Genomes & Developmental Control

Gene expression changes at metamorphosis induced by thyroid hormone in *Xenopus laevis* tadpoles

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Abstract

Thyroid hormone (TH) controlled gene expression profiles have been studied in the tail, hind limb and brain tissues during TH-induced and spontaneous *Xenopus laevis* metamorphosis. Amplified cRNA probes mixed with a universal standard were hybridized to a set of 21,807-sense strand 60-mer oligonucleotides on each slide representing the entries in *X. laevis* UniGene Build 48. Most of the up-regulated genes in hind limb and brain are the same. This reflects in part the fact that the initial response to TH induction in both tissues is cell proliferation. A large number of up-regulated genes in the limb and brain programs encode common components of the cell cycle, DNA and RNA metabolism, transcription and translation. Notch is one of the few genes that is differentially expressed exclusively in the brain in the first 48 h of TH induction studied in these experiments. The TH-induced gene expression changes in the tail are different from the limb and brain programs. Distinct muscle and fibroblast programs were identified in the tail. Dying muscle fibers in tail (marked by active caspase-3) up-regulate a group of genes that include proteolytic enzymes. At the climax of metamorphosis, tail muscle down-regulates more than half of the genes that encode the glycolytic enzymes in the cytoplasm and the tricarboxylic acid pathway and all five complexes of the electron transport system in mitochondria. These changes in gene expression precede the activation of caspase-3. Some of these same energy metabolism-related genes are up-regulated in the limb and brain programs by TH. A prominent feature of the tail fibroblasts is the down-regulation of several collagen and other extra cellular matrix genes and the up-regulation of hydrolytic enzymes that are responsible for dissolving the notochord and resorbing the tail.
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Introduction

During amphibian metamorphosis, thyroid hormone (TH) controls developmental changes that range from complete organ growth such as limb development to cell death in the gills and tail (Dodd and Dodd, 1976). Many tadpole organs are induced by TH to remodel including the intestine (McAvoy, 1977), pancreas (Dodd and Dodd, 1976), liver (Cohen, 1970), and brain (Kollros, 1981). TH functions by binding to thyroid hormone receptors that belong to the nuclear receptor family (Sap et al., 1986; Weinberger et al.,

1986). These receptors function as transcription factors. Therefore, changes in gene expression are presumed to be at the heart of the remarkable developmental changes that occur during amphibian metamorphosis. The control of so many diverse developmental programs by a single small molecule makes it a tractable developmental system to study using molecular tools. We have shown previously that the thyroid receptors are essential for many if not all of these developmental programs (Schreiber et al., 2001).

More than 10 years ago, we analyzed TH-induced gene expression changes in tail (Brown et al., 1996; Wang and Brown, 1993), limb (Buckbinder and Brown, 1992), intestine (Shi and Brown, 1993) and cultured cells (Kanamori and Brown, 1993) by a subtractive hybridization method (Wang

and Brown, 1991). In this paper, we have examined the TH-induced global gene expression changes in three different developmental programs (tail, hind limb and brain) using oligonucleotide microarrays designed to contain representative sequences from all of the *X. laevis* clusters (Build 48) in the NCBI UniGene database. This global approach has identified many new TH-regulated genes. Clustering of functionally related genes that are differentially expressed reveal insights into the biological changes induced by TH at metamorphosis.

Materials and methods

Design of oligonucleotide microarray using the UniGene database for Xenopus laevis cDNA sequences

Agilent Technologies (Palo Alto, CA) prepared the micro array slides using sequences from *X. laevis* UniGene Build 48 (February 2004). Each slide has a capacity for 22,543 oligonucleotides and included 21807 entries representing 21654 UniGene clusters. Each oligonucleotide is in the sense direction of the mRNA and 60 nucleotides in length. We added a second oligonucleotide for a set of 153 genes to serve as internal control. The duplicated genes include all of the up- and down-regulated genes that had been identified in the TH-induced tail subtractive hybridization studies (Brown et al., 1996), cell cycle-related genes, and genes involved in major signal transduction pathways. UniGene has many duplicate entries because the *X. laevis* genome is pseudotetraploid. In addition, Agilent includes 736 oligonucleotides on each slide as positive and negative hybridization controls. These *X. laevis* arrays are available from Agilent Technologies. The original design which was used in these experiments has the reference number

AMADID #012454. The 5445 oligonucleotides that hybridized with intensity below 2.3 in these experiments have been changed in a second version of *X. laevis* arrays (AMADID #013214).

RNA sample collection, probe preparation, and in situ hybridization

NF54 pre-metamorphic tadpoles (Nieuwkoop and Faber, 1956) were treated in their rearing water with 100 nM T3 for 24 h and 48 h. Tail, hind limb and brain tissues were isolated (Fig. 1A). The tadpoles collected for brain and hind limb but not for the tail samples had been pretreated in 1 mM methimazole for 1 week to reduce the endogenous levels of TH so that the response to TH is solely from the externally added hormone (Cai and Brown, 2004). An additional TH-induced early time point (14 h) was analyzed for the limbs to identify possible direct response genes. Tails at the climax of spontaneous metamorphosis (NF62) were also collected. Total RNA was isolated from the dissected tissue samples using the TRIZOL (Invitrogen) method according to manufacturer's protocol. Three separate groups of animals were treated identically with TH, and tissue samples were taken for each time point. Six tadpoles were sacrificed for each tail sample and 12 tadpoles for each of the limb and brain samples. The brain and limb samples were collected from the same tadpoles. cDNA was prepared from the total RNA of each sample. Then, cRNA labeled with Cy3 CTP (Perkin Elmer Cat #NEL 580) was prepared using a linear amplification and labeling method (Agilent Kit #5184-3523). This experimental cRNA probe was mixed with Cy5 CTP (Perkin Elmer, Cat #NEL 581) labeled universal standard cRNA. The standard cRNA probe was prepared from total RNA that had been isolated from whole tadpoles at NF stages 50, 52, 54, 56, 58, 60, 62, 64, 66 and juvenile frogs and then combined in equal amounts. We prepared enough universal standard RNA so that it can be used for future metamorphosis array experiments permitting a comparison of data from different time-series and tissues.

In situ hybridization on tissue sections used DIG-labeled RNA probes (Cai and Brown, 2004).

Results

Statistical analysis, filtering and GO mapping

Data from all of the replicates were subjected to correlation matrix analysis, and replicates with a correlation coefficient less than 0.95 were disregarded in further analysis (we removed one sample each from Tail 24 h T3 treatment group, tail NF62 group and one sample from brain 48 h T3 treatment group). We also disregarded the data from 5448 spots that hybridized with mean log-intensity values of less than 2.3 for the Cy5 labeled universal standard cRNA. Differential expression differences in pairwise comparisons used a False Discovery Rate (FDR) method. Gene expression changes are considered statistically significant when their FDR is <5% using an ANOVA-FDR test (Benjamini and Hochberg, 1995; Sharov et al., 2005) (http://lgsun.grc.nia.nih.gov/ANOVA/). All data have been submitted to GEO (NCBI) database (GEO Accession for tail series GSE3405, for limb series GSE3404 and brain series GSE3402). The ANOVA output including the lists of pairwise comparisons of treatment groups, hierarchical clustering, Principal component analyses within each tissue can be accessed at these addresses: Tail data: http://lgsun.grc.nia.nih.gov/ANOVA/output/DBrown-TailArray.html; limb data: http://lgsun.grc.nia.nih.gov/ANOVA/output/DBrown-LimbArray.html; brain data: http://lgsun.grc.nia.nih.gov/ANOVA/output/DBrown-BrainArray.html.
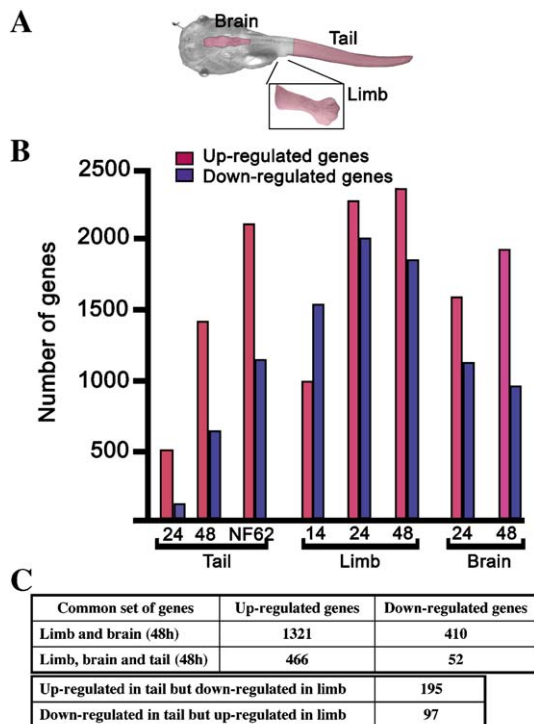

Fig. 1. Overview of the three programs. (A) NF53 tadpole showing the organs in red that was selected to make probes for hybridization. (B) Graph showing the number of up- and down-regulated genes that are differentially expressed at each time point. (C) Tabular comparison of shared differentially expressed genes.

