TTTTTAACAA   TGTTCTACTT
        E  N  Y      V  Q  I  D      I  T  R      V  F  N      N  V  L  L

1351  CAGCAAACTC   AACATTTAGA   CAGCCATGGA   GAGCCAACAA   TCACCAGTTT
        Q  Q  T      Q  H  L      D  S  H  G      E  P  T      I  T  S

1401  ATACACTAAT   TGGTATTTGG   AAACAAAACT   GAGGCATGTA   AGCAATGGAC
        L  Y  T  N      W  Y  L      E  T  K      L  R  H  V      S  N  G

1451  ATATAGCCTG   TTTCCCAGCA   ATGAAAGCAT   TTGTAAACCT   GCCTGGTGAG
        H  I  A      C  F  P  A      M  K  A      F  V  N      L  P  G  E

1501  AATGAGCCTA   CTTTTAATGC   AGAAGAGTAT   CCGATGAGAG   CACTCTCTGA
        N  E  P      T  F  N      A  E  E  Y      P  M  R      A  L  S

1551  GCTGCTGGGA   CCATATGGCA   TGAAGTTTCT   GAGTGAGAGC   CTCATGTGGC
        E  L  L  G      P  Y  G      M  K  F      L  S  E  S      L  M  W

1601  ATATTTCCTC   ACAGGTGGCT   GAGCTTAAGA   AACTTGTGGT   GGAGAATGTT
        H  I  S      S  Q  V  A      E  L  K      K  L  V      V  E  N  V

1651  GATGTCTTAA   CCCAAATGAG   GACAAGTTTT   GATAAGCCAG   AGCAGATGGC
        D  V  L      T  Q  M      R  T  S  F      D  K  P      E  Q  M

1701  AGCCCTTTTC   AAAAGGCTTA   CATCCGTTGA   CAGTGTCTTG   AAGAGGGTCA
        A  A  L  F      K  R  L      T  S  V      D  S  V  L      K  R  V
```

```
1751 CCATTATTGG  AGTTATATTG  TCTTTCCGAT  CTCTGGCACA  GGAAGCACTG
       T  I  I     G  V  I  L     S  F  R     S  L  A     Q  E  A  L

1801 AGAGATGTTT  TGTCTTACCA  CATTCCTTTT  CTTGTAAGCT  CTGTTGAAGA
       R  D  V     L  S  Y     H  I  P  F     L  V  S     S  V  E

1851 CTTCAAGGAT  CACATTCCTA  GAGAGACGGA  CATGAAGGTT  GCAATGAATG
       D  F  K  D     H  I  P     R  E  T     D  M  K  V     A  M  N

1901 TGTATGAGTT  ATCATCAGCA  GCTGGATTGC  CCTGTGAAAT  AGACCCTGCC
       V  Y  E     L  S  S  A     A  G  L     P  C  E     I  D  P  A

1951 TTGGTTGTGG  CACTGTCCTC  TCAAATAGCC  TGTCTGCTCA  TGGTGTTTGT
       L  V  V     A  L  S     S  Q  I  A     C  L  L     M  V  F

2001 GGCTGTTTCT  ACGTCAACGT  TGGCCAGCAA  CGTCATGTCG  CAATACAGTC
       V  A  V  S     T  S  T     L  A  S     N  V  M  S     Q  Y  S

2051 CTGCAATAGA  AGGTCATTGC  AACAACATAC  ACTGTTTGGC  AAAGGCATCA
       P  A  I     E  G  H  C     N  N  I     H  C  L     A  K  A  S

2101 ACCAAATTGG  CGGCAGCACT  ATTTACCATT  CACAAAGGGG  CATTGAAGGA
       T  K  L     A  A  A     L  F  T  I     H  K  G     A  L  K

2151 TCGTCTGAAA  GAATTCTTGG  CGCTTGCATC  CTCCAGCCTA  CTAAAGATTG
       D  R  L  K     E  F  L     A  L  A     S  S  S  L     L  K  I

2201 GCCAAGAGAC  TGATAAAACT  ACTACAAGAA  ACAGGGAATC  TGTTTATTTG
       G  Q  E     T  D  K  T     T  T  R     N  R  E     S  V  Y  L

2251 CTGCTAGATA  TGATTGTGCA  AGAATCGCCA  TTCCTGACCA  TGGATCTGCT
       L  L  D     M  I  V     Q  E  S  P     F  L  T     M  D  L

2301 GGAGTCCTGC  TTCCCATACG  TCTTACTGAG  AAATGCGTAC  CACGCGGTTT
       L  E  S  C     F  P  Y     V  L  L     R  N  A  Y     H  A  V

2351 ACAAACAGAG  TGTCACATCC  TCTGCATAAA  TATTTACTTT  GCGGAACAAA
       Y  K  Q     S  V  T  S     S  A

2401 CCAGCGCTCA  GTTGAAATGC  CTCAATTTTC  CCCAGAAACT  GTGGTGGAGT

2451 ACTTTTACTG  AATGGTTTGG  AAAAACAAAA  CAAACAAACA  AAAAC
```

FIG 21. Alignment of the amino acid sequences of all known Hem family proteins. Amino acid sequence of *xth-2* and Hem-2 and alignment of sequences of all known members of the Hem family. Each lane contains 50 residues aligned with each other. Potential membrane-spanning segments are in bold blue for other members of Hem family. Clone *xth-2* sequence is on top in bold red. hem1, Hematopoietic Protein; mh19, House mouse brain protein; dhem2, Fruit Fly (*Drosophila melanogaster*), Nap1, Norway Rat (*Rattus norvegicus*) (for Nck associated protein).

```
          1                                                  50
xth2      .......... .......... .......... .......... ..........
hem1      .......... .......... .......... .......... ..........
mh19      .......... .......... .......... .......... ..........
dhem2     MARPIFPNQQ KIAEKLIILN DRGLGILTRI YNIKKACGDT KSKPGFLSEK
Nap1      ........ML RGTEIVYIKF VLKFFKRNSL YNIKKACGDP KAKPSYLIDK

          51                                                 100
xth2      .......... .......... .......... .......... ..........
hem1      .......... .......... .......... .......... ..........
mh19      .......... .......... .......... .......... ..........
dhem2     SLESSIKFIV KRFPNIDVKG ....LNAIVN IKAEIIKSLS LYYHTFVDLL
Nap1      NLESAVKFIV RKFPAVETRN NNQQLAQLQK EKSEILKNMA LYYFTFVDVM

          101                                                150
xth2      .......... .......... .......... .......... ..........
hem1      .......... .......... .......... .......... ..........
mh19      .......... .......... .......... .......... ..........
dhem2     DFKDNVCELL TTMDACQIHL DITLNFELTK YYLDLVVTYV SLMIVLSRVE
Nap1      DLRDHVCDLL NTIAVCQVFF DITVNFDLTK NYLDLTVTYT TLMILLSRIE

          151                                                200
xth2      .......... .......... .......... .......... ..........
hem1      .......... .......... .......... .......... ..........
mh19      .......... .......... .......... .......... ..........
dhem2     DRKAVLGLYN AAYELQNNQA DTGFPRLGQM ILDYEVPLKK LAEEFIPHQR
Nap1      GRKAIIRLYN YAHEMTHGGS DREYPRLGQM IVDYEHPLKK MMVEFVPHSK
```

```
          201                                                        250
xth2      .......... .......... .......... .......... ..........
hem1      .......... .......... .......... .......... ..........
mh19      .......... .......... .......... .......... ..........
dhem2     LLTSALRSLT SIYALRNLPA DKWREMQKLS LVGNPAILLK AVRTDTMSCE
Nap1      SLSDALISLQ MVYPRRNLSA DQWRNAQLLS LISAPSTMLN PAQSDTMPCE

          251                                                        300
xth2      .......... .......... .......... .......... ..........
hem1      .......... .......... .......... .......... ..........
mh19      .......... .......... .......... ......QSSS CLSLFRDEVF
dhem2     YISLEAMDRW IIFGLLLNHQ MLGQYPEVNK IWLSALESSW VVALFRDEVL
Nap1      YLSLDAMEKW IIFGFILCHG MLNTEATALN LWKLALQSSS CLSLFRDE.F

          301                                                        350
xth2      .......... ......NKRI NDIRECKENA VSHAGSTHRE RRKFLRSALK
hem1      .......... .......... .......... .......... ..........
mh19      HIHKAAEDLF VNIRGYNKRI NDIRECKEAA VSHAGSMHRE RRKFLRSALK
dhem2     QIHQYIQATF DGIKGYSKRI GEVKEAYNTA VQKAALMHRE RRKFLRTALK
Nap1      HIHKAAEDLF VNIRGYNKRI NDIRECKEAA VSHAGSMHRE RRKFLRSALK

          351                                                        400
xth2      ELATVLADQP GLLGPKALFV FMALSFARDE IIWLLRHADN IP.....KKF
hem1      .......... .......... .......... .......... ..........
mh19      ELATVLSDQP GLLGPKALFV FMALSFARDE IIWLLRHADN MP.....KKS
dhem2     ELALIMTDQP GLLGPKAIFI FIGLCLARDE ILWLLRHNDN PPLLKNKGKS
Nap1      ELATVLSDQP GLLGPKALFV FMALSFARDE IIWLLRHADN MP.....KKS

          401                                                        450
xth2      ADDFMDKHIA ELIFYMEELR AHVRKYGPVM QRYYVQYLSG FDAVVLNELV
hem1      .......... .......... .......... .......... ..........
mh19      ADDFIDKHIA ELIFYMEELR AHVRKYGPVM QRYYVQYLSG FDAVVLNELV
dhem2     NEDLVDRQLP ELLFHMEELR ALVRKYSQVM QRYYVQYLSG FDATDLNIRM
Nap1      ADDFIDKHIA ELIFYMEELR AHVRKYGPVM QRYYVQYLSG FDAVVLNELV
```

```
          451                                                        500
xth2      QNLSVCPEDE SIIMSSFVNT MTSLCVKQVE DGEVFDFRGM RLDWFRLQAY
hem1      .......... .......... .......... .......... ..........
mh19      QNLSVCPEDE SIIMSSFVNT MTSLSVKQVE DGEVFDFRGM RLDWFRLQAY
dhem2     QSLQMCPEDE SIIFSSLYNT AAALTVKQVE DNELFYFRPF RLDWFRLQTY
Nap1      QNLSVCPEDE SIIMSSFVNT MTSLSVKQVE DGEVFDFRGM RLDWFRLQAY

          501                                                        550
xth2      TSVSKASLSL ADHRELGKMM NTIIFHTKMV DSLVEMLVET SDLSIFCFYG
hem1      .......... .........M NLIVFHSRML DSVEKLLVET SDLSTFCFHL
mh19      TSVSKASLSL ADHRELGKMM NTIIFHTKMV DSLVEMLVET SDLSIFCFYS
dhem2     MSVGKAALRI AEHAELARLL DSMVFHTRVV DNLDEILVET SDLSIFCFYN
Nap1      TSVSKASLSL ADHRELERMM NTIIFHTKMV DSLVEMLVET SDLSIFCFYS

          551                                                        600
xth2      RAFEKMFQQC LELPSQSRYS IPFPLLCTHF MSCTHELCPE ERHHIGDRSL
hem1      RIFEKMFAMT LEESAMLRYA IAFPLICAHF VHCTHEMCPE EYPHLKNHGL
mh19      RAFEKMFQQC LELPSQSRYS IAFPLLCTHF MSCTHELCPE ERHHIGDRSL
dhem2     KMFDDQFHMC LEFPAQNRYI IAFPLICSHF QNCTHEMCPE ERHHIRERSL
Nap1      RAFEKMFQQC LELPSQSRYS IAFPLLCTHF MSCTHELCPE ERHHIGDRSL

          601                                                        650
xth2      SLCYMFLDEM AKQARNLITD ICTEQCTLCD QLLPKHCAKT ISQAVN...K
hem1      HHCNSFLEEL AKQTSNCVLE ICAEQRNLSE QLLPKHCATT ISKAKNKKTR
mh19      SLCNMFLDEM AKQARNLITD ICTEQCTLSD QLLPKHCAKT ISQAVNKKSK
dhem2     SVVNIFLEEM AKEAKNIITT ICDEQCTMAD ALLPKHCAKI LSVQSARKKK
Nap1      SLCNMFLDEM AKQARNLITD IYTEQCTLSD QLLPKHCAKT ISQAVNKKSK

          651                                                        700
xth2      K..QTGKKGE PEREKPGVES MRKNRLVVTN LDKLHTALSE LCFSINYAPN
hem1      KQRQTPRKGE PERDKPGAES HRKNRSIVTN MDKLHLNLTE LALTMNHVYS
mh19      K..QTGKKGE PEREKPGVES MRKNRLVVTN LDKLHTALSE LCFSINYVPN
dhem2     D..KSKSKHF DDIRKPGDES YRKTREDLTT MDKLHMALTE LCFAINYCPT
Nap1      K..QTGKKGE PERKSPGVES MRKNRLVVTN LDKLHTALSE LCFSINYVPN
```

```
          701                                                       750
xth2      MVVWEHTFTP REYLTSNLEI RFTKSIVDMT MYNQVTQEIA KPSELFTSVR
hem1      FSVFEHTIFP SEYLSSHLEA RLNRAIVWLA GYNATTQEIV RPSELLAGVK
mh19      MAVWEHTFTP REYLTSHLEI RFTKSIVGMT MYNQATQEIA KPSELLTSVR
dhem2     VNVWEFAFAP REYLCQNLEH RFSRDLVGMV MFNQETMEIA KPSELLASVR
Nap1      MAVWEHTFTP REYLTSHLEI RFTKSIVDMT MYNQATQEIA KPSELLTSVR

          751                                                       800
xth2      AYMTVLQSIE NYVQIDITRV FNNVLLQQTQ HLDSHGEPTI TSLYTNWYLE
hem1      AYIGFIQSLA QFLGADASRV IRKPLLQQTQ PLDSCGEQTI TTLYTNWYLE
mh19      EYMTVLQSIE NYVQIDITRV FNNVLLQQTQ HLDSHGEPTI TSLYTNWYLE
dhem2     AYMNVLQTVE NYVHIDITRV FNNCLLQQTQ ALDSHGEKTI AALYNTWYSE
Nap1      AYMTVLQSIE NYVQIDITRV FNNVLLQQTQ HLDSHGEPTI TSLYTNWYLE

          801                                                       850
xth2      TKLRHVSNGH IACFPAMKAF VNLPGENEGL FNAEEYP... .MRALSELLG
hem1      SLLRQASSGT IILSPAMQAF VSLPREGEQN FSAEEFSDIS EMRALAELLG
mh19      TLLRQVSNGH IAYFPAMKAF VNLPTENELT FNAEEYSDIS EMRSLSELLG
dhem2     VLLRRVSAGN IVFSINQKAF VPISPEGWVP FNPQEFSDLN ELRALAELVG
hem2      TLLRQVSNGH MPYFPAMKAF VNLPTENELT FNAEEYSDIS EMRSLSELLA

