

Thyroid Hormone-dependent Gene Expression Program for *Xenopus* Neural Development*

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Although thyroid hormone (TH) plays a significant role in vertebrate neural development, the molecular basis of TH action on the brain is poorly understood. Using polymerase chain reaction-based subtractive hybridization we isolated 34 cDNAs for TH-regulated genes in the diencephalon of *Xenopus* tadpoles. Northern blots verified that the mRNAs are regulated by TH and are expressed during metamorphosis. Kinetic analyses showed that most of the genes are up-regulated by TH within 4–8 h and 13 are regulated by TH only in the brain. All cDNA fragments were sequenced and the identities of seven were determined through homology with known genes; an additional five TH-regulated genes were identified by hybridization with known cDNA clones. These include five transcription factors (including two members of the steroid receptor superfamily), a TH-converting deiodinase, two metabolic enzymes, a protein disulfide isomerase-like protein that may bind TH, a neural-specific cytoskeletal protein, and two hypophysiotropic neuropeptides. This is the first successful attempt to isolate a large number of TH-target genes in the developing vertebrate brain. The gene identities allow predictions about the gene regulatory networks underlying TH action on the brain, and the cloned cDNAs provide tools for understanding the basic molecular mechanisms underlying neural cell differentiation.

Thyroid hormone (TH)¹ plays a critical role in the development of the vertebrate brain and peripheral nervous system (1–4). Thyroid hormone deficiency during the fetal and neonatal period produces neurodevelopmental defects. This condition, known as cretinism, results in severe mental retardation and defects in skeletal growth (5). The cytoarchitectural changes in brain development that are influenced by TH have been studied primarily in rats (4). Lack of TH during fetal life leads to abnormal neuronal maturation, neurite outgrowth, synapse formation, neuroglial cell development, and subsequent myelination (4). However, despite the profound effects that the hormone has on the developing brain, little is known

about the molecular mechanisms of TH action in neural development.

Although attention has been focused on rodent models for TH action in neural development, the classical vertebrate model for hormone action in development is the amphibian tadpole (6). Thyroid hormone controls amphibian metamorphosis and thus plays an important role in the developmental changes in the nervous system that occur during metamorphosis. These changes involve extensive remodeling of regions of the central nervous system that underlie development of sensory and motor systems that are necessary for the shift from one life history stage (*i.e.* the aquatic, fish-like, herbivorous larvae) to another (*i.e.* the terrestrial (in many species), tetrapodal, carnivorous adult frog). Metamorphosis of the amphibian tadpole is one of the few vertebrate systems in which the effects of a hormone on neural cell proliferation, differentiation, and apoptosis can be directly correlated with functional changes that lead to the development of adult behaviors.

Recent work on tadpole metamorphosis using molecular biological approaches has provided basic mechanistic information on diverse developmental processes, such as tissue remodeling, growth and differentiation, and apoptosis (6–8). The metamorphic process is controlled by thyroxine (T₄), which is converted to a more active form (T₃; 3,5,3'-triiodothyronine) by monodeiodinases in target tissues (T₃ will be used to refer to thyroid hormone throughout this paper). The thyroid status of tadpoles can be easily altered by the addition of hormone or T₃ synthesis inhibitors to water in which the animals are raised. Thyroid hormone is thought to control metamorphosis by regulating gene expression. T₃ receptors are members of the steroid receptor superfamily and function as ligand-dependent transcription factors. *Xenopus* has two T₃ receptor α (TR α) and two TR β genes (owing to its pseudotetraploidy), each of which is expressed during metamorphosis. The T₃-TR complex interacts with specific T₃ response elements (TREs) present in the target gene and thus can either enhance or repress gene transcription (8, 9). The TRs bind TREs as either homo- or heterodimers; although TR can heterodimerize with receptors for retinoic acid, vitamin D, and retinoid X, the most effective dimerization partner is the retinoid X receptor, which binds 9-*cis* retinoic acid (10, 11). Because T₃ regulates gene expression and can induce very different developmental changes in diverse tissues (*e.g.* degeneration of tail (*i.e.* apoptosis), remodeling of brain and gut, and growth and differentiation of limbs), it is predicted that the hormone induces tissue-specific genetic programs. Recent studies using a subtractive hybridization approach have isolated T₃-responsive genes in various tadpole tissues (*e.g.* tail (12, 13), hind limb (14), and intestine (15)). These analyses have shown that some of the early T₃-response genes are common to all tadpole tissues, whereas others are tissue-specific (13, 16, 17).