Common set of genes	Up-regulated genes	Down-regulated genes
Limb and brain (48h)	1321	410
Limb, brain and tail (48h)	466	52
Up-regulated in tail but down-regulated in limb		195
Down-regulated in tail but up-regulated in limb		97

Table 1
Most differentially regulated genes in the tail program

GenBank accession number	Gene name	Fold change		Reference
		48 h	NF 62	
(A) Up-regulated genes				
U41856	fibroblast activation protein-alpha	71.7	93.7	*
U37376	gene B MAM domain	62.1	109.8	*
L49412	MMP-13 (collagenase 3)	53.6	104.4	*
U41856	fibroblast activation protein-alpha	51.6	78.6	*
U41858	CRF binding protein	51.6	48.4	*
U41824	MMP-13 (collagenase 3)	51.4	63.2	*
U37376	gene B MAM domain	40.2	61.1	*
BC043797	gap junction channel protein-beta 6	34.3	77.1	
U41855	gene 12-b (no open reading frame gene)	29.7	14.2	*
U41858	CRF binding protein	26.3	35.3	*
U41854	gene 12-3 (no open reading frame gene)	19.6	9.2	*
AF513854	RAS-like GTP-binding protein	16.7	25.2	
U41824	MMP-13 (collagenase 3)	16.4	22.8	*
U41855	gene 12-b (no open reading frame gene)	15.5	8.3	*
BC043635	arginase type I	12.8	5.7	Xu et al. (1993)
CB201454	Ring finger protein	12.6	2.2	
BC046378	hyaluronoglucosaminidase 2	11.8	7.9	
BC059296	sox 4	11.7	6.5	
BC057739	RUNX-1	10.9	13.5	
BC042245	glycine dehydrogenase	10.2	10.2	
BC045103	solute carrier family 43	10.1	5.5	
BC054153	glutamine synthase	9.9	7.1	Veldhoen et al. (2002)
BC054233	MMP-13 (collagenase 3)	9.4	9.8	*
U37377	alpha-aspartyl dipeptidase (gene D)	7.4	5.9	*
BC054947	MMP-2 (collagenase 4)	7.3	17.9	Jung et al. (2002)
CF271248	galectin 1	7.2	18.1	
U08407	arginase type II	6.9	1.9	Patterton and Shi, 1994
AB037269	biglycan	6.9	12.9	
U47622	FGF 9 (glia-activating factor)	6.7	4.3	
L28111	iodothyronine deiodinase, type III	6.7	NS	*
AB107220	C/EBP delta-1	6.3	4.3	
L28111	iodothyronine deiodinase type III	6.2	NS	*
BC057736	protein with phospholipase domain	6.1	5.8	
Y08932	dipeptidylpeptidase 4	6.1	13.3	
BC043760	alkaline phosphatase	6.0	5.2	
U37377	alpha-aspartyl dipeptidase (gene D)	5.7	6.9	*
(B) Down-regulated genes				
U41839	gene 18 (skin specific)	0.05	NS	*
U41839	gene 18 (skin specific)	0.06	NS	*
U41861	gene 19 (skin specific)	0.06	NS	*
U41860	gene 17 (skin specific)	0.09	0.4	*
U41861	gene 19 (skin specific)	0.09	NS	*
U41860	gene 17 (skin specific)	0.09	0.5	*
AW768217	lectin	0.1	NS	
U46576	gene 20 (skin specific)	0.13	0.42	*

Annotation of Xenopus Unigene clusters and functional clustering using Gene Ontology mapping

Annotation of most of the *X. laevis* UniGene clusters relies on their homology with known human genes identified in the Homologene database and tBLASTx searches done using the Human RefSeq database (Maglott et al., 2000). Of the 16359 spots having 2.3 or greater log-intensity value for the Universal standard hybridization, 8447 spots on the array have a human homolog (represented by 8380 UniGene clusters, since we included more than one oligonucleotide for some clusters). These genes have 6429 unique human homologs of which 5469 have ontology terms associated with them in the Gene Ontology (GO) database (Ashburner et al., 2000) according to the GOMiner (Bussey et al., 2003; Zeeberg et al., 2003) tools. These human RefSeq homologs helped us to use "GO-Mapping" and other annotation tools for *X. laevis* that has very few genomic tools available. We have prepared 14 supplementary tables that list the differentially expressed genes in selected GO categories. A complete list of all of the GO categories that are statistically significant to a probability of 0.05 or less can be found on our web site (http://www.ciwemb.edu/brownlab). Full data sets for each tissue and other supplementary data is also available at this website.

General features of the three programs

The number of statistically significant differentially expressed genes (using pairwise comparison to corresponding untreated NF54 tissue at ≤0.05 FDR) in the tail, hind limb and brain programs are presented in Fig. 1. The kinetic response of a gene is a clue to its potential significance. Previous experiments found that TH needs to be present for 48 h in order to induce a visible morphological change (Wang and Brown, 1993). Direct response genes of TH have a lag of several hours after the addition of TH and then reach a maximal induction within the first 24 h (Wang and Brown, 1993). Most of the genes that are up- or down-regulated in 48 h TH-treated tails are similarly but more dramatically regulated in the NF62 tail (Table 1). Therefore, NF62 represents a later phase of the TH-induced tail resorption program than 48 h TH treatment. By NF62, the climax of metamorphosis, endogenous TH has been elevated for at least 1 week.

In Fig. 1C, we have compared the regulated genes that are shared between programs. The limb and brain programs share many of the same regulated genes. Surprisingly, as many genes are differentially expressed at 24 h in the brain and limb as there are at 48 h. Previous experiments (Buckbinder and Brown, 1992) have shown that terminally differentiated genes such as

Notes to Table 1:
*Genes found in the subtractive hybridization screen (Wang and Brown, 1993; Brown et al., 1996). Entries that have no reference have not been described previously to be regulated by TH. Fold change values are in comparison to untreated NF 54 tail.
NS means Fold change value is not statistically significant (FDR > 0.05).

myosin and keratin require several days of TH to be induced in the limb. Although there are many genes regulated in all three programs, the tail (death) program is very different from the limb and brain programs (compare Tables 1 and 4). In fact, we will point out below some genes and even groups of functional genes that have opposite regulation in the tail and limb.

The tail program

Analysis of the tail program serves as an excellent control for the quality of the data. Table 1 lists in descending order the most differentially expressed up- and down-regulated genes in the 48 h TH-treated tail found by arrays. Twelve of the top 14 genes up-regulated after 48 h in TH had been identified previously in the subtractive hybridization screen using the same conditions and similar developmental stages (Wang and Brown, 1993). Since *X. laevis* is pseudotetraploid there are two copies of many genes in the database. As mentioned before, we added additional 60-mers for the TH-regulated genes previously identified in the array design. All four MMP-13 (collagenase-3) oligonucleotides on the array hybridize with similar values. The 17 TH up-regulated genes found in the subtractive hybridization screen were up-regulated significantly in the array of 48 h TH-induced RNA. In addition to the TH-induced tail program at NF54 we included an analysis of tails at the climax of spontaneous metamorphosis (NF62) when the endogenous TH concentration is highest (Leloup and Buscaglia, 1977), and TH-responsive genes are expressed at their maximum levels (Wang and Brown, 1993). The tails at this stage have not shortened, and the muscle appears normal histologically. Tails analyzed at the climax of spontaneous metamorphosis (NF62) have the same genes at the top of the differentially expressed list of up-regulated genes including two newly identified genes (a connexin (BC043797) and a ras-related gene (AF513854) (Table 1)). Of the top 50 up-regulated genes after 48 h of TH all but three are also up-regulated spontaneously at NF62. Two of the 3 oligonucleotides with different profiles encode iodothyronine deiodinase type III known to be a direct target of TH but down-regulated in the tail at metamorphic climax (Wang and Brown, 1993). All but one of the 117 most differentially up-regulated genes in the tail after 24 h of TH treatment are also up-regulated at 48 h. 100 of these same genes are also up-regulated at NF62. Likewise seven out of eight of the most down-regulated genes at 48 h in the array (Table 1B) were those that had been found by subtractive hybridization. Three of the four larval-specific skin genes are not down-regulated significantly in the tail at NF62 a paradox that was noted by Veldhoen et al. (2002). Whatever the explanation by the end of metamorphosis their mRNA is not detectable in any region of the froglet skin. The genes that are most down-regulated at NF62 are very different from those down-regulated by 48 h of TH-induction. Only a small subset of the genes that are differentially up-regulated in the tail are also up-regulated in the brain or the hind limb.