          851                                                       900
xth2      PYGMKFLSES LMWHISSQVA ELKKLVVENV DVLTQMRTSF DKPEQMAALF
hem1      PYGMKFLSEN LMWHVTSQIV ELKKLVVENM DILVQIRSNF SKPDLMASLL
mh19      PYGMKFLSES LMWHISSQVA ELKKLVVENV DVLTQMRTSF DKPDQMAALF
dhem2     PYGIKTLNET LMWHIANQVQ ELKSLVSTNK EVLITLRTSF DKPEVMKEQF
Nap1      PYGMKFLSES LMWHISSQVA ELKKLVVENV DVLTQMRTSF DKPDQMAALF

          901                                                       950
xth2      KRLTSVDSVL KRMTIIGVIL SFRSLAQEAL RDVLSYHIPF LVSSVEDFKD
hem1      PQLTGAENVL KRMTIIGVIL SFRAMAQEGL REVFSSHCPF LMGPIECLKE
mh19      KRLSSVDSVL KRMTIIGVIL SFRSLAQEAL RDVLSYHIP. ..SS......
dhem2     KRLQDVDRVL QRMTIIGVII CFRNLVHEAL VDVLDKRIPF LLSSVKDFQE
Nap1      KRLSSVDSVL KRMTIIGVIL CFRSLAQEAL RDVLSYHIPF LVSSIEDFKD
```

```
          901                                                       950
xth2      KRLTSVDSVL KRMTIIGVIL SFRSLAQEAL RDVLSYHIPF LVSSVEDFKD
hem1      PQLTGAENVL KRMTIIGVIL SFRAMAQEGL REVFSSHCPF LMGPIECLKE
mh19      KRLSSVDSVL KRMTIIGVIL SFRSLAQEAL RDVLSYHIP. ..SS......
dhem2     KRLQDVDRVL QRMTIIGVII CFRNLVHEAL VDVLDKRIPF LLSSVKDFQE
Nap1      KRLSSVDSVL KRMTIIGVIL CFRSLAQEAL RDVLSYHIPF LVSSIEDFKD

          951                                                      1000
xth2      HIPRETDMKV AMNVYELSSA AGLPCEIDPA LVVALSSQ.. ..........
hem1      FVTPDTDIKV TLSIFELASA AGVGCDIDPA LVAAIANLKA DTSSPEEEYK
mh19      .......... .......... .......... .......... ..........
dhem2     HLPGGDQIRV AS...EMASA AGLLCKVDPT LATTLKSK.. KPEFDEGEHL
Nap1      HIPRETDMKV AMNVYELSSA AGLPCEIDPA LVVALSSQNQ RTLVQEEDIK

          1001                                                     1050
xth2      IACLLMVFVA VSMPTLASNV MSQYSPAIEG HCNNIHCLAK ASTKLAAALF
hem1      VACLLLIFLA VSLPLLATDP SSFYSIEKDG YNNNIHCLTK AIIQVSAALF
mh19      .......... .......... .......... .......... ..........
dhem2     TACLLMVFVA VSIPKLARNE NSFYRATIDG HSNNTHCMAA AINNIFGALF
Nap1      IACLLMVFVA VSLPTLASNV MSQYSPAIEG HCNNIHCLPK PSTKLLLLCL

          1051                                                     1100
xth2      TI.HKGALKD RLKEFLALAS SSLLKIGQET DKTTTRNRES VYLLLDMIVQ
hem1      TLYNKN.IET HLKEFVVVAS VSLLQLGQET DKLKTRNRES ISLLMRLVVE
mh19      .......... .......... .......... .......... ..........
dhem2     TICGQSDMED RMKEFLALAS SSLLRLGQES DKEATRNRES IYLLLDEIVK
Nap1      QF.TKEALKD RLKEFLALAS SSLLKIGQET DKTTTRNRES VYLLLDMIVQ