Some of the most prominent non-random functional clusters of regulated genes in the tail program are listed in Table 2. A complete list of these functional clusters can be

Table 2
Gene ontology categories enriched significantly in tail up- and down-regulated programs

		Total genes	48 h up	NF 62 up	48 h down	NF 62 down
Number of unique GO annotated genes		5580	744	923	330	570
GO Function	GO ID					
Biological process						
Signal transduction	0007165	916	155 [a]	188 [a]	32	53
TGFβ pathway	0007179	15	6 [a]	5 [b]	0	0
Insulin receptor signaling	0008286	7	5 [a]	2	0	0
GTPase mediated signaling	0007264	115	26 [a]	29 [a]	5	6
Rho mediated signaling	0007266	13	5 [a]	6 [a]	3	1
Energy pathways	0006091	129	13	23	24 [a]	47 [a]
Glycolysis	0006096	28	1	3	6 [a]	14 [a]
TCA cycle	0006099	19	1	1	2	9 [a]
Cell Death	0008219	190	35 [a]	39 [b]	10	17
Molecular function						
Hydrolase activity	0016787	817	136 [a]	146 [b]	47	76
Peptidase activity	0008233	233	44 [a]	44 [b]	17	18
Cell component						
Proteasome	0000502	38	14 [a]	3	3	1
Mitochondrion	0005739	355	15	29	68 [a]	105 [a]
Mitochondrial electron transport chain	0005746	75	0	4	28 [a]	40 [a]
Muscle fiber	0030484	26	4	6	7 [a]	14 [a]
Myofibril	0030016	19	3	4	7 [a]	13 [a]
Lysosome	005764	53	13 [a]	21 [a]	6	3

[a] Calculated P values are smaller than 0.05 and so these GO categories are significantly enriched.
[b] Calculated P values are greater than 0.05 and so this GO categories are not significantly enriched, although the genes that are differentially expressed are very similar.

found at our web site. In situ hybridization of representative genes comprising a function shows that in most cases they are expressed together in a single tail cell type either in muscle or fibroblasts. Some of these functions will be discussed in the next sections.

The tail hydrolytic program

Previous experiments have shown that tail muscle and fibroblasts respond independently to TH (Das et al., 2002). Muscle death by apoptosis is cell autonomous and marked by activation of caspase-3, while the fibroblasts were identified as the source of multiple proteolytic enzymes that play a role in the dissolution of the tail (Berry et al., 1998). Most of the genes found by subtractive hybridization in the tail that are up-regulated in the second kinetic wave from 24 to 48 h after TH were expressed in fibroblasts.

These microarray experiments have identified genes encoding many different kinds of hydrolases including proteases that are up-regulated in the tail as an integral part of the resorption process. The TH-regulation of many of these genes has not been reported previously. Table 3 summarizes the genes that

Table 3
Selected hydrolases and extracellular matrix (ECM) genes that change significantly in the tail program

GenBank accession number	Gene name	Fold change		Expression profile
		NF 62	48 h	
(A) Hydrolases including proteases				
BC061653	aminoacylase 1	2.3	1.7	
BC043760	alkaline phosphatase	5.2	6.0	
BC043635	arginase I	5.7	12.8	
U08407	arginase II	1.9	6.9	
BX848880	arylsulfatase B	1.9	1.9	
BC056060	lysosomal ATPase accessory protein 2	3.4	2.1	
BC041732	lysosomal ATPase V0 subunit A1	2.2	1.6	
BC054258	lysosomal ATPase V0 subunit C	1.9	1.4	
CA792621	lysosomal ATPase V0 subunit 2	1.8		
BC046738	lysosomal ATPase V1 subunit B2	1.7		
BX849380	lysosomal ATPase V1 subunit F	2.0	1.9	
BX851010	complement component 1(r subcomponent)	3.4		
M94264	cdc 25C	3.3	3.6	
BU900523	tripeptidyl-peptidase I	2.1	0.7	
BC056069	nonspecific cytosolic dipeptidase	11.1	5.7	M
BC041315	carboxypeptidase A6	6.4	3.9	F
BC059995	carboxypeptidase N (polypeptide 1)	8.5	4.1	M
BC046667	cathepsin B	3.1	4.2	M
BC061685	cathepsin D (lysosomal aspartyl protease)	2.3	2.1	
BC056059	cathepsin S	2.3		
CB944759	cathepsin Z	2.0	2.2	
U26349	desert hedgehog (*Drosophila*)	2.4	1.7	
Y08932	dipeptidylpeptidase 4	13.3	6.1	
AJ320159	dual specificity phosphatase 1	10.7	4.3	
X69977	ERCC5 (XPGC-related factor)	1.9	1.9	
BC060330	coagulation factor X	2.7	1.6	
U41856	FAPα	93.8	71.6	F [a]
BC042266	alpha-L-fucosidase1	3.1	1.7	
BX846788	Galactosylceramidase		2.1	
BC042274	glucosamine (N-acetyl)-6-sulfatase	1.9		
BC046378	hyaluronoglucosaminidase 2	7.9	11.8	
BC056842	legumain	4.1	3.1	
Z27093	matrix metalloproteinase 11 (stromelysin 3)	7.2	5.5	F [a]
L49412	matrix metalloproteinase 13 (collagenase 3)	104.4	53.6	F [a]
BC054947	matrix metalloproteinase 2	18.0	7.3	
BC061659	matrix metalloproteinase 28	1.6	2.2	
BX849421	nardilysin (N-arginine dibasic convertase) [b]	0.6		
BC059352	platelet-activating factor (acetylhydrolase)	2.0	1.6	
CA983237	proprotein convertase (subtilisin/kexin type 5)	2.2	1.8	
BC054994	peptidase D	3.8	2.6	
BC056041	phospholipase A2	1.8	1.9	
BC061654	plasminogen activator	3.8	2.4	
L20816	phospholipase C beta 3 (phosphatidylinositol-specific)	5.1	3.3	
AF029404	Serine protease 8 (prostasin)	2.6		
L33099	protein tyrosine phosphatase non-receptor (type 9)	2.1	2.2	
U09135	protein tyrosine phosphatase receptor (type A)	1.6		

Table 3 (*continued*)

GenBank accession number	Gene name	Fold change		Expression profile
		NF 62	48 h	
(A) Hydrolases including proteases				
AF197945	protein tyrosine phosphatase, receptor (type F)	1.9		
AB038498	XMT-SP1 serine protease	1.6		
BC048222	Thrombospondin [b]	0.5		
CD324947	tissue inhibitor of metalloproteinase 2	2.0	2.6	
BC060022	ubiquitin specific protease 16	1.7	1.6	
BC042353	ubiquitin specific protease 4	1.6		
X54240	valosin-containing protein	1.3	2.2	
B ECM genes:				
CB198061	collagen, type XI, alpha 1	0.129	0.371	F
AB047066	collagen, type XVIII, alpha 1	0.576	0.545	F
M63596	collagen, type II, alpha 1	0.305	0.485	F
BC046861	collagen, type IX, alpha 1	0.047	0.155	F
BC045013	secreted protein, acidic, cysteine-rich (osteonectin)	0.6	0.6	
BC046711	spondin 1 (f-spondin) extracellular matrix protein	0.5		
BC043890	chondromodulin	0.1	0.2	F
BC054282	Lumican	0.6	0.5	

Blank spaces in fold change columns means that the fold change is not statistically significant (FDR > 0.05) for that condition.
"M" means expression in dying muscle (active caspase-3 positive) as shown by in situ hybridization (Fig. 3).
"F" means expression in fibroblasts as shown by in situ hybridization (Fig. 2).
[a] Berry et al. (1998).
[b] Up-regulated in limb and brain programs.

encode TH up-regulated genes for hydrolytic enzymes in the tail. The cell type of expression of several of them has been identified by in situ hybridization. The fibroblast specific proteases include multiple secreted MMPs, membrane bound serine proteases, and lysosomal cathepsins. Hyaluronidase (BC046378), a lysosomal enzyme, is dramatically up-regulated in fibroblasts (Fig. 2). TH up-regulates 40% of the genes encoding lysosomal enzymes that are present on the array (Suppl. Table S1). In situ hybridization localizes these lysosomal genes and other TH-induced up-regulated genes to tail fibroblasts. These include three genes previously identified in the subtractive hybridization screen (Berry et al., 1998) integrin alpha-1 (U44025), fibronectin (M77820), and an MAM domain containing protein (U37376). Three newly identified genes that are up-regulated in tail fibroblasts are biglycan (AB037269), calponin 2 (BC046257), and tropomyosin 3 (BC054220).

A major program that is down-regulated in tail fibroblasts is the synthesis of several kinds of collagen and extra cellular matrix proteins (Fig. 2; Table 3B). The most obvious TH-induced change in tail fibroblasts is from a synthetic to a hydrolytic profile.