          1101                                  1137
xth2      ESPFLTMDLL ESCFPYVLLR NAYHAVYKQS VTSSA*..
hem1      ESSFLTLDML ESCFPYVLLR NAYREVSRAF HLN*....
mh19      .......... .......... .......... ........
dhem2     QSPFLTMDLL ESCFPYVLIR NAYHGVYKQE QILGLAL*
Nap1      ESPFLTMDLL ESCFPYVLLR NAYHAVYKQS VTSSA*..
```

FIG 22. Comparison of amino acid sequences of members of the Hem family as a matrix of identical amino acid residues. hem 1, Hematopoietic Protein; mh19, House mouse brain protein; dhem2, Fruit Fly (*Drosophila melanogaster*), Nap1, Norway Rat (*Rattus norvegicus*) (for Nck associated protein). Protein *xth-2* is in red bold.

| | Hem1 | mh19 | dhem2 | Nap1 | **xth-2** |
|---|---|---|---|---|---|
| Hem1 | 100 | 58 | 48 | 59 | 53 |
| mh19 | | 100 | 60 | 99 | 94 |
| dhem2 | | | 100 | 58 | 60 |
| Nap1 | | | | 100 | 91 |
| **xth-2** | | | | | 100 |

## 5.7. Summary and Discussion

The differential display clone xL52 was used as a probe to screen *Xenopus* cDNA kidney library based on the signals detected by RT-PCR assay when various *Xenopus* adult tissues like (brain, kidney, liver and muscle) were used as templates for analysis by RT-PCR in an attempt to isolate longer sequences, which include the coding region. The results obtained from this analysis indicated that clone xL52 was represented in all the tissues examined with slightly lower level in muscle tissue. On the primary screen approximately half a million plaques were plated which produced 7 positive plaques. This gives a representation of approximately 1 in 7000 or 0.001% clone xL52 recombinants in the library. One positive plaque was isolated to plaque purity with the following length: ~2.5kb, which will be used later on in our discussion as *xth-2*. Simple restriction digests have been used to produce a rudimentary restriction map to aid in the sequencing of clone *xth-2* cDNA.

The entire clone was completely sequenced and its restriction map was determined. Sequence analysis using the incomplete open reading frame from *xth-2* clone at the amino acid level revealed matches to a recently discovered family of tissue-specific transmembrane proteins which are conserved from invertebrates through mammals ``The Hem protein family''. The level of similarity of *xth-2* to mammalian Hem proteins was more than 90%.

A human cDNA was recently cloned, termed Hem-1, which showed an expression pattern restricted to blood cells (Hromas *et al.,* 1991). Kato (1990) reported a murine partial cDNA clone termed mh19, that showed a specific expression pattern in mouse brain. When comparing the predicted sequences of these two proteins, a high degree of similarity was noticed suggesting that they belong to a family of proteins with distinct expression patterns.

Extending the search for further members, six members from *Caenorhabditis elegans* to humans were identified. This indicates that this small family, termed the Hem family is conserved to a high degree from invertebrates through mammals. Moreover, members appear to be specifically expressed in distinct tissues, such as in the hematopoietic or central nervous system. In addition, the patterns of expression are developmentally regulated. This analysis suggests that the primary sequence has been retained and that the function may be conserved. One member of this family *dhem-2* is found in *Drosophila.* It has an essential function in oogenesis. P-element mutants of *dhem-2* are maternally lethal (Baumgartner *et al.*, 1995).

Considering these protein homology comparisons, it appears that the Hem family consists of at least two types of proteins, with one type including Hem-2, *dhem-2*, humtag, the *C. elegans* ESTs (i.e., expressed sequence tags), and mh19, and the other including Hem-1. Moreover, their distinct tissue expression clearly allows

discrimination between the two types of family members (Baumgartner *et al.*, 1995).

Although all Hem family proteins are remarkably rich in leucine residues (average 13%), as compared with an average value of 8% in *Drosophila* and vertebrate proteins (Smoller *et al.*, 1990), none of these residues conforms to known conserved leucine motifs. Several cysteine residues are strictly conserved indicating that the overall folding pattern may be similar in all members. The main characteristic of the common protein structure is the presence of several hydrophobic regions indicative of membrane-spanning domains. Thus, it is probable that the proteins transverse the cell membrane several times. This hypothesis is supported by the finding that polyclonal rabbit antisera against human Hem-1 peptide recognises the cell membrane by immunohistochemical analysis. No member of the family revealed a clear signal cleavage site near the predicted initiator methionine residue. It is not known, however, whether the N terminus is on the extracellular side of the membrane or whether it is intracellular.

Expression analysis of vertebrate Hem genes showed that Hem-1 was previously found to be expressed preferentially in cells of hematopoietic origin (Hromas *et al.*, 1991). Hem-1 was expressed in the myeloid leukaemia line HL-60, the erythroid leukaemia line HEL and the monocytic leukaemia line U937. It was not expressed in human brain, HepG2 hepatoma cells or human pancreatic carcinoma cells.

Thus, Hem-1 expression was tightly restricted to blood cells (Baumgartner *et al.*, 1995).

Hem-2 was expressed in a wider range of tissues. It was most highly expressed in brain, heart, liver and testis. Within the brain, it was also widely expressed. However, there was somewhat higher expression in the amygdala, hippocampus and thalamus (Baumgartner *et al.*, 1995).

The function of the Hem-2 genes may be conserved during evolution. It has been shown that *dhem-2* is expressed maternally. Furthermore, one lethal P-element insertion in the *dhem-2* 5'untranslated region was shown to lead to female sterility indicating that the *dhem-2* gene is essential for *Drosophila* oogenesis and early embryogenesis.

Many genes appear to have been assembled from a pool of modules that are widely shared (Doolittle, 1992). It appears that there are convenient structural units that are used over and over again, the same modules sometimes being used to perform different tasks. Genes can be grouped into families based on these shared structural modules.

The idea that many proteins are constructed from various modules, each identifiable by a consensus sequence that is sometimes related to the exon structure

of the gene, is becoming increasingly familiar (Bork, 1992). However, there is no evidence yet that domains of the Hem family of proteins are shared with other proteins. Rather, it appears to constitute a family that has retained its autonomy in the protein world. The function of this family remains an intriguing question. Since it does not share domain homology to other protein families, there are few clues as to its function in cellular metabolism.

### 5.8. The Hem family of conserved proteins during evolution

It was confirmed the existence of a small family of proteins that is highly conserved from invertebrates through mammals. To date, the family consists of two types of proteins, which are expressed in distinct tissues. One type is preferentially expressed in the central nervous system and oocytes, while the other is preferentially expressed in hematopoietic cells (Baumgartner *et al.*, 1995).

Related protein sequences may be classified into two types: orthologous sequences, which are found indifferent species where the differences reflect species divergence, and paralogous sequences, which are derived from a gene duplication, typically before separation of the species. Hence, it was proposed that *dhem-2*, mh19, Hem-2, humtag and the two *C.elegans* ESTs (i.e., expressed sequence tags) are respective orthologues, while Hem-1 is the corresponding paralogue. This implies that a common ancestor species existed, which may have harboured a prototype of a Hem gene. Since the nematodes harbour a copy of the Hem-2

orthologue, it follows that this ancestral type must have existed very early. Subsequently, gene duplications events, in combination with the development of tissue specification, such as the formation of the central nervous system, or blood cells could have led to the appearance of two protein types (Baumgartner *et al.*, 1995).

## 5.9. Isolation of Hem Proteins

### 5.9.1. Isolation of Hem-1 protein

This was isolated through screening a large number of polymerase chain reaction generated products. A sequence that was was expressed in only hematopoietic cells. Using this fragment, a complete Hem-1 cDNA was cloned (Hromas, *et al.*, 1991).

### 5.9.2. Isolation of mh19 protein

This is a partial cDNA clone randomly selected from mouse cerebellar cDNA libraries (Kato. 1990).

### 5.9.3. Isolation of *dhem-2* protein

During the course of the analysis of the $ten^{m}$ gene (Baumgartner *et al.*, 1994), noticed the presence of a gene immediately 3′ of the $ten^{m}$ transcription unit. The analysis of partial sequences revealed a high degree of similarity to the human gene Hem-1, which has expression restricted to blood cells. Subsequently, a terminal fragment from the 14kb *EcoRI* subclone was used to screen 8 to 12 hour cDNA library (Brown and Kafatos, 1988) yielding four cDNA clones of which *dhem-2*

turned out to be the largest and it was chosen for further analysis (Baumgartner, 1995).

### 5.9.4. Isolation of *Nap1* protein

A specific 559 bp cDNA fragment, corresponding to nucleotide 862 to 1420 of the mouse mh19 cDNA (Kato, 1990), was amplified by PCR. This fragment was labelled and used to screen a rat brain cDNA library (constructed in the λZAP2 vector) by colony hybridisation (Kitamura, 1996). All protein sources, sizes, tissue specificity and expression pattern are indicated in Table 10.

**Table 10.** The Hem Family (Transmembrane Proteins)

| Name | Source | Tissue-Specificity | Protein Size in kD | Function | Reference |
|---|---|---|---|---|---|
| xth-2 | African Clawed Frog Xenopus laevis | Brain, Kidney Liver and Muscle. | 84.0 kD | Not Known | 1 |
| Hematopoietic Protein Hem-1 | Human Homo sapiens | Blood. Expressed only in cells of hematopoietic origin. | 118 kD | Not known | 2 |
| Membrane-associated Protein Hem-2 (Brain Protein H19) (mh19) | House mouse Mus musculus | Brain. High expression in cerebral cortex. | 74.0 kD | Not Known | 3 |
| Membrane-associated Protein Hem-2 (Nap1 Protein) | Norway Rat Rattus norvegicus | Brain. Preferentially expressed in brain, heart, liver and testis. | 128 kD | Associates preferentially with the first SH3 domain of Nck protein. | 4 |
| Membrane-associated Protein Hem-2 (dhem-2) | Fruit Fly Drosophila melanogaster | Expressed maternally in the oocyte and shows uniform expression during the first half of embryogenesis, but becomes restricted to the brain and the nervous system during late embryogenesis. | 129 kD | Plays a role during growth of the oocyte. | 5 |

Table References:


1. This thesis, (1998)
2. Hromas *et al.*, (1991)
3. Kato, K. (1991)
4. Kitamura *et al.*, (1996)
5. Baumgartner, S. (1995)

# CHAPTER 6

**Detection of clone *xth-2* transcripts in *Xenopus laevis* embryos by RT-PCR assays**

---

It was decided to assay for *xth-2* transcripts in developing *Xenopus laevis* embryos using RT-PCR assays, in order to obtain a developmental profile of when and at what levels this transcript is expressed.

## 6.1. Developmental profile of expression of clone *xth-2* transcript

*Xenopus laevis* embryos were cultured under standard conditions and at a various developmental stages embryos were collected and frozen in liquid nitrogen. Subsequently, these embryos were homogenised and nucleic acid extracted for analysis by RT-PCR assay. Four developmental stages were analysed in this way representing points from the gastrula (stage 13) and stages 14, 20 and 25.

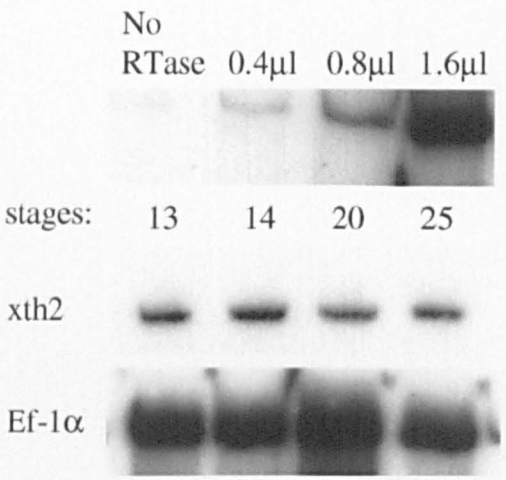
RNA extracted from these embryos was reverse transcribed and the synthesised cDNA was then subjected to PCR using a pair of gene specific primers:

(A) 5' GCTTACATCCGTTGCAGTGT 3' (B) 5' CAGATTGGTGCTGGATATGC 3'. The results obtained (see Figure 23) showed the presence of four bands of the expected size and almost of similar intensity which means that the developmental stage series suggests that the *xth-2* transcript is present at all stages of development and showed the same level of expression. Assays were performed on comparable samples using Ef-1α primers: Ef-1α Upstream primer 5' GGCAATCCAGCTGCTGATGA 3'

Ef-1α Downstream primer 5' CACTGCCTTGATGAC 3'

FIG 23. The temporal pattern of expression of clone *xth-2* was determined by reverse transcription followed by (RT-PCR), using RNA extracted from different stages of *Xenopus* development. Gene specific primers were used for the temporal expression. PCR cycles were carefully performed on *Xenopus* embryos of stages 13, 14, 20, 25 cDNAs. To ensure integrity and cDNA quality, range of cDNA concentrations (0.4μl, 0.8μl, 1.6μl) were included: (No reverse transcriptase as a negative control is also included.

The *xth-2* clone is expressed and maintained throughout development. The level of expression of *xth-2* clone was abundant in all the selected developmental stages as shown in the Figure. Elongation factor-1α was used as a control for these preparations.


No
RTase
0.4µl
0.8µl
1.6µl
stages:
13
14
20
25
xth2
Ef-1α