The tail muscle program

Whereas most of the TH-induced up-regulated proteases are expressed in fibroblasts the NF62-tail array reveals genes that
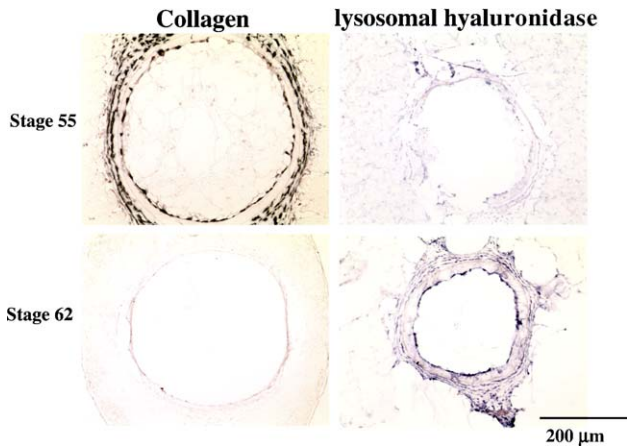
Fig. 2. In situ hybridization of tail cross sections with a collagen (BG553552) and a hyaluronidase (BQ735978) probe at NF55 and NF62. The notochord is surrounded and lined with fibroblasts.

are regulated in the muscle program including several proteases. One previously identified muscle-specific peptidase was called "gene D" (U37377). Proteolytic enzymes expressed in tail muscle include carboxypeptidase N small active subunit (BC059995), cytosolic dipeptidase (BC056069), and cathepsin B (BC046667) (Table 3). The expression profiles of all of the genes that are up-regulated by TH in tail muscle are identical to that shown by UCP2 in Fig. 3A. These genes are expressed first in peripheral muscle fibers that are the most advanced toward cell death. Their fibers are visibly disrupted and positive for active caspase-3 (Fig. 3B). As metamorphosis advances the expression of these genes continues to coincide with the activation of caspase-3 and the appearance of dying muscle fibers. Muscle death progresses from lateral to medial fibers. In addition to the three genes shown in Fig. 3 several other genes are up-regulated specifically in the same dying muscle fibers.

These include galectin-1 (AB056478), glycine dehydrogenase (BC042245), annexin A4 (BC060389), and annexin A2 (BC041306). We have not found any gene encoding a proteolytic enzyme that is expressed in both fibroblasts and muscle.

Previously, we had only identified genes that are down-regulated by TH in the apical cell layer of the tail epidermis (Furlow et al., 1997). These skin genes are down-regulated throughout the body including the limb and tail. Identification of down-regulated genes requires that the preexisting mRNA be degraded after the cessation of transcription of the gene. This lag in mRNA degradation explains why TH-induced down-regulation is more exaggerated in NF62 tails compared to 48 h of TH-induction. Some of the functional categories of genes that are down-regulated in the tail at metamorphic climax are summarized in Table 2. The array contains 335 nuclear encoded genes for proteins that localize in mitochondria (Suppl. Table S2). More than 20% of these genes are down-regulated in the tail at climax. Representatives from all 5 complexes of the electron transport system including five of the eight genes involved in ATP synthesis in the mitochondria are down-regulated (Fig. 4A). Of the 75 total genes encoding proteins in the electron transport chain 32 of the 40 genes that are down-regulated in the tail at NF62 are up-regulated in the limb after 48 h of TH-induction (Fig. 4B). Also down-regulated in the tail at NF62 are 9 of 17 genes encoding tricarboxylic acid cycle (TCA) enzymes (Suppl. Table S3). 8 TCA genes are up-regulated in the limb and 6 of these are amongst those that are down-regulated in the tail. Additionally, half of the 32 genes in the cytoplasm-localized glycolytic pathway are down-regulated in NF62 tails (Suppl. Table S4). In the hind limb the 4 glycolytic pathway genes that are up-regulated are down-regulated in the tail. A total of 13 of the genes involved in energy metabolism and down-regulated in NF62 tails have been tested by in situ
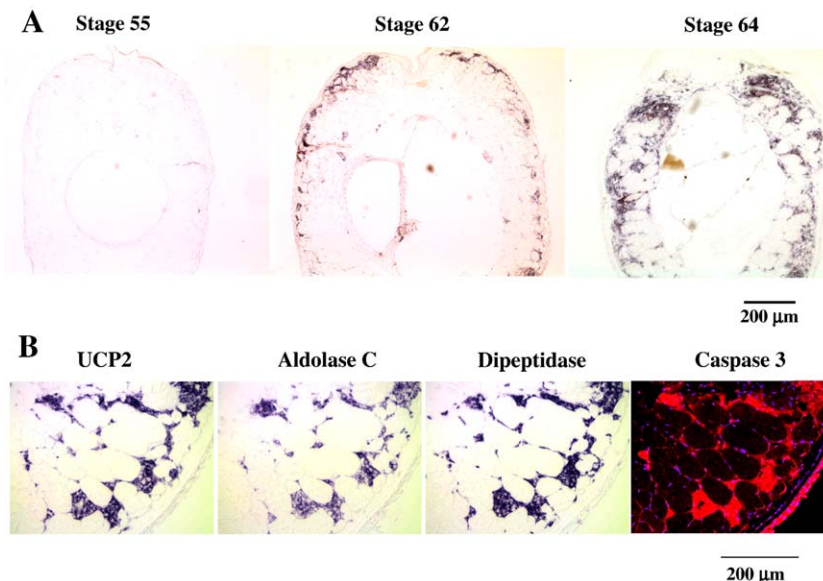

Fig. 3. In situ hybridization of tail cross sections using probes expressed in muscle. (A) Mitochondrial uncoupling protein-2 (UCP-2, BC044682) at 3 stages of development. (B) UCP-2, aldolase C (BC054264), and a dipeptidase (BC056069) are up-regulated at NF62 in dying muscle. They have been hybridized to adjacent tail sections at NF62. The section used for the dipeptidase was immunostained for active caspase-3.
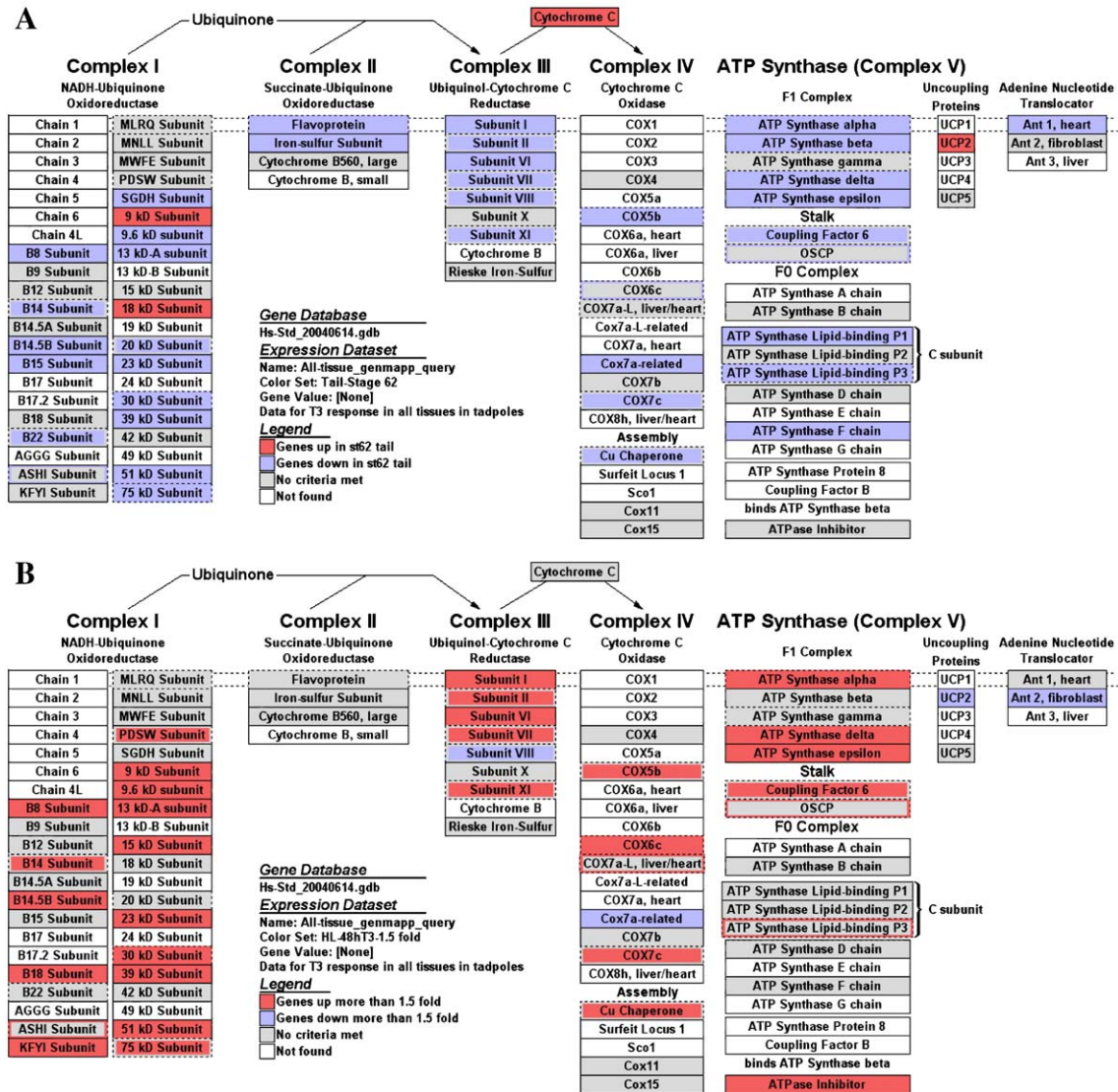
Fig. 4. Summary of mitochondrial electron transport genes in the (A) tail at NF62 and (B) hind limb after 48 h of TH. Significantly up-regulated genes are red; significantly down-regulated genes are blue. In each case, they are compared to NF54 control tail and limb. Genes that are present on the array but not significantly regulated are light yellow. Genes in open boxes are not present in the array.