## 6.2. Spatial distribution of *xth-2* in normal *Xenopus* embryos by whole mount *in situ* hybridisation

### 6.2.1. Introduction

Three techniques have been used to analyse localised gene expression in *Xenopus* embryos. Since the embryos are large, microdissected tissues can be assayed biochemically for the presence of specific RNAs (Mohun *et al.*, 1984); skilled dissection can yield a high degree of spatial resolution (Hopwood *et al.*, 1989) but can not provide information at the level of single cells. Protein products can be localised by immunological methods, and analysis of embryos by whole-mount immunohistochemistry provides both high resolution and three-dimensional information (Dent *et al.*, 1989; Hemmati-Brivanlon and Harland, 1989), however, after isolating a gene of interest it takes considerable time and effort to raise and purify specific antibodies. *In situ* hybridisation is a powerful technique for examining the spatial expression of RNAs in embryos, but *in situ* hybridisation to sectioned *Xenopus* embryos is laborious and not reproducible. The method has not been sufficiently sensitive to detect rare transcripts, such as those from homeobox genes, so that only moderately abundant RNAs have been analysed. Even then an exposure time of weeks (Weeks and Melton, 1987; Sato and sargent, 1989) or even months (Altaba and Melton, 1989) is often necessary. Since *in situ* hybridisation has been carried out on sectioned tissue, two-dimensional information must be reconstituted into three dimensions; this must be done either by the imagination of the investigator or by computer (Wilkinson *et al.*, 1987).

A sensitive, non-radioactive *in situ* hybridisation method has been developed for the localisation of specific RNAs in whole-mount *Drosophila* embryos (Tautz and Pfeifle, 1989). This has been the basis of the whole mount technique for *Xenopus* embryos (Harland, 1991), the non-radioactive method is rapid, sensitive and allows staining of whole embryos.

### 6.2.2. *In situ* assays

RT-PCR analysis on RNA extracted from the whole *Xenopus* embryos at various developmental stages demonstrated that *xth-2* transcript was expressed during embryogenesis. In order to localise *xth-2* transcript to specific cell types, and investigate its expression pattern, the technique of non-radioactive whole mount *in situ* hybridisation (Harland, 1991) was carried out on albino embryos (stages 20-29). The detailed protocol is described in the Methods .3.29.

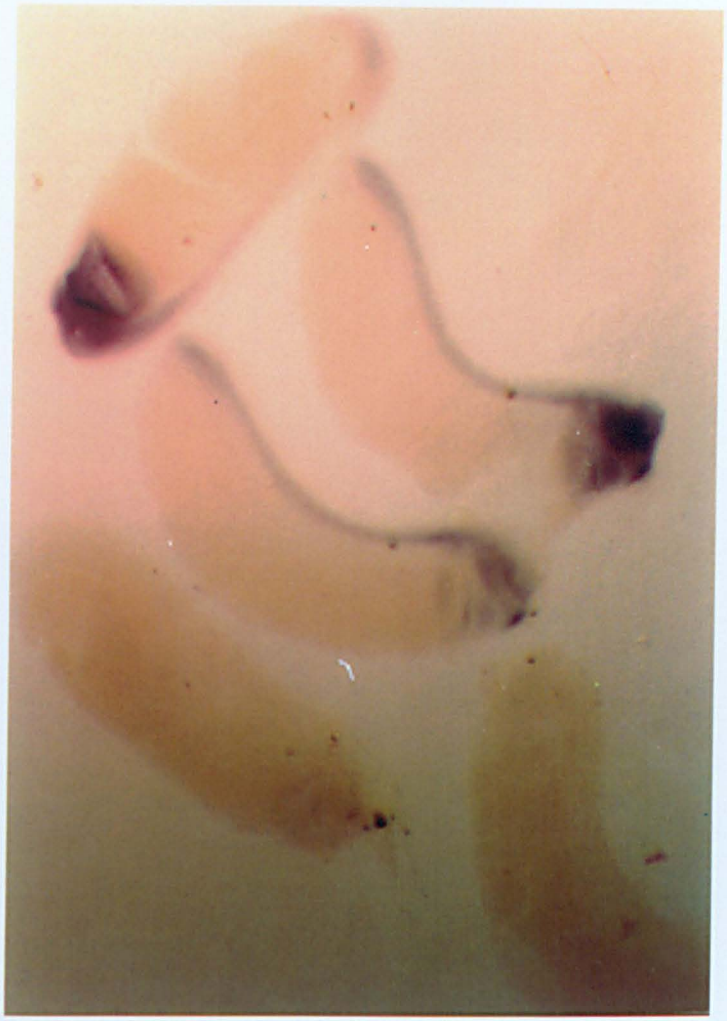
At tailbud stage *xth-2* is expressed in restricted parts of the brain, in and around the eye, in the developing branchial arches, and in the somites (Figure 24) Panels: A–C. Panel C, shows a stage 26 embryos stained for a prolonged period to show the specificity of somite staining, however, other embryos were stained for a shorter period to reveal that expression pattern extended throughout the central nervous system (CNS) to the tailbud.

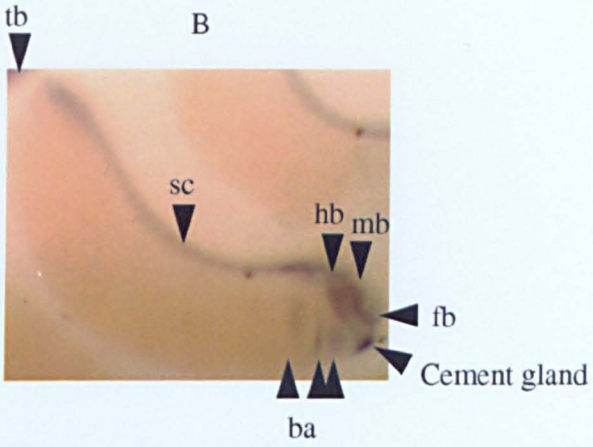
FIG 24. Whole mount *In situ* hybridisation on *Xenopus laevis* embryos showing the expression pattern using a digoxygenin labelled antisense RNA probe transcribed with T7 RNA polymerase from *xth-2* clone into pBluescript (SK) linearised with *SmaI*. *In situ* analysis was performed as described by Harland (1991).

A field of embryos shows the hybridisation pattern (panel A). Control embryos (no probe) are also shown.

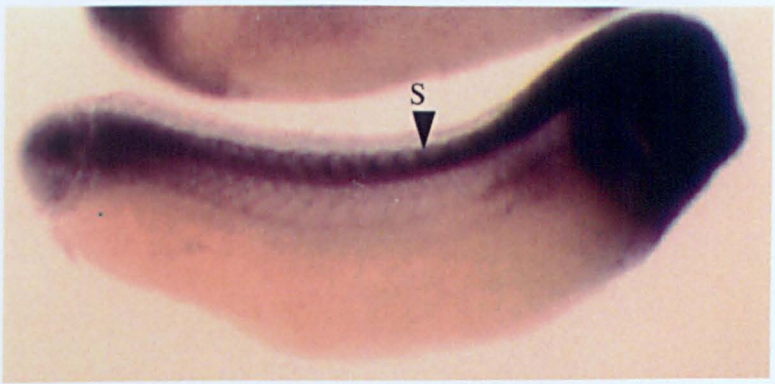
*xth-2* mRNA is detected along the anterio-posterior region of the late tailbud embryos including (the brain:fore brain (fb); mid brain (mb); and hind brain (hb); branchial arches (ba) tail bud (tb) in panel B, and the somites (S) in panel C which showed prolonged staining.

A






C



## 6.3. Summary and Discussion

Temporal and spatial distribution of the transcript *xth-2* by RT-PCR and whole mount *in situ* hybridsation analysis revealed that this clone was uniformly expressed in the stages examined:13,14,20,25. The transcription of the gene *xth-2* therefore, appears to be regulated during development and triggered at the beginning of neurulation. This is because *in situ* hybridisation was also performed on early stages of *Xenopus* embryos and no signal was detected or too weak to be detected. However, the majority of the early stage's embryos were broken while performing the procedure, although it was repeated several times and therefore, were unable to take any photgraph (data not shown). The expression pattern extended along the anterio-posterior region. The level of expression was high in the brain (CNS) and the tailbud.

Inspection of the anterior domain of *xth-2* expression in tailbud embryos indicates that expression is predominantly in the visceral arches and the otic vesicle. The term visceral arches is used instead of the commonly used ``branchial arches'' as in aquatic species this term refers to only the gill arches and excludes the mandibular and hyoid arches. The striped expression pattern observed in the visceral arch region is a result of expression of *xth-2* being stronger in the arches than in the grooves in between. This may be a result the greater depth of the expressing tissue, rather than expression levels.

This level of expression extended throughout to the tailbud region where the *Xenopus* tailbud is not composed of homogeneous cell population, and consists of distinct cell populations which differ by lineage and expression of marker genes.

It would be very interesting to look at the expression pattern of *xth-2* during amphibian metamorphosis as well, however, because of the time available, I was unable to carry out these investigations, but, this will be one of my priorities in the future work.

# CHAPTER 7

**Protein Analysis**

## 7.1. Introduction

Although the cDNA of *xth-2* appears to be not full length, there is an incomplete coding region, predicted to start from the first in frame AUG codon at the 166nt position. This needed to be confirmed in an *in vitro* cell-free translation system. This was essential as protein secondary structure predictions, characteristics and database searches are dependent on having the correct amino acid sequence.

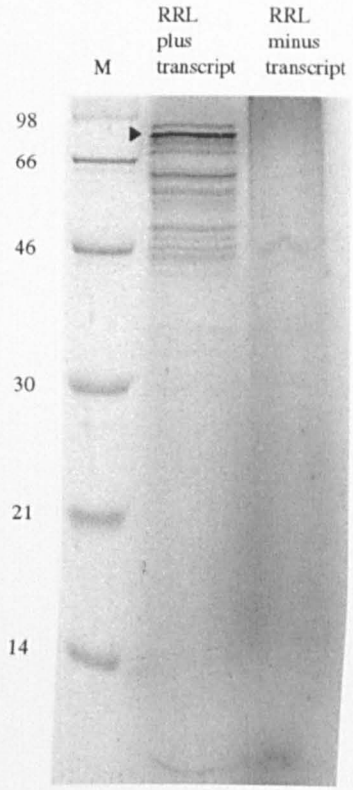
## 7.2. *In vitro* translation of clone *xth-2* cDNA

The predicted molecular weight of the translation product of *xth-2* starting from the AUG start site at the 166nt position is ~84.0 kD. To confirm this, *xth-2* cDNA was used as a template for *in vitro* transcription to generate mRNA to be used in a rabbit reticulocyte cell free translation system.

1μg of *XhoI* linearised pBluescript containing *xth-2*, was used to generate mRNA using T3 RNA polymerase in a 20μl final reaction volume (see Methods 3.26.1.). When the reaction was complete, 1μl of the reaction mixture was electrophoresed on 1% (w/v) agarose gel to confirm the reaction had worked, producing an undegraded mRNA of the expected length.

1μl of the transcription reaction was then used in the *in vitro* translation reaction as described in Methods 3.27.), using [$^{35}$S]-methionine to label the protein product. When the reaction was complete a quarter of the reaction mixture was electrophoresed on a 10% SDS-polyacrylamide gel. The gel was fixed, dried and exposed to X-ray film overnight. The autoradiograph showed several bands (see Figure 25 ), of which one strongly labelled band of the predicted molecular weight ~84.0kD. The appearance of the other smaller secondary bands after analysis of a translation reaction by SDS-PAGE could be due to an internal start sites which may be present in the sequence. Another possibility that if the translated protein contains a large number of the amino acid used for labelling (e.g., methionine) and the amount of label is used up too quickly, premature termination may result, producing secondary bands upon analysis. Also, post-translational modifications, such as isoprenylation or myristylation, may occur in reticulocyte translations and can affect the rate of migration of the translated product in the SDS-PAGE. Because these processes may only affect a percentage of the molecules, two or more bands may be apparent on SDS-PAGE. Becuase of the large amount of globin in reticulocyte lysates, proteins of approximately the same size may migrate abnormally. In addition, proteases in reticulocyte lysates can cause degradation to the translated protein as a result truncated products will be observed on SDS-PAGE.

FIG 25. *In vitro* translation of synthetic capped transcripts derived from pBth-2 template linearised with *XhoI*. 0.5μg of transcript was introduced into a rabbit reticulocyte lysate (RRL from Gibco). The product was analysed by SDS-PAGE (10%). A single product of about ~84.0 kD is indicated. A control of translation without added transcript is also included.


M
RRL plus transcript
RRL minus transcript
98
66
46
30
21
14

An alternative vector (s) used for producing synthetic mRNAs is an SP6 cloning vectors into which any protein coding cDNA clone can be inserted for the purpose of synthesising functional mRNA (see Appendix C ).

Messenger RNAs produced by pSP6 in *in vitro* transcription of cDNA clones are effective templates for translation. The SP6 derived mRNAs are translated as efficiently as native mRNAs in injected oocytes and in wheat germ extracts. This transcription system produces large amounts (micrograms) of mRNAs in a single enzymatic reaction and does so in the absence of other unwanted transcriptional events (such as transcribing both strands of the DNA template). pSP6 transcription of the recombinant pSP64T plasmids will produce synthetic mRNAs that contain 5' and 3' flanking regions, including a poly A tail. These 5' and 3' flanking regions, which are derived from globin mRNA, allow for the efficient translation of the inserted protein coding region both in injected oocytes and in wheat germ extracts. Krieg and Melton (1984) suggested that this procedure can be used to identify and synthesise the protein encoded by any cloned cDNA. This may therefore be an attractive alternative to so called hybrid selected translation assays when the mRNA of interest is rare. In addition, it may be possible to use this method to produce sufficient amounts of mRNA and subsequently protein in order to generate antibodies against the products of cloned cDNAs. Finally, as an alternative to DNA expression vectors, it should be possible to inject synthetic SP6 mRNAs into

cells in order to direct the synthesis of specific proteins and mutants thereof (Krieg and Melton, 1984).

### 7.3. Dominant negative approach

A dominant negative approach has been successfully used to identify the developmental roles of many genes. For example, the elimination of fibroblast growth factor (FGF) signalling in the embryos of *Xenopus laevis*, using a truncated form of *Xenopus* FGFR-1 with dominant negative activity has generated greater understanding of the mechanisms of mesoderm induction and patterning in *Xenopus* and a good example of the use of dominant negative approach. The modified receptor, called XFD, is deleted in the cytoplasmic tyrosine kinase signalling domain, and capable of inhibiting the response of the wild type receptor to FGF (Amaya *et al.*, 1991). Embryos expressing XFD show relatively normal head development, but posterior and trunk structures are severely reduced, or vestigial, with reduction deletion of axial structures posterior to the hindbrain (Amaya *et al.*, 1991). These phenotypic defects can be mostly easily explained as the result of loss, or severe reduction of trunk mesoderm and inhibition of tail extension, and suggest a role for FGF in induction of posterior axial mesoderm in *vivo*. XFD can block the induction of mesoderm by bFGF and XeFGF, but can also block induction by bVgI and activin in animal cap assays (Cornell and Kimelman, 1994; LaBonne and Whitman, 1994). However, activin induction of a subset of markers of mesoderm is unaffected by XFD, suggesting that functional FGF

signalling is not required for the induction of all mesoderm (LaBonne and Whitman, 1994; Cornell and Kimelman, 1994).

Mice which are homozygous null for the FGFR-1 gene are embryonic lethals, dying just after gastrulation and exbibiting growth and patterning defects (Deng *et al.*, 1994). Although the mesoderm does develop in these embryos, anterior-posterior axis extension is incomplete, the notochord is expanded and somites are missing. FGFR-1 is therefore proposed to be involved in receiving a signal which modulates the competence of anterior streak cells to respond to a node-derived organiser signal. Therefore, in the absence of FGFR-1, normal paraxial mesodermal fates are respecified to axial fates in the mouse.

Since the phenotype generated by XFD was revealed, several subsequent studies have identified dominant negative and constitutively active mutations of components of the classical MAP kinase cascade in *Xenopus*, generating trunkless embryos with normal heads, and blocking convergence and extension movements in animal cap explants treated with FGF or activin. The high level of understanding of the signal transduction pathway controlled by FGF has enabled LaBonne and colleagues to propose a model to explain the effect of XFD on a subset of mesodermal markers induced by activin in animal caps and the requirement for an activin-like signal in mesoderm induction by FGF (LaBonne *et al.*, 1995). MAP kinase is activated rapidly in animal caps by FGF signalling but

not by activin (Hartley *et al.,* 1994; LaBonne and Whitman, 1994), and XFD, inhibits this. In addition, a low level of active MAP kinase detected in untreated animal caps was aslso inhibited by XFD, suggesting the existence of a low level (sub-inducing) FGF signal (LaBonne *et al.,* 1995). XFD was also shown to prevent immediate early induction of a mesodermal marker by activin, suggesting that the purpose of sub-inducing signal was to enable induction of mesoderm by activin.

### 7.4. Clone *xth-2* protein sequence

The predicted amino acid sequence of the *in vitro* translation product is shown in figure, starting from the AUG start codon at the 166nt position and ending at the TAA stop codon at 2377nt position and codes for a protein of 737 amino acid residues. The amino acid sequence of *xth-2* is shown in Figure 26.

FIG 26. Amino acid sequence of *xth-2* protein, 737 amino acids