hybridization. The expression of all of them is localized in tail muscle (Fig. 5). One third of the genes on the array that are in involved in muscle contraction are down-regulated by NF62 in the tail.

Differentially expressed genes in the hind limb and brain metamorphic programs

TH-induced gene expression profiles were carried out on hind limbs of NF54 tadpoles treated with 100 nM TH for 14, 24, 48 h and brains of the same animals induced for 24 and 48 h. As different as the brain and limb TH-induced programs will become, their first response is the same, an increase in cell proliferation. Cell proliferation in these tissues begins between 24 h and 48 h after the administration of exogenous TH (Cai and Brown, 2004). Then, hind limbs grow and within 3 to 4 days expression of terminally differentiated muscle and fibroblast

genes is detected (Buckbinder and Brown, 1992). Less is known about the fate of the newly replicated cells in the brain. However, the kinetics of TH-induced DNA replication in the brain is the same as that of the hind limb. Table 4 lists the most differentially up-regulated genes in descending order for the limb and next to each entry the result for the brain. The two programs are remarkably similar containing many cell cycle-related genes. Table 5 summarizes some functional clusters of limb and brain TH-induced genes. Most of the statistically significant differentially expressed genes in the hind limb program are also differentially expressed in the brain program. Out of 955 genes that are up-regulated in the brain all but 72 genes are also up-regulated in the limb. Of the 199 genes that are down-regulated in brain, all but 22 are also down-regulated in the hind limb after 48 h of TH treatment. Most of the cell cycle-related genes that are differentially expressed in the limb and brain in the first 48 h after TH are the same (Fig. 6). Genes involved in every step of
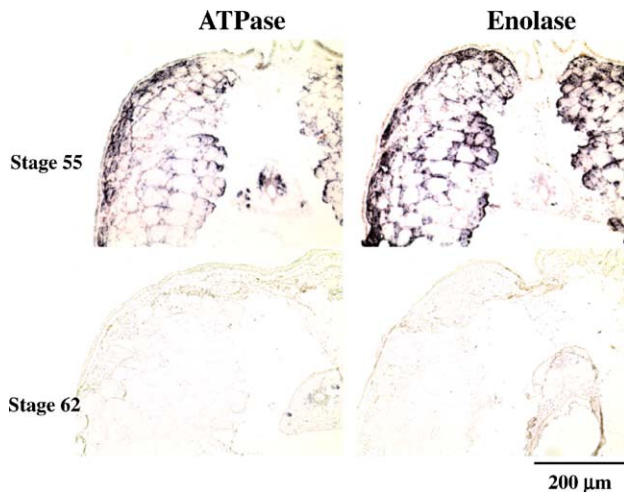
Fig. 5. In situ hybridization of NF55 and 62 tail cross sections using probes for two down-regulated genes. ATPase (BQ383639)is a mitochondrial gene; enolase (BQ736040) is a gene from the glycolysis pathway.

the cell cycle are TH-regulated (Suppl. Table S5). The most dramatically TH-induced genes are the members of the mini-chromosome maintenance complex (MCM). Five of the six MCM genes on the *X. laevis* array are up-regulated after 48 h of TH induction in the limb and the brain. In the limb TH induces all cell types to up-regulate the cell cycle genes (Brown et al., 2005) (Fig. 7). Induction in the brain is limited to the cells that line the ventricles, the same cells that are stimulated by TH to replicate (Cai and Brown, 2004).

Genes in functional categories related to cell growth such as RNA metabolism, and translation activity (Suppl. Table S6) are preferentially induced in the limb bud and to a lesser extent in the brain. The high representation of enriched gene products that reside in the nucleolus (Suppl. Table S7) are related to this increase in translation-related proteins. The genes that encode cysteinyl, tryptophanyl, tyrosyl and phenylalanyl tRNA aminoacylation are up-regulated. Half of the genes in the array that encode protein-folding proteins are up-regulated in the hind limb (Suppl. Table S8) including chaperone proteins. Many of the components that are involved in the ubiquitination and proteosome pathways are up-regulated especially in the limb program (Suppl. Table S9A and S9B).

Of the 72 genes that are up-regulated in brain but not in limb only five are increased in expression greater or equal to 2 fold over control NF54 brain (Suppl. Table S10). Two of these genes are the duplicate entries of Notch. This gene has already been shown to be expressed in *X. laevis* retina in the cells that undergo TH-induced replication (Marsh-Armstrong et al., 1999). TH induces cells that line the brain and spinal cord ventricles to replicate and express Notch. A transcription factor that is only up-regulated in the brain is the otx2 homeobox protein (AW200443).

Transcription regulation

One functional group of proteins that we have analyzed is transcription regulators looking for candidate genes that

are downstream from the thyroid receptors and will direct the specialized expression characteristic of one of these developmental programs. The array contains 728 genes that encode proteins that are involved in regulation in transcription of which 372 are differentially expressed at one or more time points of the three programs (Suppl. Table S11). Kinetic considerations help to narrow this list to genes of biological interest. Genes that are induced at early time points are more likely to be direct response genes down stream from the thyroid hormone receptors. There are 221 genes concerned with the regulation of transcription that are elevated at the earliest time point studied, which is the limb after 14 h of TH treatment. If a gene expression profile returns to normal levels by 24 and 48 h of induction, we disqualify it as an interesting candidate since the continuous presence of TH for 48 h is needed to induce lasting morphological changes (Wang and Brown, 1993). In Table 6, we have selected 36 differentially regulated genes that regulate transcription. These genes were chosen either because they are substantially induced by TH in one or more organ or they are known to play a role in one of the predominant functions that play a role in metamorphosis (i.e., cell cycle, cell death). This list includes proteins that play a role in the regulation of transcription and are also involved in DNA replication (CDK2, FUBP1, HMGB3, 5 members of the MCM complex, MXD4, and cMYC). Amongst the genes implicated in apoptosis are DATF1, and SOX4. Two transcription factors that are expressed in muscle that are up-regulated in the tail program are RUNX and TRMM55 while MyoD is up-regulated in the limb muscle. Several genes that affect chromatin modification are regulated by TH. DNA methylase (DNMT1) is up-regulated in the limb and brain but not in the tail. Induction with added TH has a 2 to 4 h lag before any new mRNA has been detected indicating remodeling of chromatin occurs prior to a change in gene expression. Some of the highest regulated genes whose product are involved in chromatin modification are BAF53, BAZ1B, CHD4, DNMT1, HDAC1 and 2. Co-repressors and co-activators that are differentially expressed include ASCC2, EZH2, HES1, NCoR1, NCoR2, TRIP3. There are several genes that have opposite expression patterns in the growth (limb) compared to the death (tail) programs. Any one of these genes might control the opposite expression patterns in these two programs that has been described above. These include CEBPD, DNMT1, HBP1, HDAC9, HES1, MYOD1, TRMM55, and ZFP36.