```
1   MALSFARDEI IWLLRHADNI PKKFADDFMD KHIAELIFYM EELRAHVRKY
51  GPVMQRYYVQ YLSGFDAVVL NELVQNLSVC PEDESIIMSS FVNTMTSLCV
101 KQVEDGEVFD FRGMRLDWFR LQAYTSVSKA SLSLADHREL GKMMNTIIFH
151 TKMVDSLVEM LVETSDLSIF CFYGRAFEKM FQQCLELPSQ SRYSIPFPLL
201 CTHFMSCTHE LCPEERHHIG DRSLSLCYMF LDEMAKQARN LITDICTEQC
251 TLCDQLLPKH CAKTISQAVN KKQTGKKGEP EREKPGVESL RKNRLVVTNL
301 DKLHTALSEL CFSINYAPNM VVWEHTFTPR EYLTSNLEIR FTKSIVDMTM
351 YNQVTQEIAK PSELFTVLEA YMTVLQSIEN YVQIDITRVF NNVLLQQTQH
401 LDSHGEPTIT SLYTNWYLET KLRHVSNGHI ACFPAMKAFV NLPGENEPTF
451 NAEEYPMRAL SELLGPYGMK FLSESLMWHI SSQVAELKKL VVENVDVLTQ
501 MRTSFDKPEQ MAALFKRLTS VDSVLKRVTI IGVILSFRSL AQEALRDVLS
```

551 YHIPFLVSSV EDFKDHIPRE TDMKVAMNVY ELSSAAGLPC EIDPALVVAL

601 SSQIACLLMV FVAVSTSTLA SNVMSQYSPA IEGHCNNIHC LAKASTKLAA

651 ALFTIHKGAL KDRLKEFLAL ASSSLLKIGQ ETDKTTTRNR ESVYLLLDMI

701 VQESPFLTMD LLESCFPYVL LRNAYHAVYK QSVTSSA*.

FIG 27. Amino acid composition of *xth-2* protein. The amino acid composition is shown in the Figure below which indicates that leucine and serine at 11.8 and 8.3 mole-% respectively, to be the most abundant in the molecule. All amino acids are present in the protein. The predicted molecular weight is ~84.0kD, which corresponds closely with the results from the *in vitro* translation analysis.

| Residue | Number | Mole Percent |
|---|---|---|
| Ala (A) | 50 | 6.7% |
| Arg (R) | 32 | 4.3% |
| Asn (N) | 28 | 3.8% |
| Asp (D) | 32 | 4.3% |
| Cys (C) | 21 | 2.8% |
| Gln (Q) | 29 | 3.9% |
| Glu (E) | 54 | 7.2% |
| Gly (G) | 21 | 2.8% |
| His (H) | 23 | 3.1% |
| Ile (I) | 37 | 5.0% |
| Leu (L) | 88 | 11.8% |
| Lys (K) | 39 | 5.2% |
| Met (M) | 33 | 4.4% |
| Phe (F) | 38 | 5.1% |
| Pro (P) | 29 | 3.9% |
| Ser (S) | 62 | 8.3% |
| Thr (T) | 46 | 6.2% |
| Trp (W) | 5 | 0.7% |
| Tyr (Y) | 24 | 3.2% |
| Val (V) | 54 | 7.2% |

## 7.5. Protein database searches

The most obvious first stage in the analysis of any new sequence is to perform comparisons with sequence databases to find homologues. Searches were performed using the FASTA and BLAST programs, with the GCG default parameters and a word size of 2, to search the non-redundant protein databases. Searches performed on the full 737 amino acid sequence, produced similarities to a recently discovered tissue-specific transmembrane proteins called "the Hem family".

## 7.6. Motifs

The following motifs were found using the MotifFinder program on which searches the prosite database of protein domains:

| | Start | End | Site sequence |
|---|---|---|---|
| **N-glycosylation site** | 85 | 88 | NLSV |
| **cAMP and cGMP-**dependent protein kinase phosphorylation site | | | |
| | 280 | 283 | KKQT |
| | 525 | 528 | KRLT |
| | 535 | 538 | KRMT |
| **Protein kinase C phosphrylation site** | | | |
| | 63 | 65 | TQR |
| | 283 | 285 | TGK |
| | 298 | 300 | SMR |
| | 337 | 339 | TPR |
| | 545 | 547 | SFR |
| | 654 | 656 | STK |
| | 691 | 693 | TDK |
| | 695 | 697 | TTR |

| | | | |
|---|---|---|---|
| **Casein kinase II phosphorylation site** | | | |
| | 72 | 75 | SGFD |
| | 142 | 145 | SLAD |
| | 165 | 168 | SLVE |
| | 260 | 263 | TLCD |
| | 307 | 310 | TNLD |
| | 337 | 340 | TPRE |
| | 344 | 347 | SNLE |
| | 375 | 378 | TVLE |
| | 412 | 415 | SHGE |
| | 512 | 515 | TSFD |
| | 528 | 531 | TSVD |
| | 567 | 570 | SSVE |
| | 568 | 571 | SVED |
| **Tyrosine kinase phosphorylation site** | | | |
| | 38 | 46 | KhiaElify |
| | 697 | 703 | RnrEsvY |
| **N-myristoylation site** | | | |
| | 356 | 361 | GMTCTN |
| | 541 | 546 | GVILSF |
| **Amidation site** | 282 | 285 | TGKK |

## 7.7. Isoelectric point

The isoelectric point is the pH at which the protein has no net charge. Using Isoelectric, on the GCG (Wisconsin) package, the *xth-2* protein is shown to be a neutral protein with an isoelectric point of ~6.0.

## 7.8. Functional analysis of clone *xth-2* in embryo system

### 7.8.1. Introduction

An indication of the function and any developmental implications of a new clone can be addressed by microinjecting the clone in developing *Xenopus* embryos and noting any phenotypic effects. It was decided to express *xth-2* clone in developing embryos to see if this could give further clues to the functioning of the protein.

## 7.9. Microinjection of *Xenopus* embryos

Embryos were generally injected bilaterally (both blastomeres) with mRNA at the two cell stage or unilaterally (one blastomere). Gene expression can then be assayed by whole-mount *in situ hybridisation* Prior to injection, embryos were transferred to 5% Ficoll (w/v) to reduce cytoplasm leakage. After injection, the embryos were maintained in Ficoll until stage 6, after which they were cultured in 0.1x BX to avoid exogastrulation.

*Xenopus* eggs freshly laid into Barth X were fertilized artificially by squeezing the testis of a freshly killed *Xenopus* male. Fertilised eggs were then de-jellied using freshly made 2% cystine for about 10 minutes prior to micro-injection. Bilateral injection was performed at two-cell stage *Xenopus* embryos of synthetic capped transcript. Injected embryos were divided into two duplicate batches: batch 1 included controls or uninjected embryos; batch 2 a full concentration of the synthetic message (10ng). Each batch contained about 20 embryos; (i.e., 80

embryos in total). They were then incubated at 14°C overnight, having been transferred to 0.1Barth X following recovery from injection.

### 7.10. Embryonic effects of expression of clone *xth-2*

Following microinjection of *xth-2* clone on *Xenopus* embryos, there was no clear indication of whether this clone had an effect on developing embryos. All the uninjected embryos (i.e., 40 embryos) developed normally (see Figure 28 A). However, few of the injected embryos (6 embryos out of 40) in batch 2 developed with minor abnormalities, most typically a reduction in the posterior structures (see Figure 28 B). These embryos (i.e., full concentration injected message), were unable to elongate during neurula stages. The anterior region was fairly normal with well-developed head, a good eye and cement gland. There were no obvious signs of abnormalities during the early stages of development (i.e., blastula or gastrula). As a result of proper involution of the most anterior dorsal mesoderm, the head structures appeared normal, whereas posterior reductions were due to the improper involution of posterior lateral and ventral mesoderm. Expression of *xth-2* therefore, does not provide information on the or clues to its specific role during embryonic patterning events. This experiment was repeated in an attempt to confirm if these abnormalities could occur again. The number of abnormal phenotypes seen in the second experiment was more or less the same as the first one (i.e., all the embryos were quite normal), and therefore this is the reason why

we could not consider this small number of tail-less phenotypes as a significant change.

FIG 28. Expression of *xth-2* in developing *Xenopus laevis* embryos.

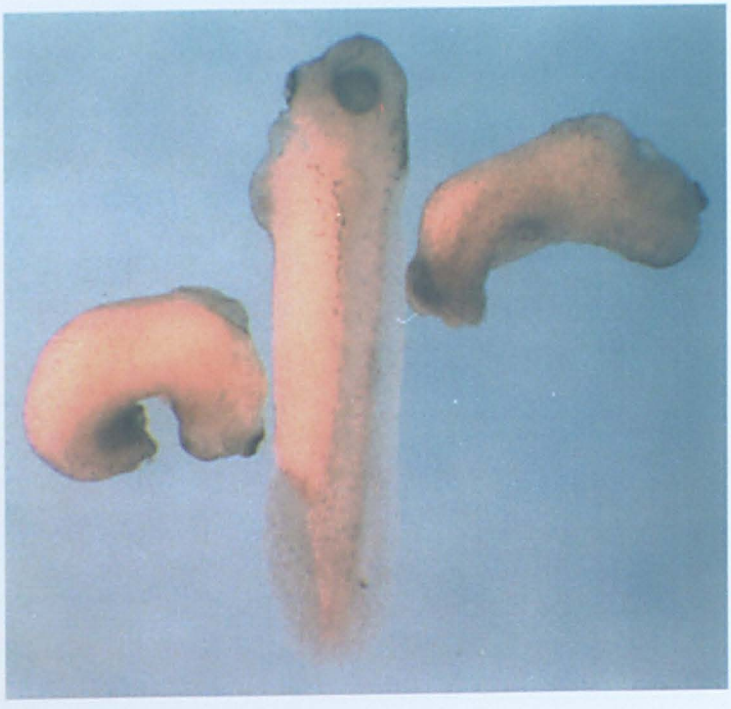
Panel A. Shows field of uninjected embryos which developed normally.

Panel B. Shows two *Xenopus* embryos demonstrating the effects induced by injection of (10ng) *xth-2* and uninjected embryos that were allowed to develop until free living stage was reached.