Discussion

A dramatic biological problem associated with amphibian metamorphosis that has interested biologists since the discovery of the role of TH in 1914 is how one simple hormone can control so many different developmental programs. The ultimate fate of different tadpole tissues and organs, each composed of multiple cell types, is determined during embryogenesis. TH has never been implicated in the determination of a cell type only in its differentiation. The

Table 4
Most differentially up-regulated genes in the limb and brain programs after 48 h of TH treatment

GenBank accession number	Gene name	Fold change		Reference
		Limb	Brain	
Z85983	nucleophosmin/nucleoplasmin (chaperone)	20.7	7	
BF611398	UDP-glucose ceramide glucosyltransferase	19.9	4.5	
BC041200	heat shock 70 kDa protein	18.3	5.4	Helbing et al. (2003)
U62807	heat shock 70 kDa protein	17	3.5	
BC060456	transforming growth factor beta regulator 4	16	8.3	
U51234	MCM7	13.9	9.4	
BC043837	hypoxia up-regulated gene	13.9	5.7	
L28111	iodothyronine deiodinase type III	13.1	6.8	Wang and Brown (1993)
BC041192	heat shock 60 kDa protein (chaperonin)	12.9	3.5	Buckbinder and Brown (1992)
BC059298	ribosome binding protein	12.8	2	
AF143494	t-complex protein	12.6	3.5	
BX849421	nardilysin (N-arginine dibasic convertase)	12.4	3.4	
BC044001	tubulin alpha-2	12.4	9	
BC063726	fatty acid desaturase-2	12.2	3.5	
AW200067	protein serine racemase	12	4.2	
BC044018	peptidylprolyl isomerase D (cyclophilin D)	11.2	5.2	
BC044073	myo-inositol1-phosphate synthaseA1	11	2.9	
BC060415	3′-phosphoadenosine 5′-phosphosulfate synthase	11	2.1	
AF351126	enhancer of zeste	10.9	5.5	
BC049389	lysophosphatidic acid G-protein-coupled receptor	10.8	2.2	
BC044051	MCM3	10.4	3.9	Buckbinder and Brown (1992)
BU913725	mitochondrial DNA specific single-stranded DNA binding protein	10.4	2.1	
U44048	MCM5	10.2	12.6	
BC044673	t-complex protein	10.2	3.7	
U41855	gene 12-b (No ORF gene)	10.2	15.8	Wang and Brown (1993)
BC046867	protein disulfide isomerase-related protein	10.1	2.3	
Z14253	eukaryotic translation termination factor 1	10.1	4.6	
U66710	MCM7	10.1	4.6	
BQ399739	unknown EST	10.1	2.7	
U51234	MCM7	9.9	4.3	
BX848752	Hypothetical protein with Sas10/Utp3 domain (regulator of chromatin silencing)	9.8	3	
AB085173	HMT1 hnRNP methyltransferase-2	9.6	3.2	
BC041223	nucleolar protein NOP5/NOP58	9.4	5.4	
U44047	MCM2	9.2	4.4	
X88927	nucleolar and coiled-body phosphoprotein	9.2	3.9	
U44048	MCM5	9.1	4.9	

Entries that have no reference have not been previously shown to be regulated by TH. Fold change values are in comparison to corresponding methimazole treated NF 54 control tissues.

discovery in 1986 that TH functions by binding to a nuclear receptor that functions as a transcription factor (Weinberger et al., 1986) focused TH research on changes in gene expression as an explanation for hormone driven changes. Although some TH controlled genes had been identified by subtractive hybridization (Wang and Brown, 1991), functional studies of genes in metamorphosis did not begin until the development of transgenesis in 1997 (Kroll and Amaya, 1996). Transgenic experiments confirmed that the diverse programs of metamorphosis all begin with the same thyroid receptors

Table 5
Gene ontology categories enriched significantly in limb and brain up-regulated programs

		Total genes	Limb 48 h up	Brain 48 h up
Number of unique GO annotated genes		5580	1254	816
GO Term	GO ID			
Biological process				
Nucleic acid metabolism	0006139	1392	355 [a]	302 [a]
mRNA metabolism	0016071	124	50 [a]	45 [a]
rRNA metabolism	0016072	24	11 [a]	9 [a]
tRNA metabolism	0006399	42	16 [a]	14 [a]
DNA repair	0006281	111	42 [a]	33 [a]
DNA recombination	0006310	36	18 [a]	10 [a]
Translation	0043037	102	31 [a]	32 [a]
Protein folding	0006457	123	58 [a]	45 [a]
Ubiquitin-dependent protein catabolism	0006511	71	20 [a]	21 [a]
Intracellular transport	0046907	275	90 [a]	71 [a]
Cell cycle	0007049	385	147 [a]	127 [a]
DNA replication and chromosome cycle	0000067	8	5 [a]	4 [a]
M phase	0000279	138	78 [a]	60 [a]
Mitotic checkpoint	0007093	11	6 [a]	6 [a]
Mitotic spindle assembly	0007052	9	6 [a]	3 [b]
G1/S transition of mitotic cell cycle	0000082	36	20 [a]	18 [a]
Regulation of cyclin dependent protein kinase activity	0000079	24	13 [a]	10 [a]
G2/M transition of mitotic cell cycle	0000086	28	15 [a]	11 [a]
G1 phase of mitotic cell cycle	0000080	8	5 [a]	4 [a]
Traversing start control point of mitotic cell cycle	0007089	6	4 [a]	3 [a]
Cellular component				
Endoplasmic reticulum	0005783	242	86 [a]	71 [a]
Nucleolus	0005730	55	24 [a]	20 [a]
Endomembrane system	0012505	111	47 [a]	36 [a]
Nuclear envelope-endoplasmic reticulum network	0042175	44	16 [a]	13 [a]
Nuclear membrane	0005635	46	23 [a]	20 [a]
Golgi membrane	0000139	24	11 [a]	5
Endoplasmic reticulum	0005783	242	86 [a]	71 [a]
Proteasome complex	0000502	37	19 [a]	15 [a]
Mitochondrion	0005739	369	116 [a]	103 [a]
Mitochondrial electron transport chain	0005746	75	32 [a]	24 [a]
Chromosome	0005694	114	51 [a]	49 [a]
Kinetochore	0000776	14	11 [a]	9 [a]
Replisome	0030894	15	7 [a]	8 [a]

[a] Calculated P values are smaller than 0.05 and so these GO categories are significantly enriched.
[b] Calculated P values are greater than 0.05 and so this GO categories are not significantly enriched, although the genes that are differentially expressed are very similar.
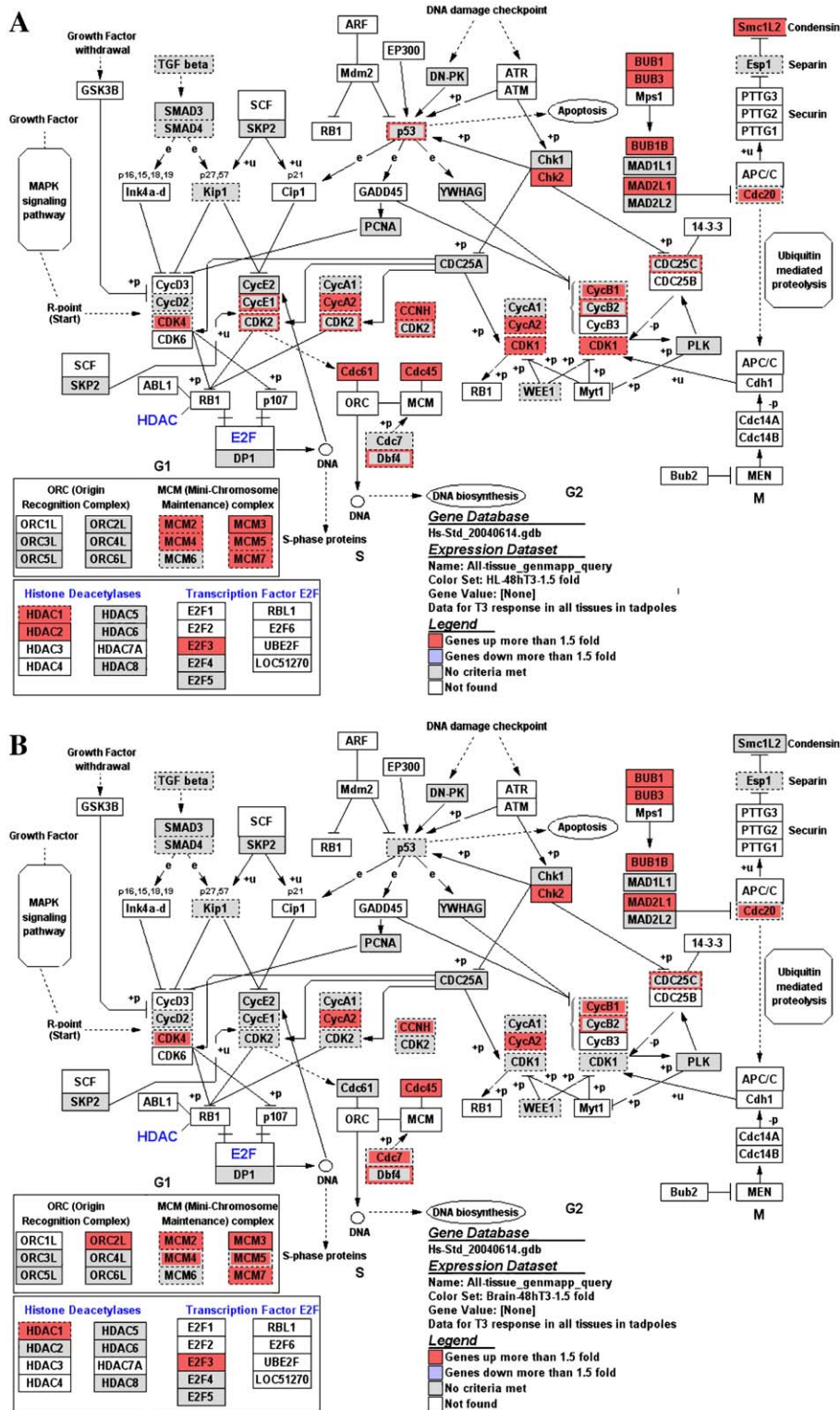
Fig. 6. Summary of cell cycle genes in (A) hind limb and (B) brain after 48 h of TH. Significantly up-regulated genes are red; significantly down-regulated genes are blue. In each case, they are compared to NF54 control limb and brain. Genes that are present on the array but not significantly regulated are light yellow. Genes in open boxes are not present in the array.

(Schreiber et al., 2001). A major challenge is to identify downstream genes in each of the many programs and to sort TH responsive genes into biological functions. The screens reported here are comprehensive enough to reveal for the first

time groups of TH-induced genes that are involved in the same or a related function. The complete list of all functional clusters for the three programs, determined by the GoMiner software, can be found at our web site. We have selected
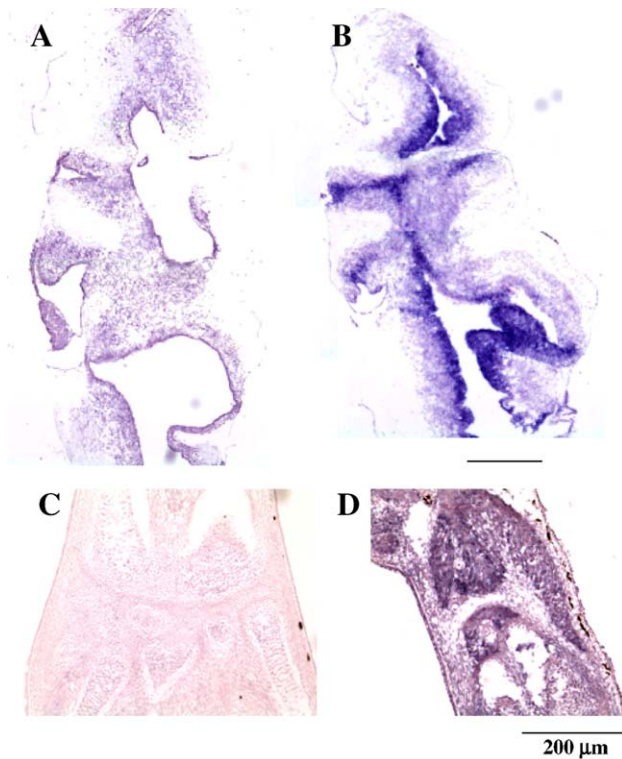
Fig. 7. In situ hybridization of frontal sections of NF54 brain (A, B) and hind limb (C, D) control (A, C) and 3 day treatment with 5 nM T3 (B, D). The probe is MCM 7 (U66710).

some of these to emphasize (Tables 2 and 5) mainly because we can imagine a role for activating and repressing these pathways in a particular program. The second goal of these comprehensive screens has been to reveal candidate genes for future study that might play a central role in the downstream pathways of differentiation controlled by TH.

Comparison of these data with other metamorphosis gene expression profiles

The first profiles of TH-induced gene expression in *X. laevis* were carried out by subtractive hybridization (Wang and Brown, 1991). We identified 17 up- and 4 down-regulated genes in NF54 tails that had been induced with TH for 48 h. Our array experiments show these genes to be the most extremely regulated genes in the UniGene based microarray (Table 1). We estimated from a statistical analysis that there would be no more than 35 up- and 10 total down-regulated genes in the tail resorption program (Wang and Brown, 1993) and greater than 120 up-regulated genes in the limb program after 24 h of TH-induction (Buckbinder and Brown, 1992). The subtraction method cleaved cDNAs into small intragenic fragments with restriction enzymes so that separate pieces of the same differentially expressed cDNA were enriched independently. The array method has found many more expressed genes in the tail. Clearly the subtractive hybridization procedure identified multiple fragments of the most differentially expressed genes in the program but failed to identify most of the less dramatically induced genes. It correctly predicted that the limb program

would be larger than the tail program with less exaggerated gene regulation.

Gene expression profiles of TH-induced tail genes in *X. laevis* have been reported using a cDNA array of 420 selected cDNAs from *Rana catesbeiana* and *X. laevis* (Helbing et al., 2003; Veldhoen et al., 2002). The probes were isolated from different time points up to 72 h of TH-induction to analyze the kinetics of mRNA change and various stages of *X. laevis* tadpole tail development. The cDNAs on the array were chosen by their availability and included known TH-regulated genes so that functional clustering is not likely to be statistically significant.

Functional and cell type specific programs in the metamorphosing tails

There are at least two independent programs that are initiated by TH in the tail. Muscle is a direct cell autonomous target of the hormone and dies by apoptosis (Das et al., 2002; Nakajima and Yaoita, 2003; Yaoita and Nakajima, 1997). The notochord and fins are composed of fibroblasts that are induced by TH to change from a collagen-synthesizing program in pre-metamorphic tadpole to one that synthesizes and secretes hydrolytic enzymes that will dissolve the tail (Table 3 and Fig. 2). The notochord is a collagen rich structure synthesized by fibroblasts that must be dissolved for the tail to shorten. Of the 4 proteolytic enzymes that we found by subtractive hybridization 3 were expressed in fibroblasts (Berry et al., 1998). Many additional proteolytic enzymes are up-regulated in these fibroblasts at the climax of metamorphosis (Table 3). They consist of serine proteases, MMPs, intracellular and secreted proteases. Many genes whose products are expressed in lyososomes are up-regulated by TH (Suppl. Table S1) including one encoding hylaruronidase (BC046378) and several cathepsins. Multiple forms of collagens and extra cellular matrix components are highly expressed in the tail of the growing tadpole and then down-regulated in tail fibroblasts at climax (Table 3). The transformation from synthesis to degradation that occurs in fibroblasts is an essential part of the tail resorption program. Suppl. Table S12 lists differentially regulated peptidases in all three programs.

One of the most significant functional clusters of up-regulated genes is related to signal transduction. This category includes a wide variety of very different signaling systems. (Suppl. Table S13) Representative genes in this category tested by in situ hybridization are expressed in fibroblasts (data not shown). Genes mediating signaling could be involved in cytoskeletal changes in fibroblasts that accompany the invasion of collagen lamella by these cells. However, we do not know the biological significance of such a large representation of genes involved in signal transduction in the TH-induced tail program.

In the earlier subtractive hybridization study carried out in this lab, only one gene (an aspartyl dipeptidase called Gene D (Berry et al., 1998) was identified to be up-regulated in muscle. The discovery in these experiments of additional TH up-regulated genes in tail muscle has provided new insight into the process of tail death and resorption. Muscle death monitored by