Injection of *xth-2* resulted in a mild abnormality at the posterior end. There were no clear real tail structures. These tail-less phenotypes represented only a small proportion of the injected embryos (see text), and therefore, were considered insignificant.

A

B



### 7.11. Summary and Discussion

*In vitro* translation studies have shown that the *xth-2* cDNA produces several protein products, of which one main protein product of ~84.0kD. This protein is produced when translation is initiated at the 166nt AUG codon and terminated at the 2377nt stop codon. This produces a protein 737 amino acids in length, but it must be taken into consideration that this result is from *in vitro* system. *In vivo* the protein product produced may be the same or translation may initiate from any of the upstream AUG sites in reading frame.

Using the isoelectric on GCG program (Wisconsin) package, the protein is predicted to be with an isoelectric point of ~6.0., suggesting the protein to be neutral and nuclear in location.

Protein database searches using incomplete open reading frame from *xth-2* clone at the amino acid level have revealed matches to a recently discovered family of tissue-specific transmembrane proteins which are conserved from invertebrates through mammals ``The Hem protein family''. The level of similarity of *xth-2* to mammalian Hem proteins was more than 90%.

Functional analysis addressed by microinjection of clone *xth-2* into *Xenopus* developing embryos has not revealed any significant effects that might be

considered as clues to its function. This could be attributed to the fact that this protein might interact with other proteins to exert its effect.

# CHAPTER 8

## 8.1. Discussion and Conclusions

The technique of differential display RT-PCR has been applied to RNA extracted from thyroid hormone treated *Xenopus laevis* tadpole tails in an attempt to isolate and then characterise genes regulated by thyroid hormone.

The banding patterns generated from different primer sets in this method produced ten products that appeared to be obviously differentially expressed. Of these ten bands, eight were up-regulated by thyroid hormone and two were down-regulated. However, it was only possible to follow up one of these differential bands to any extent, this clone appeared to be up-regulated by thyroid hormone. Library screens allowed the isolation of longer sequences from coding region of this clone, termed *xth-2*. The coding region of this clone was completely sequenced and the predicted amino acid sequence from the longest open reading frame was determined.

Since the development of the differential display technique several improvements and modifications have been made to the procedure (Liang *et al.*, 1993; Chen and Peck, 1996).

The isolation of differentially amplified products and their identification and confirmation as fragments from a differentially regulated RNA (the labour-

intensive step following differential display) has too been improved, producing greater efficiency and reliability of results (Vogeli-Lange *et al.*, 1996). The technique has developed, in the last four years and represents a breakthrough as a method to isolate genes. The differential display technique decribed in this project was performed during 1993 at a time when only 13 publications were available on the technique. Therefore, the reaction protocol was taken directly from the Liang and Pardee (1992) publication. To date over 340 publications have described the use of the differential display technique and therefore, the methodology would be modified accordingly if these experiments were repeated.

### 8.2. *In vitro* translation of *xth-2*

*In vitro* translation studies in rabbit reticulocyte lysates have shown that *xth-2* RNA produces several products of which ~84.0kD is the main product. This protein is produced when transalation is initiated at the 166nt AUG codon and terminated at the 2377nt stop codon, producing a protein 737 amino acids in length.

The sequence context around the $^{166}$AUG codon in *xth-2*, determined by *in vitro* translations, does not adhere strictly to the consensus sequence for initiation (Kozak, 1987). The sequence lacks a purine in the -3 position but possesses a G in the +4 position which may indicate why it has a higher efficiency than predicted.

In the *xth-2* cDNA there are no stop codons upstream to the largest ORF. By comparison with known Hem proteins, part of the coding region is missing, and therefore, *xth-2* cDNA does not represent a full length and the predicted initiative codon at 166nt is in fact, not the authentic AUG codon as *in vitro* translation suggests. The question raised is how accurate may the *in vitro* translation experiments represent the *in vivo* translation of *xth-2*.

### 8.3. Further discussion and Possible role of Hem proteins

Functional analysis of *xth-2* following its microinjection to the developing *Xenopus laevis* embryos, has not shown a clear phenotypic effect or clue to the function of this protein. Despite the high degree of sequence similarity between *xth-2* protein and other members of the Hem proteins and from the data surveyed, the function of the Hem family to which *xth-2* protein belongs is not yet known.

Searching for clues as to the function of this protein, recent studies revealed that another member of Hem proteins was identified and termed *Nap1* (for Nck associated protein), and is known to be involved in signalling transduction through its association with the first SH3 domain of *Nck* protein. In addition, it has recently been found that *Nap1* showed an indirect interaction with activated GTP-binding protein Rac. Moreover, the 140kD Rac-binding protein is a potential candidate for a link that connects *Nap1* to Rac. The multimolecular complex comprising Rac, *Nap1* and probably the 140kD protein might mediate some of the biological effects

transmitted by the multipotent GTPase (Kitamura *et al.*, 1997). *xth-2* protein has shown an overall identity of 91% at the amino acid level to *Nap1* protein, therefore, we could speculate that the function of *xth-2* protein is similar to the *Nap1* protein.

Also, the results obtained from sequence analysis have shown that *xth-2* is of membrane protein, where we could assume that *xth-2* might be a membrane receptor. As we know, receptors are proteins located either in the cell's plasma membrane or inside the cell. If this is the case, where a receptor is located in the cell's plasma membrane, then a possible model of how *xth-2* protein might be involved in signal transduction is through a direct interaction between the receptor (*xth-2*) and the growth factors.

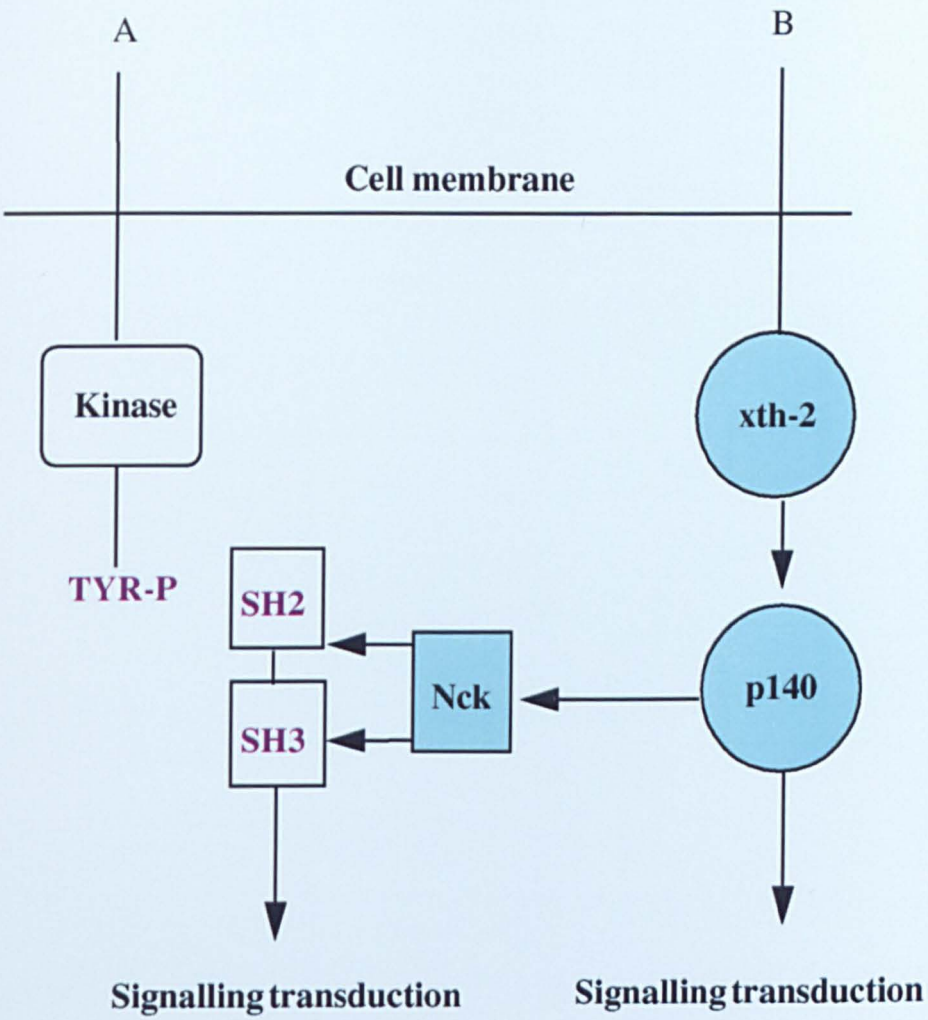
The combination of the growth factors with the membrane receptor *xth-2* causes a conformational change of the receptor, and this step, known as receptor activation, is always the initial one leading to the cell's ultimate responses to the messenger, which can be (1) changes in the membrane permeability, in the rates at which it transports various substances, or in its electrical state (2) changes in the rate at which a particular substance is synthesised or secreted by the cell; or (3) changes in the strength of contraction, if the cell is a muscle cell.

Certain messengers involved in growth and development bind to their receptors, the receptors themselves become active protein kinases. The receptors then

phosphorylate specific cytosolic and plasma-membrane proteins including themselves. This provides one way of translating the first messenger's signal into cellular responses. These protein kinases that function in this way belong to the family known as tyrosine kinases, because they phosphorylate specifically the tyrosine portions of proteins. Despite the seeming variety of these ultimate responses, there is a common denominator: they are all due directly to alterations of particular cell proteins.

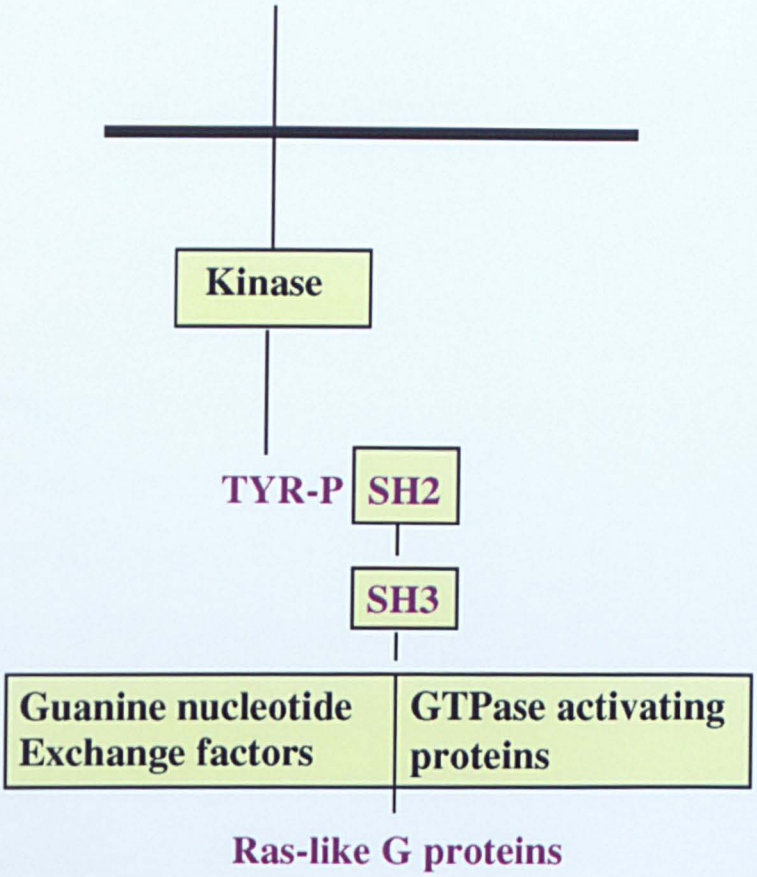
Another possible model is that protein *xth-2* could interact indirectly with other proteins like p140 that was recently cloned and found to be associated with SH3 domain of *Nck* protein. This protein p140 could be a potential linker between *xth-2* protein and *Nck* protein. Signal transduction in this case could be either through the interaction of *xth-2* and p140 which can then transmit the signal through its association or activation of other cellular proteins (one possible pathway), or the signal can be transmitted through a multistep interactions of *xth-2* with p140 and *Nck* (another possible pathway), illustration of such hypothetical model is shown in Figure 29.

FIG 29. A hypothetical model of how *xth-2* protein might be involved in signalling transduction. A: SH2-SH3 domains mediate protein-protein interactions that co-ordinate the activation of signalling pathways by tyrosine kinases. B: The 140 kDa protein that associates with *Nck* is a potential linker between *xth-2* and *Nck* and may therefore shed light on the interaction of *xth-2* with *Nck* as well as on signalling downstream of *Nck*.


A
B
Cell membrane
Kinase
xth-2
TYR-P
SH2
SH3
Nck
p140
Signalling transduction
Signalling transduction

To summarise, signal transduction is often mediated by specific protein-protein complexes. In many cases, the formation of such complexes is controlled by small modular domains termed the *Src* homology 2 and 3 (SH2 and SH3) domains (see Figure 30), which are found in a wide variety of proteins (Mayer and Baltimore, 1993; Pawson, 1995; Pawson and Gish, 1992). SH2 domains have been shown to bind specifically and with high affinity to tyrosine-phosphorylated proteins and are thought to mediate the association of signalling proteins in response to tyrosine phosphorylation (Anderson *et al.*, 1990; Margolis *et al.*, 1990; Matsuda, 1991; Mayer *et al.*, 1991). SH3 domains bind to specific proline-rich sites on target proteins (Pawson, 1995; Ren *et al.*, 1993) These observations suggest that SH2-SH3 domains mediate a network of phosphorylation-dependent protein-protein interactions that co-ordinate the activation of signalling pathways by tyrosine kinases. These complex interactions may be especially important in integrating signals in the animal, where any cell is potentially exposed to stimuli from multiple sources, such as soluble hormones, adjacent cells and the extracellular matrix (Kitamura *et al.*, 1996).

FIG 30. Postulated role for SH3 domains in coupling tyrosine kinases to small G proteins. According to this scheme, proteins with SH2, SH3 and catalytic domains would be capable of multiple interactions with tyrosine kinases, Ras-like small G protein pathways and their enzymatic substrates (Pawson and Gish, 1992).


Kinase
TYR-P
SH2
SH3
Guanine nucleotide Exchange factors
GTPase activating proteins
Ras-like G proteins

## 8.4. Possible role of Hem proteins in metamorphosis

The signalling pathways that lead through apoptosis into cell death as an important feature of amphibian metamorphosis need to be determined. In many cases of programmed cell death (PCD) during postembryonic development, the signals activating the process may induce a few early gene products that would lead to a cascade of proteins to be synthesised sequentially, including some that are components of the apoptotic apparatus. Progress is so rapid in this field that it is difficult to make any predictions. However, current knowledge of immediate-early genes and protein kinase pathways provide important clues as does the determination of the morphology seen in apoptosis. A number of protein kinase and phosphatase genes have recently been identified and proposed a morphologic/functional model of signalling that lead to cell death. Therefore, we can not exclude the possibility that Hem proteins may take part in signalling transduction that lead to cell death during amphibian metamorphosis.

## 8.5. Future Work

### A. Protein expression of *xth-2* cDNA

Cellular localisation of the *xth-2* protein would provide invaluable information regarding the possible function of the protein. Questions such as whether the protein has a role during amphibian metamorphosis or it is translated during metamorphosis to have a role in tail resorption, could be answered. As antibodies to the cloned *xth-2* cDNA product were not available, a logical step is produce a

fusion protein for use as antigen for the production of polyclonal antibodies. Expression of large amounts of fusion protein from a cloned gene introduced into *E.coli* is a fast method from which the protein can be easily purified and likely to elicit an antibody response.

**B. Role in apoptosis**

Experiments can also be designed to see if *xth-2* protein is associated with cell death when expression is induced.

**C. Dominant negative**

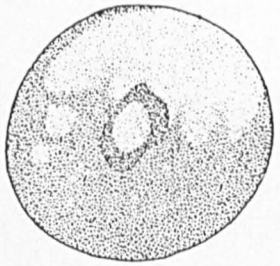
The dominant negative approach has been successfully used to identify the developmental roles of many genes. Using a truncated form of *xth-2* will generate greater understanding of its possible role.

**D. Thyroid hormone inducibility**

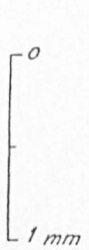
To see if *xth-2* might be thyroid hormone inducible (i.e., whether it is a direct or indirect interaction).

## Appendix A

The stages of *Xenopus* embryonic development. Normal Table of *Xenopus laevis* (Daudin) Edited by P.D. Nieuwkoop and J. Faber First edition 1956, Second edition 1967. Republished 1994, with a new foreword by John Gerhart and Marc Kirschner Garland Publishing Inc, New York.

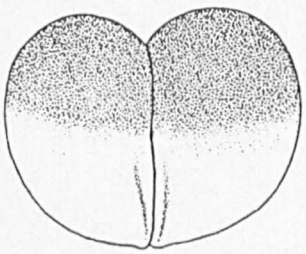


St. 1 — Animal view (An.)
Magnification 24x

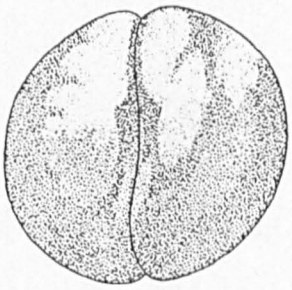



St. 1 — Dorsal view (Dors.)

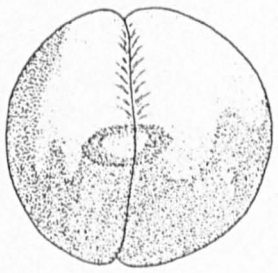
St. 1 — Ventral view (Ventr.



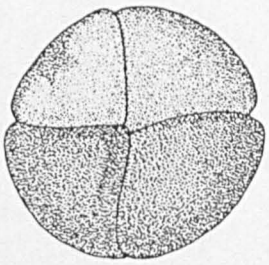
St. 2 — Ventr.



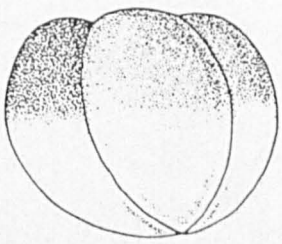
St. 2 — An.



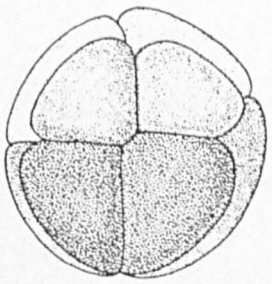
St. 2 — An.



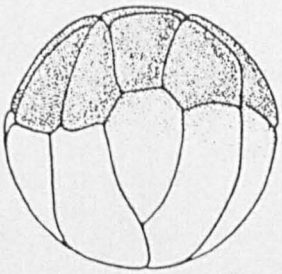
St. 3 — An.



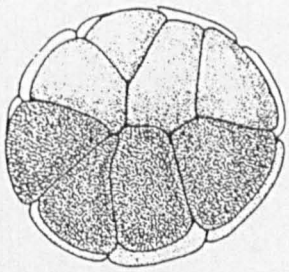
St. 3 — Dorso-lateral view (Dors.-lat.)



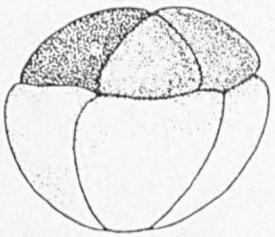
St. 4 — An.



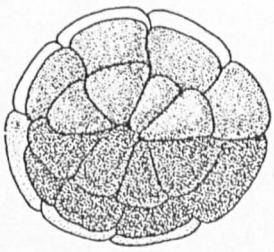
St. 5 — Dors.



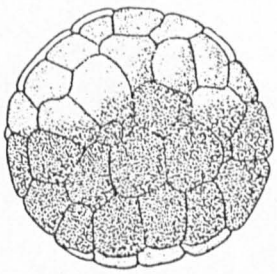
St. 5 — An.



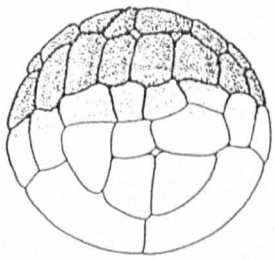
St. 4 — Dors.-lat.



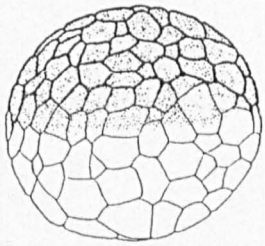
St. 6 — An.



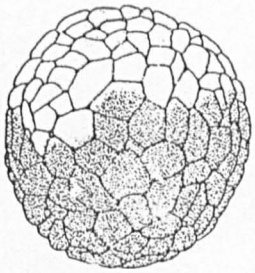
St. 6½ — An.



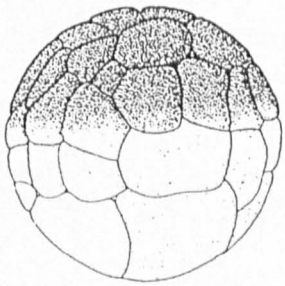
St. 6½ — Dors.



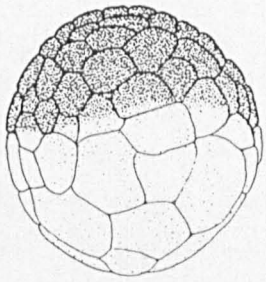
St. 7 — Dors.



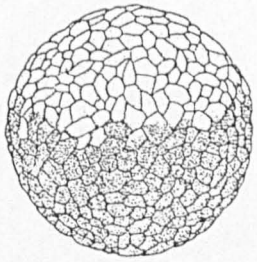
St. 7 — An.



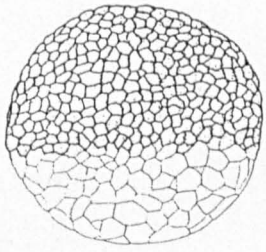
St. 6½ — Ventr.



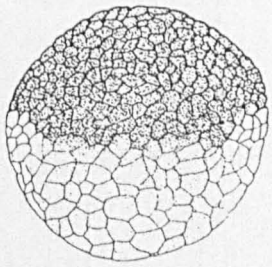
St. 7 — Ventr.



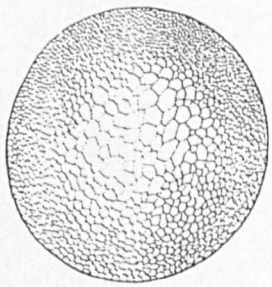