Table 6
Selected genes involved in regulation of transcription

GenBank accession number	Gene symbol	Gene name	Tail			Limb			Brain		Possible role in metamorphosis
			24 h	48 h	NF62	14 h	24 h	48 h	24 h	48 h	
BC043771	ACTL6A	BAF53	2.1	2.5			5.4	6.9	1.9	2.6	chromatin-mediated transcription
BC044110	ASCC2	ASC-1 complex subunit P100					2.6	3.0	1.3	1.4	Regulation of transcription
AF412333	BAZ1B	Bromo domain transcription factor	1.9	2.6			5.3	5.8	2.3	2.4	chromatin-dependent regulation of transcription
X53962	CDK7	cyclin-dependent kinase 7 MO15				1.6	3.0	2.8	1.3	1.6	regulators of cell cycle progression
AB107221	CEBPD	C/EBP delta-2	4.9	5.3	7.3 [a]	4.2	0.5	0.5	0.6		activation and/or differentiation of macrophages
BC046866	CHD4	chromodomain helicase DNA binding protein 4	1.8	2.5	2.3	2.8	8.4	7.8	2.1	2.2	chromatin reorganization
BC060442	DATF1	death associated transcription factor 1					0.5	0.4	0.8	0.8	apoptosis
D78638	DNMT1	DNA (cytosine-5-)-methyltransferase 1			0.7 [b]		5.1	5.1 [a]	1.7	1.8	epigenetic modification
AF351126	EZH2	Enhancer of zeste homolog 2	2.8	3.1	2.8	3.9	12.5	10.9	4.0	5.5	transcriptional repression
U37374	FOSL2	FOS-like antigen 2		2.8	2.8						cell proliferation
BC044277	FUBP1	far upstream element (FUSE) binding protein 1					3.1	3.1	1.7	2.1	activates c-myc
AY114105	GTF2F1	general transcription factor IIF		1.8	2.3		5.6	6.7	2.1	2.4	Initiation complex formation
BC046266	GTF2H1	general transcription factor IIH	1.6	2.1	1.5		3.6	3.8	1.9	1.9	Component of basal transcription factor
AF530514	HBP1	HMG-box transcription factor 1	2.5	2.3	1.6		0.2	0.2	0.5	0.5	Cell cycle inhibitor
BC041296	HDAC1	histone deacetylase 1		1.9	2.07 [b]	2.4	7.9	7.8 [a]	2.8	2.7	chromatin remodeling Sachs et al. (2001)
BC054208	HDAC2	histone deacetylase 2		1.9	1.5	1.5	2.4	2.4		1.4	chromatin remodeling
Z97214	HDAC9	histone deacetylase 9			2.7	0.3	0.1	0.4	0.3		chromatin remodeling
AF383160	HES1	hairy and enhancer of split 1	1.6	2.5	2.9	0.5		0.7			transcriptional repressor
BC044009	HMGB3	high-mobility group box 3	2.2	2.3	2.8		3.1	3.1	1.5	2.0	cell cycle and chromatin assembly
U35408	KLF9	basic transcription element binding protein 1	2.7	5.0		1.4	1.4	1.4 [a]			Krupple-like transcription factor
U44047	MCM2	MCM2		1.5			8.5	9.2 [a]	4.6	4.4	cell cycle
BC044051	MCM3	MCM3				1.9	8.7	10.4 [a]	3.6	3.9	cell cycle
U44049	MCM4	MCM4		1.7		1.7	4.1	4.7 [a]	1.9	2.1	cell cycle
U44048	MCM5	MCM5				2.3	9.1	10.2 [a]	11.2	12.5	cell cycle
U66710	MCM7	MCM7				2.3	9.1	10.1 [a]	3.6	4.6	cell cycle
X16106	MYOD1	myogenic factor 3			0.4	1.7	2.3	2.4			muscle specific
AF127041	MXD4	MAX dimerization protein 4		3.3	2.3						suppress c-myc dependent transformation
X56870	MYC	c-myc II		2.1	1.7	5.2	8.2	5.6	4.7	4.2	cell cycle progression, apoptosis and cellular transformation
U37375	NFIL3	TH/bZIP	3.9	5.5	1.5 [c]		1.3				bZip Transcription factor
AF495886	NCoR1	Nuclear receptor corepressor 1	2.0	2.7	1.4	1.3	1.3	1.3			NCoR 1 is involved in corepression in unliganded receptor
BC054296	NCoR2	Nuclear receptor corepressor 2					1.8	1.5	2.3	3.4	Also known as SMRT and involved corepression like NCoR1
BC046684	RBM14	RNA binding motif protein 14		1.4	1.9	1.4	5.1	5.8	2.0	2.1	no info
BC057739	RUNX1	RUNX1	4.0	10.9	13.5 [a]		1.6	1.6			alpha subunit of CBF
BC059296	SOX4	SOX4	9.2	11.7	6.5 [a]	2	1.8				apoptosis related
BC044016	TGIF	TGFB-induced factor homeodomain	2.5	3.1	3.6	1.4	1.5		1.3	1.5	transcriptional co-repressor of SMAD2
BC054280	TRIP3	thyroid hormone receptor interactor 3		1.7	2.6	1.8	3.7	3.2	1.5	1.9	Coactivator
CB201454	TRMM55	muscle-specific RING-finger	4.5	10.3	2.2		0.4	0.5	0.7		muscle specific
AF061980	ZFP36	zinc finger protein 36, C3H type	3.5	3.5	10.2		0.4	0.4			regulates response to growth factors

[a] In situ hybridization in all cell types.
[b] In situ hybridization in dying muscle.
[c] In situ hybridization in fibroblasts.

the activation of caspase-3 begins at climax in the fibers adjacent to the epidermis and proceeds medially toward the dissolving notochord (Fig. 3). All of the TH-induced up-regulated genes that we have examined that are expressed in tail muscle are localized in these same cells. Muscle fibers up-regulate at least one mitochondrial localized gene product, UCP-2, and four proteases that are candidates for catalyzing the final dissolution of muscle fibers.

Major metabolic pathways are shut off in all of the tail muscle at climax (Fig. 3) before the up-regulation of these proteolytic enzymes and the morphological evidence of dying fibers. The transcriptional repression of the majority of the genes encoding the steps leading to glycolysis (Suppl. Table S4) and the energy metabolism pathways in the mitochondria (Fig. 4) of tadpole tail muscle must play an important role in death and resorption. The down-regulation of these genes occurs throughout the tail muscle (Fig. 5) when the muscle fibers appear to be healthy. This global down-regulation precedes the more localized up-regulation pattern that coincides with the activation of caspase-3 (Fig. 3). Many genes involved in each of the five complexes of electron transfer effecting oxidative phosphorylation in the mitochondria are shut down including most of the genes that encode the ATP synthase complex. Genes for the mitochondria-localized TCA cycle are also down-regulated (Suppl. Table S3).

Recently, there have been reports (Hammerman et al., 2004; Rathmell et al., 2003) of involvement of energy metabolism, especially glycolysis, in programmed cell death. Multiple genes related to cell death are up-regulated in the tail by TH (Suppl. Table S14). CPP32 (caspase-3) gene expression was induced by TH in a tail muscle cell line and implicated by inhibitor studies in tail muscle apoptosis (Yaoita and Nakajima, 1997). Unfortunately, the oligonucleotide encoding caspase 3 on the array hybridized below the 2.3 intensity cut off. We have found that the over expression of the anti-apoptotic Bcl-2 in tail muscle inhibits TH-induced muscle death (Das et al., 2002). One transcription factor, highly expressed in the tail, that is a candidate for participating in the cell death in the tail is SOX4 (Hur et al., 2004).

Limb and brain TH dependent cell proliferation programs

A subtractive hybridization analysis of TH-induced genes in the hind limb after 24 h of TH-induction identified 14 up-regulated genes. Several of these were associated with growth functions (Buckbinder and Brown, 1992). No down-regulated genes were found. The limb and brain were included in the current micro array studies because they share the same early response to TH namely DNA replication. Yet ultimately TH will induce very different programs in these organs. Similarity in the programs should identify regulated genes in common that control DNA replication while the differences will be organ specific. The majority of the up-regulated genes in the two programs are the same and very different from those regulated by TH in the tail program (Table 5, Fig. 6). The TH-induced limb and brain programs share many functional categories. These include cell cycle (Suppl. Table S5), protein

folding (Suppl. Table S8), RNA and DNA metabolism and translation (Suppl. Table S6). In every functional category there are more genes up-regulated in the hind limb than in the brain and the extent that they are differentially regulated is usually greater. This result is due to the fact that a higher fraction of limb cells are induced by TH to replicate and grow than in the brain. Only the cells lining the lumen of the brain ventricles divide as a result of TH (Cai and Brown, 2004). We have found very few genes that are regulated by TH in the brain but not the limb (Suppl. Table S10) and these genes have a low differential expression. The two genes that stand out as possible candidates for neural specific development are Notch (M33874) and OTX2 homeobox protein (AW200443). Notch is a receptor for membrane bound ligands in the brain (Sestan et al., 1999) that plays multiple roles during development by virtue of its cleavage to form a protein fragment that acts as a transcription factor (Chan and Jan, 1998). Notch activation by TH occurs in the same replicating cells that line the brain and spinal cord ventricle. OTX2 has been implicated in mouse brain development (Boncinelli et al., 1993). We conclude that 48 h of TH treatment is too short a time to induce the major changes in gene expression that lead to adult brain differentiation.

Oppositely controlled programs in tail death and limb growth

The component genes in key functional categories are regulated in the opposite manner in tail versus limb programs. Many of the genes that encode energy pathway proteins that are localized in the mitochondria (e.g., the genes in the mitochondrial electron transport chain complex shown in Fig. 4) are down-regulated in tail muscle but up-regulated during limb growth. Several transcription factors have opposite regulation in tail compared to limb (Table 6) and are therefore candidates to regulate these genes. Alternatively, this reverse regulation might be due to different coactivators and repressors. We do not know whether any of them are direct TH response genes. However, a significant number of the genes in both pathways are up-regulated in the limb at the earliest time point, after 14 h of TH treatment, suggesting that they are direct response genes.

In this discussion, we have emphasized the information that global gene expression analysis can provide for the biology of metamorphosis. We have placed all of our microarray data on a web site recognizing the substantial unexplored information that has not been placed into a biological context. There are many differentially expressed individual genes and functional clusters of genes revealed in this study whose role in metamorphosis awaits explanation.

Appendix A. Supplementary data

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.ydbio.2005.12.032.

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