St. 8 — An.



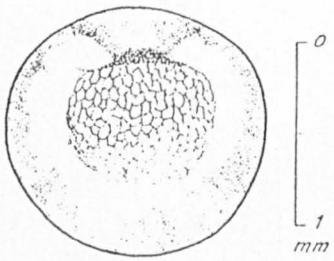
St. 8 — Dors.



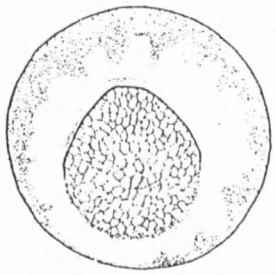
St. 8 — Ventr.



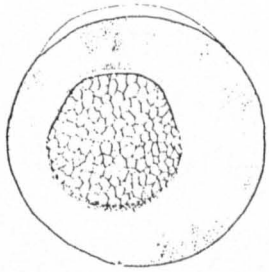
St. 9 — Vegetative view
Dorsal side above (Veg.)

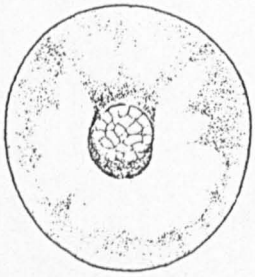



St. 10 — Veg.

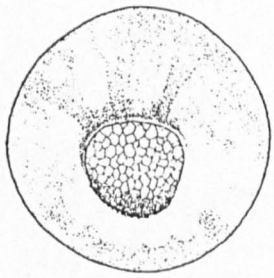


Two forms
St. 10½ — Veg.

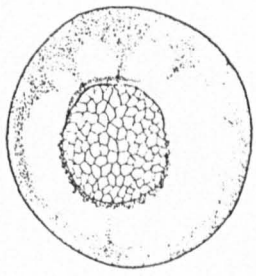




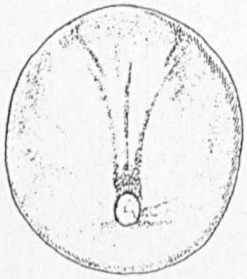
St. 12 — Veg.



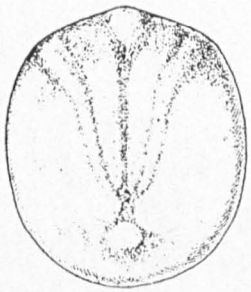
St. 11½ — Veg.



St. 11 — Veg.



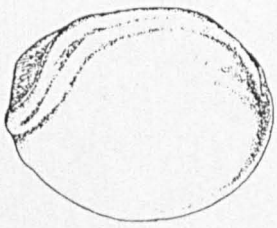
St. 12½ — Post.-dors.



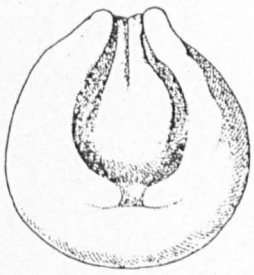
St. 13 — Posterio-dorsal view (Post.-dors.)

St. 14 — Post.-dors.

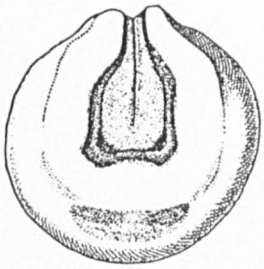
St. 15 — Post.-dors.



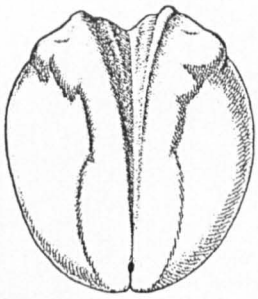
St. 14 — Lateral view (left side) (Lat.)



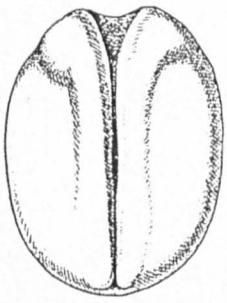
St. 15 — Anterior view (Ant.



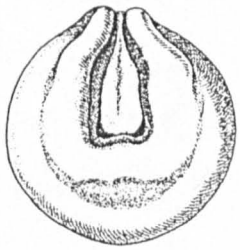
St. 16 — Ant.



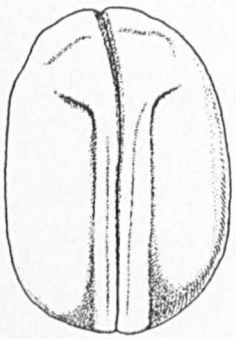
St. 16 — Post.-dors.



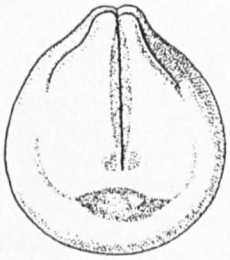
St. 17 — Dors.



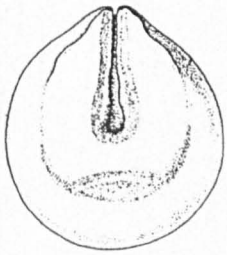
St. 17 — Ant.



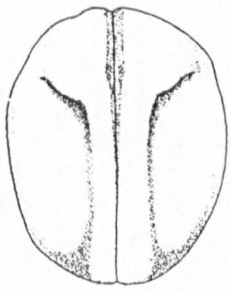
St. 20 — Dors.



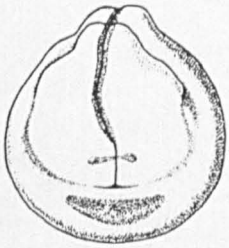
St. 19 — Ant.



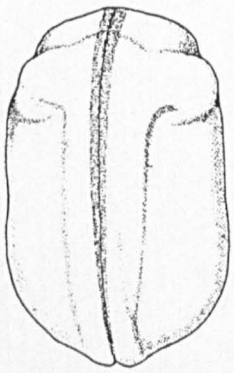
St. 18 — Ant.



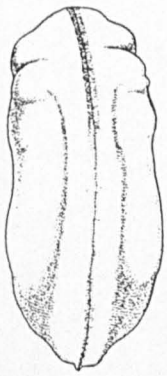
St. 19 — Dors.



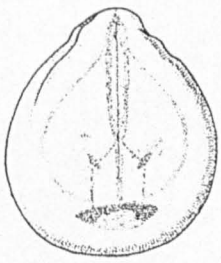
St. 20 — Ant.



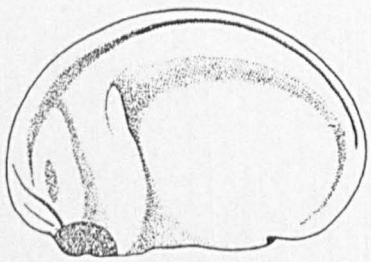
St. 21 — Dors.



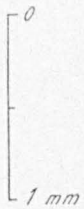
St. 22 — Dors.

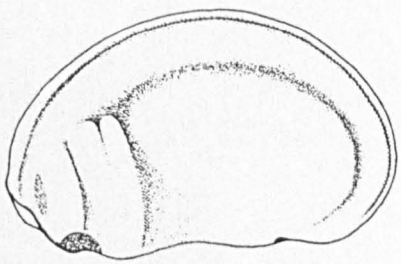


St. 21 — Ant.

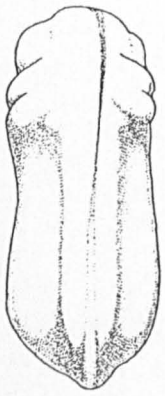


St 22 — Lat.

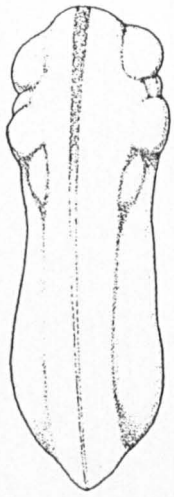


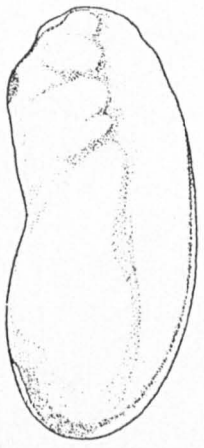
St. 23 — Lat.



St. 23 — Dors.

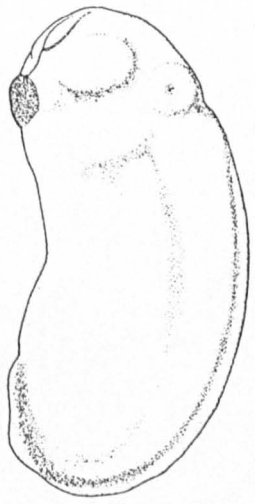


St. 24 — Dors.

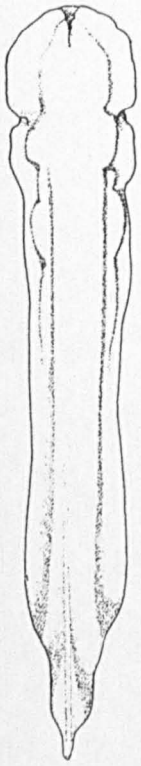


St. 24 — Lat.
(Small individual)

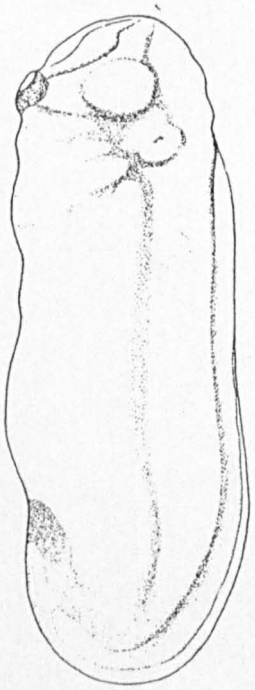
St. 25 — Dors.



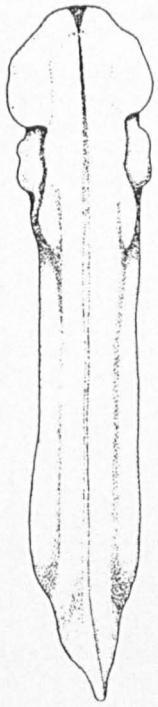
St. 25 — Lat.



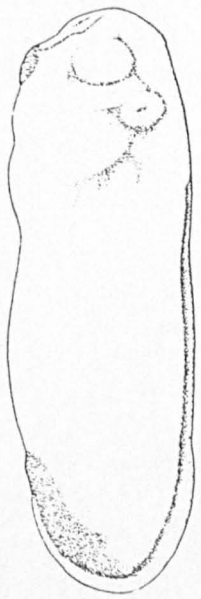
St. 28 — Dors



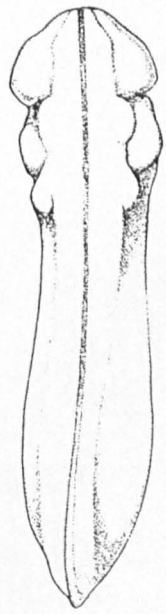
St. 27 — Lat.



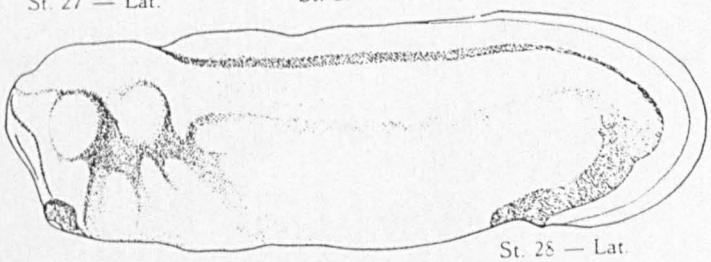
St. 27 — Dors.



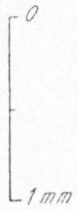
St. 26 — Lat.

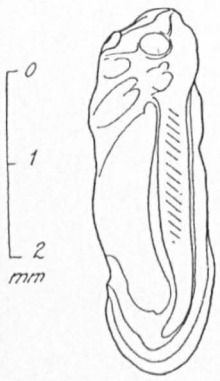


St. 26 — Dors.

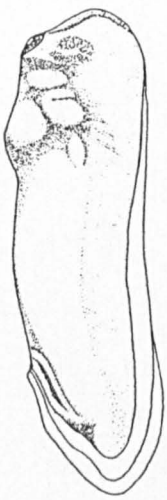


St. 28 — Lat.

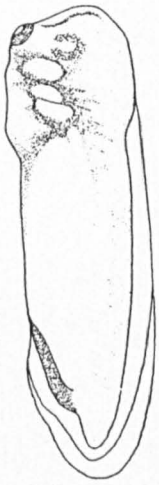





Idem Magn. 12x



St. 29/30 — Lat.



St. 31 — Lat.

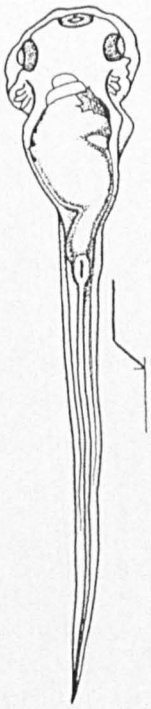
St. 32 — Lat.

St. 33/34 — Lat.

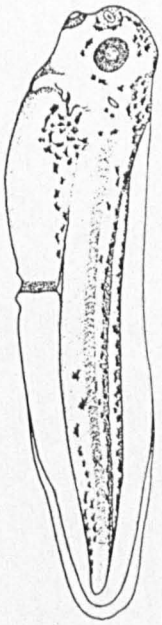
St. 35/36 — Lat.

St. 42 — Lat.

St. 41 — Lat.

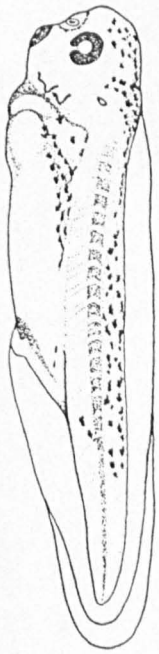


St. 41 — Ventr.

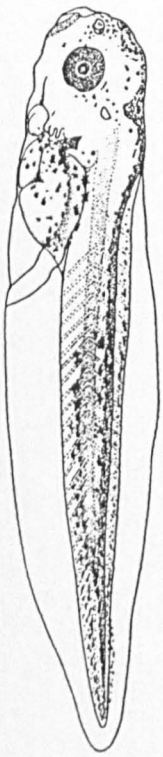


St. 40 — Lat.
(Small ind.)

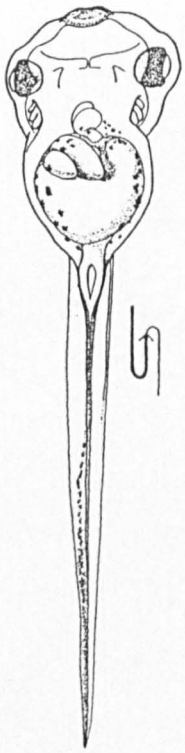
St. 39 — Lat.
(Small ind.)



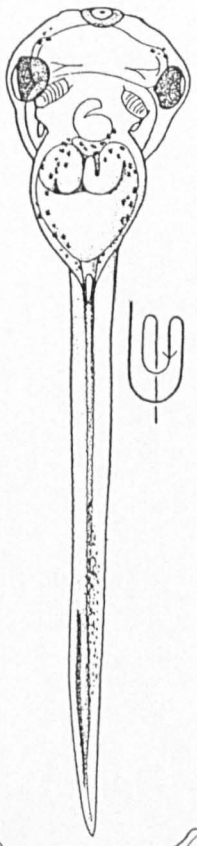
St. 37/38 — Lat.



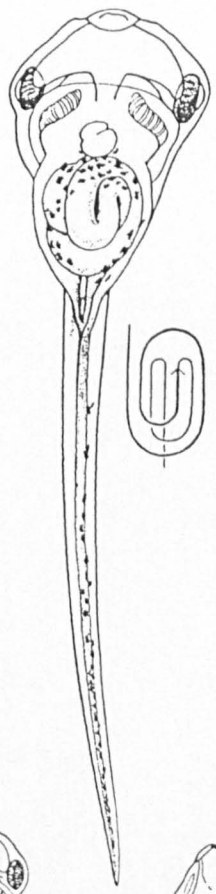
St. 43 — Lat.



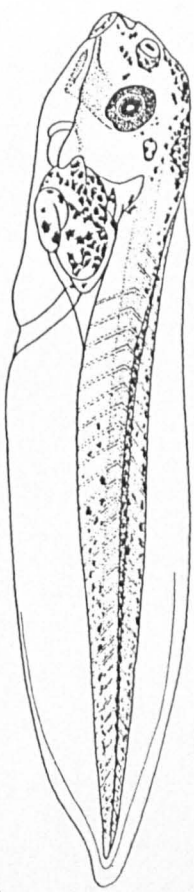
St. 43 — Ventr.



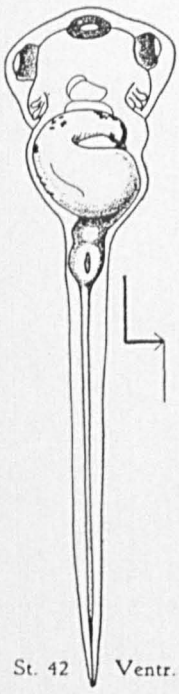
St. 44
Ventr.



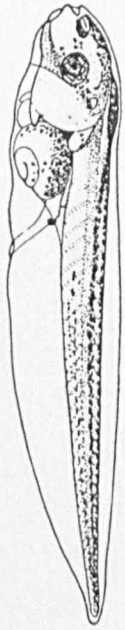
St. 45
Ventr.



St. 45 — Lat.

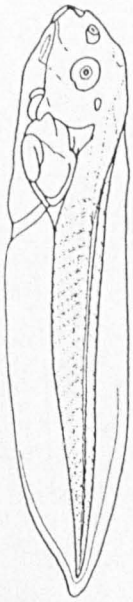


St. 42 Ventr.

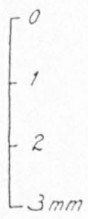


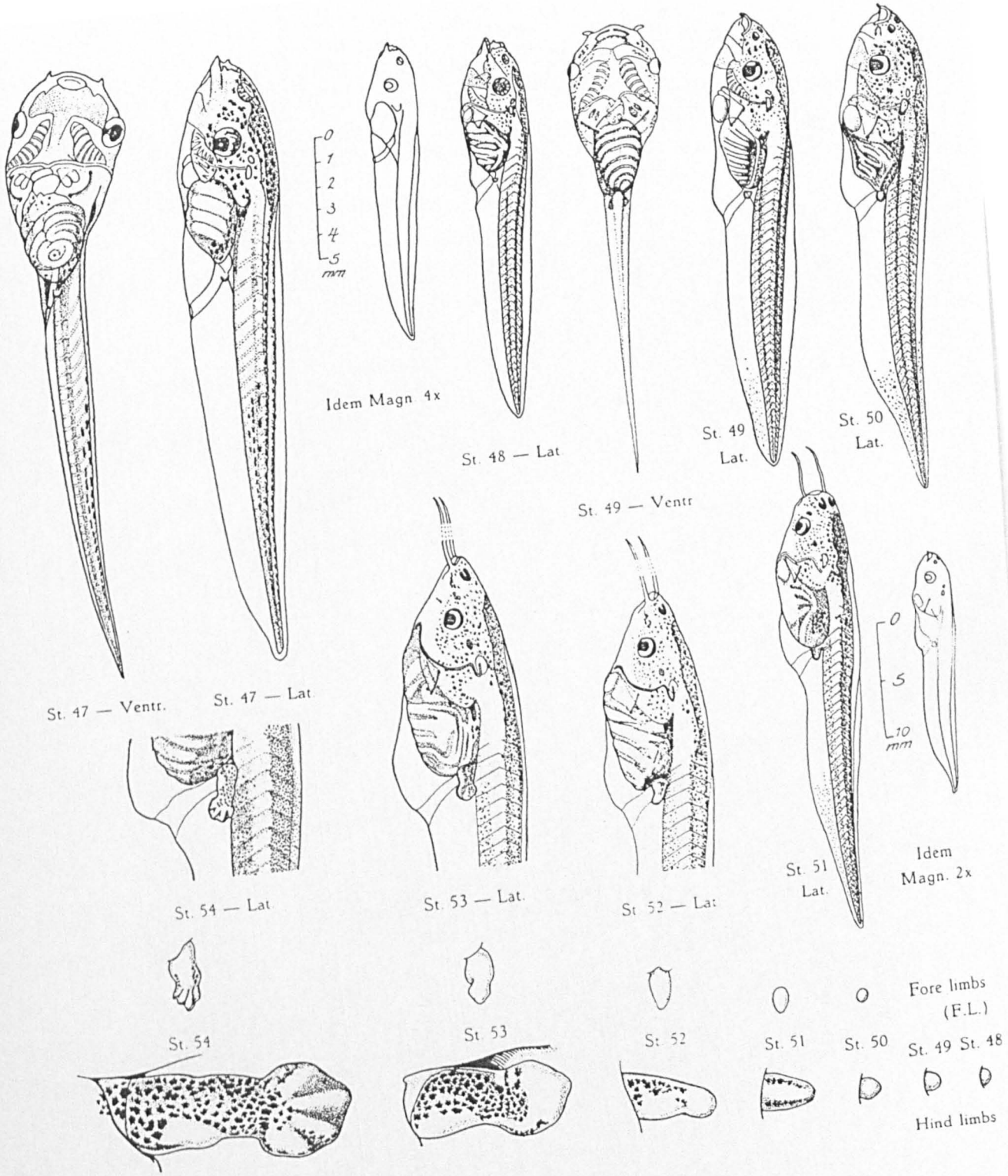
St. 46 — Lat.

St. 46 — Ventr.

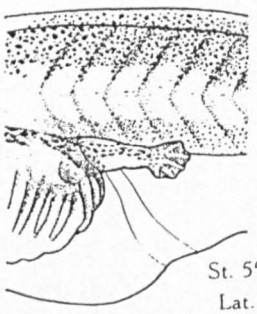


Idem Magn. 8x



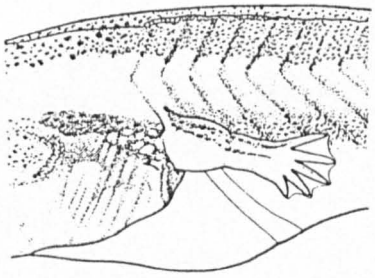

0
1
2
3
4
5
mm
Idem Magn. 4x
St. 47 — Ventr.
St. 47 — Lat.
St. 48 — Lat.
St. 49 — Ventr.
St. 49
Lat.
St. 50
Lat.
St. 54 — Lat.
St. 53 — Lat.
St. 52 — Lat.
St. 51
Lat.
0
5
10
mm
Idem
Magn. 2x
St. 54
St. 53
St. 52
St. 51
St. 50
St. 49
St. 48
Fore limbs
(F.L.)
Hind limbs

St. 55 — F.L.



St. 55
Lat.

St. 56 — F.L.



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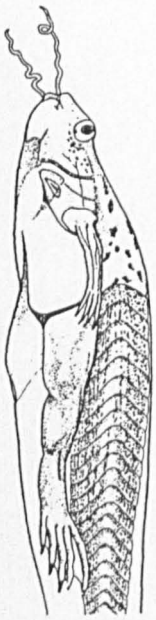
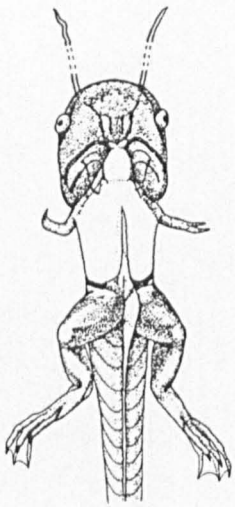


St. 60 — Lat.



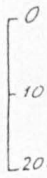
St. 59 — Ventr.

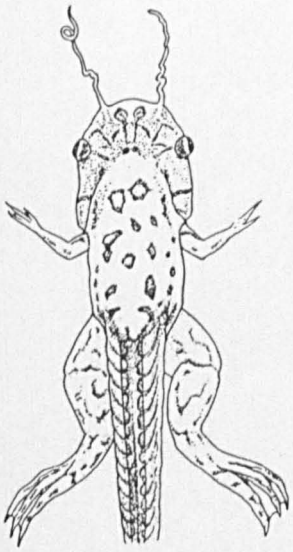
St. 59 — Lat.

St. 58 — Lat.

St. 57 — Lat.

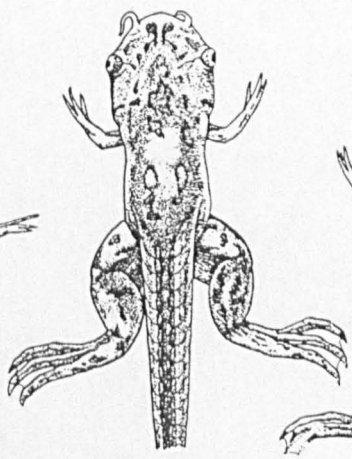
Idem Magn. 1x



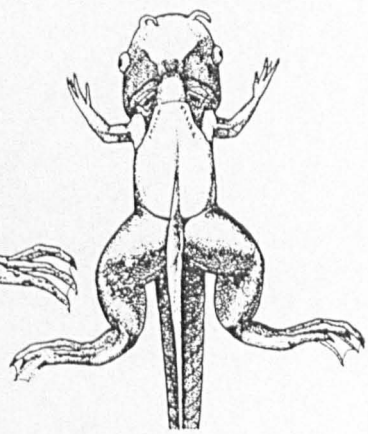



St. 60 — Dors.

St. 60 Ventr.
(Left operculum removed)



St. 61 — Dors.



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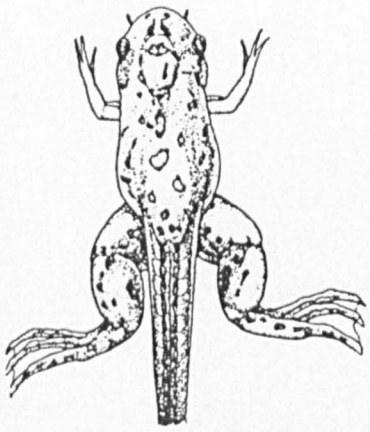
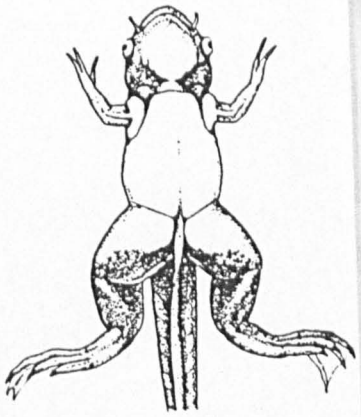


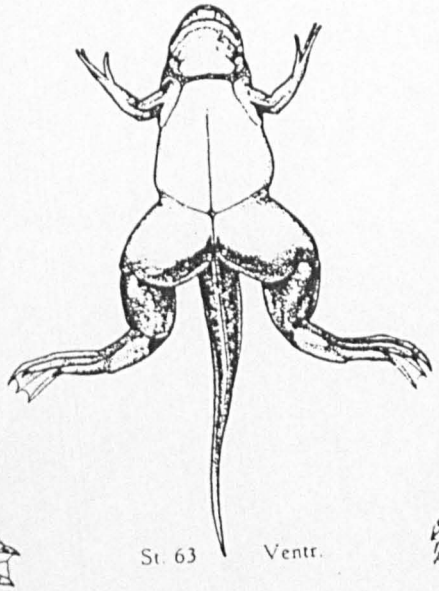
St. 62 — Dors.

St. 61 — Lat.

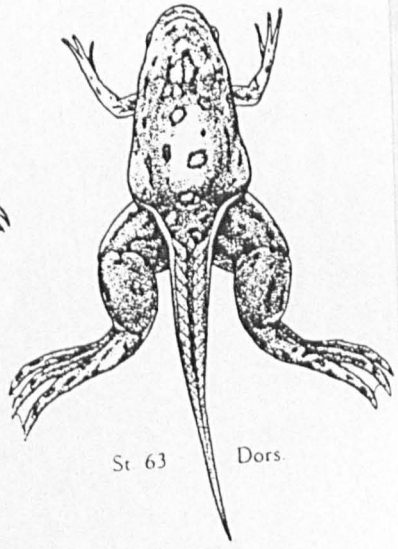
St. 62 — Lat.



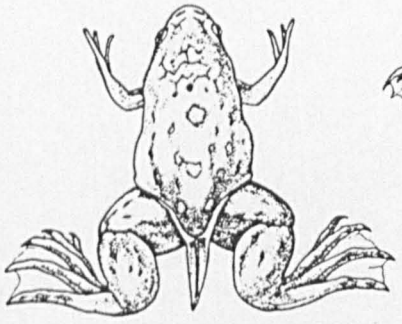
St. 62 — Ventr.



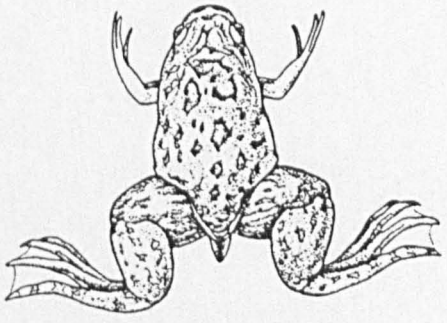
St. 63 Ventr.



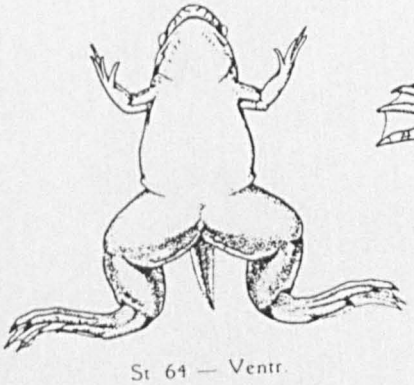
St. 63 Dors.



St. 64 — Dors.



St. 65 — Dors.



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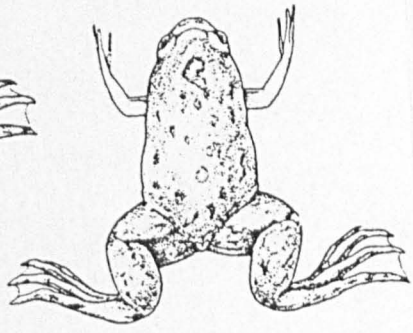


St. 66 — Dors.

## Appendix B

Partial sequences of cDNAs derived from the differential display of *Xenopus laevis* tadpole tail RNA.

## xL52

1 GAAATAGACC CTGCCTTGGT TGTGGCACTG TCCTCTCAAA TAGCCTGTCT

50 GCTCATGGTG TTTGTGGCTG TTTCTATGCC AACGTTGGCC AGCAACTCAT

101 GTGCGCAATA CAGTCCTGCA ATAGAAGGTC ATTGCAACAA CATACACTGT

151 TTGGCAAAGG CATCAACCAA ATTGGCGGCA GCACTATTTA CCATTCACAA

201 AGGGGCATTG AAGGATCGTC TGAAAGAATT CTTGGCGCTT GCATCCTCCA

251 GCCTACTAAA GATTGGCCAA GAGACTGATA AAACTACTAC AAG

## DDTA

1 GAAACCTTCC CCCTTCTTTT TTCGTACAAA AATGACTTGT CCTACGATAG

50 CAACACTGCA GACGGGTACT TTGTAAATCG CCTGTCCGTG AGGGGACTAA

101 TATTTTTCAC AACGAAACGG TCAATTAATT AATTAGATTG AAAGTATCCA

151 GCGGGATGGT TCACGAATCT ATCTCTGTTG TTATTCTGTA ACGTTTTTTA

201 GAGCTGTCTA TTCTCACGTA AAGCAAAAGT CTCTGTGTAT CGCGTGTTCC

251 AAGTGAGGAT TAAGCCTCTG CGTCGTCTAA TAACTCCTCT TGTCTGAAGA

301 AACATCACAT AAACGTCCGT TACGTGGGCC CACG

## DDTC3

1 TATATATAAA AAACAAACAA AAGTCGTAAC AAACACAGTT TGACTAGCGT

50 GGGTCAAGAC AGTCGAGAAA ATTGCGAAAA CGCTTAGACG TTAATGGACT

101 TCCATAAATA TTAACTGATG TTGTTTGAAA CGAAAACACG GAGGGACAGA

151 GACACCATTT GATGACCCAG AGAGAGTCAC TTCTAGCATC TCAAAGGAAA

201 ATCAAGATAA AGCCGAAATC TGAACTAACA CAG

## DDTD3

1 CTGTCGCCTG AGTCTCATTG TAATAATACT CCCTAATGAC CACTTAAATA

50 TATCTGGAAA GACTATTCCG AATTAATCAA AATTGGAAAG GAAGAGGAAA

101 CTTGTGTGGT ATACGACACG TAAAAAATAC GGGTAAAGGT TCGTTTAAAC

151 CTAACATTGT ACTGTCGAGA CGTTATCTCC CAACGACCAA GAAATTTTCC

201 GTCTTTATTT TGTGACCACT

## DDTE

1 TCTGAAGCTC CCCCGTATTT AACTCGTAGT ACCGTGTTCC TCCTTGTTTC

50 TGATACCTAG TTCACTGTCC CGACTGACCC CCGTTTATTT GTCCCCACTT

101 ACCCACAAGA CACATTTTCA GTCGTACTCA ATAGACACTT TTAGCAAATT

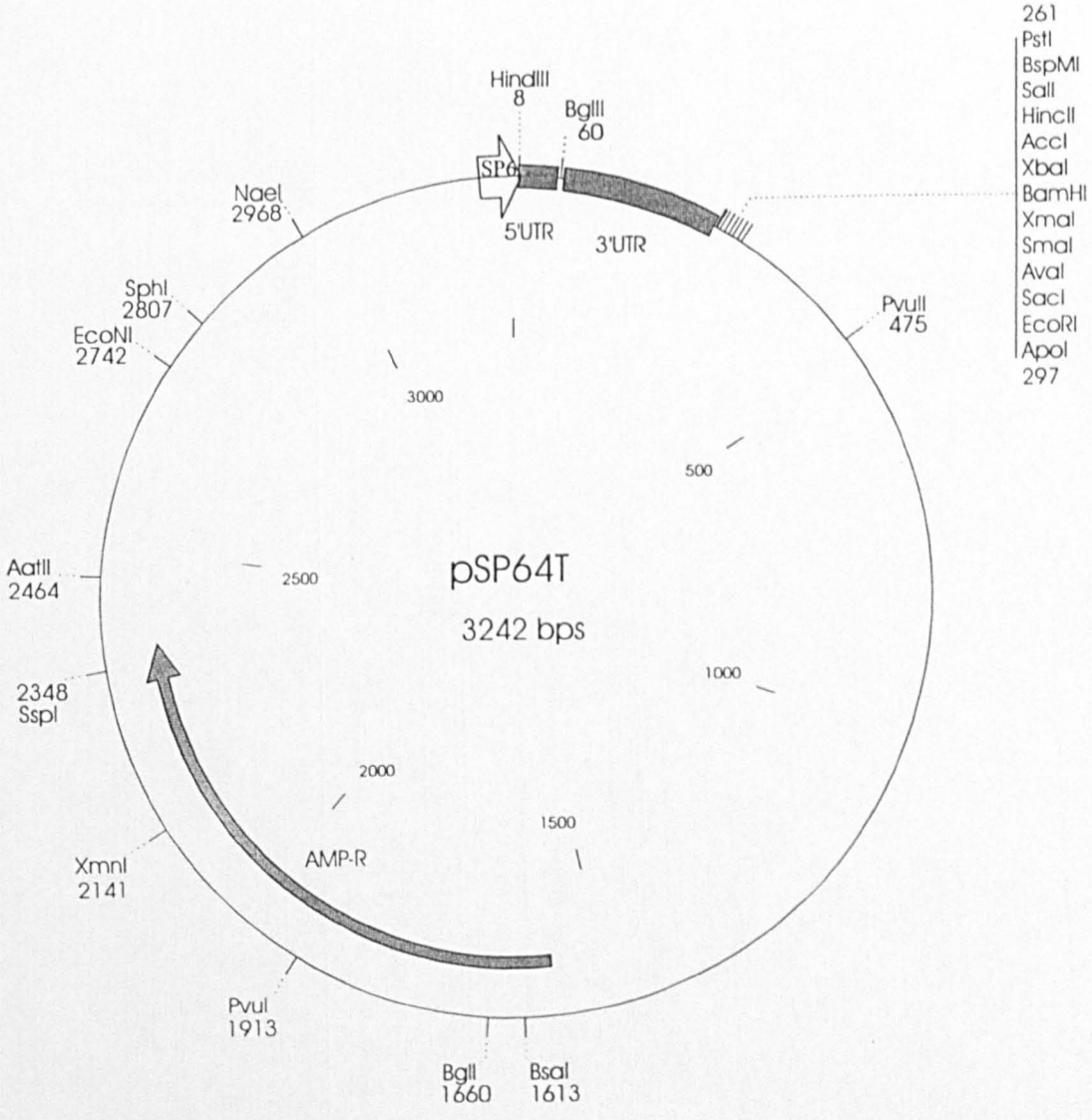
151 CATGACAAAG ACCTCTACAT TTATACTTTT ATATATGACT CGAGTTACTG

201 AGCATAGGAG TCCACAC

## Appendix C

## Plasmid vector pSP64T

pSP64T vector for synthesis of mRNA used for microinjection into *Xenopus* oocytes and embryos was proposed by Krieg and Melton (1984). The vector contains inserts of 3′ and 5′ untranslated regions (UTR) of *Xenopus* β-globin mRNA, and a *BgIII* site for cloning the inserts to be expressed. The SP6 promoter is used to transcribe the insert flanked by the globin sequences.


pSP64T
3242 bps
HindIII
8
BglII
60
SP6
5'UTR
3'UTR
261
PstI
BspMI
SalI
HincII
AccI
XbaI
BamHI
XmaI
SmaI
AvaI
SacI
EcoRI
ApoI
297
PvuII
475
NaeI
2968
SphI
2807
EcoNI
2742
AatII
2464
2348
SspI
XmnI
2141
PvuI
1913
BglI
1660
BsaI
1613
AMP-R
500
1000
1500
2000
2500
3000

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