To provide a molecular basis for understanding T₃ action on

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¹ The abbreviations used are: TH, thyroid hormone; TRE, T₃ response element; BTBE, basic transcription element binding; 5-D, 5-monodeiodinase; PDI, protein disulfide isomerase; xPDI-LP, *Xenopus* PDI-like protein; CK, creatine kinase; PCR, polymerase chain reaction.

the development of the vertebrate brain, we have cloned, by subtractive hybridization, a large number of T_3 -responsive genes in the premetamorphic *Xenopus* tadpole diencephalon. We have focused on the diencephalon (preoptic/thalamic/hypothalamic area) because this region undergoes dramatic changes in response to T_3 (*i.e.* accumulation of neurosecretory material, development of neurosecretory nerve terminals and capillaries of the median eminence; Ref. 18) and because the neurosecretory neurons of the preoptic region/hypothalamus play a central role in controlling thyroid secretory activity during metamorphosis. Releasing factors produced in these neurosecretory nuclei control secretion of pituitary thyrotropin, which stimulates thyroidal secretion resulting in the increasing plasma titers of T_4 and T_3 throughout the prometamorphic period. The presence of an intact preoptic region/hypothalamus is essential for the progression of metamorphosis because ablation or lesioning of this brain region results in metamorphic stasis (19, 20). Among the TH-regulated genes that we have isolated, several encode transcription factors that we predict are responsible for regulating a secondary wave of gene expression, the protein products of which should define the adult phenotype. Also among these genes are several cellular enzymes, one cytoskeletal protein, and two hypophysiotropic neuropeptides. The gene identities allow predictions to be made about the integrated gene regulation program underlying T_3 action on development of the vertebrate brain.

EXPERIMENTAL PROCEDURES

Hormonal and Pharmacological Treatment of *X. laevis* Tadpoles, Tissue Collection and RNA Isolation—Tadpoles were reared in dechlorinated tap water (water temperature, 20–22 °C) and fed pulverized rabbit chow. Stage 52–54 (Nieuwkoop and Faber (21)) tadpoles were treated with 3,5,3'-L-triiodothyronine (T_3 -sodium salt; Sigma) by adding it to the water to a final concentration of 5 nM. This dose was expected to produce a total plasma T_3 concentration comparable to the 8 nM concentration observed in *X. laevis* tadpoles at metamorphic climax (22), assuming complete equilibration of tadpole body fluids with the aquarium water (15). The protein synthesis inhibitors cycloheximide and anisomycin (Sigma), which together produce nearly complete inhibition of protein synthesis in *X. laevis* tadpoles (23), were added to the aquarium water to a final concentration of 20 and 25 μ g/ml, respectively. Treatment was initiated 1 h before addition of T_3 and continued for a total of 13 h. The goitrogen methimazole (1 mM; Sigma) was added to the aquarium water to inhibit T_3 synthesis at different stages of tadpole development.

For construction of subtractive cDNA libraries, 500 stage 52–54 tadpoles were treated with T_3 for 20 h before tissue collection; 500 untreated tadpoles served as controls. Tadpoles were anesthetized in 0.01% benzocaine and a portion of the brain encompassing the diencephalon was dissected. Total RNA was isolated using the guanidium thiocyanate method (24).

Construction of Subtractive cDNA Libraries—The isolation of cDNAs that corresponded to T_3 -regulated mRNAs in premetamorphic *X. laevis* tadpole diencephalon was achieved by the polymerase chain reaction (PCR)-based gene expression screen (subtractive hybridization) essentially as described by Wang and Brown (25) with modifications of Shi and Brown (15). Poly(A)⁺ RNA was purified from total RNA isolated from neural tissue of control (–) or T_3 -treated (+) tadpoles. The two starting cDNA libraries were constructed using 5 μ g of poly(A)⁺ RNA (– or +; Copy Kit II, InVitrogen).

Five rounds of subtraction were done before the enriched (–) cDNAs (down-regulated genes) and (+) cDNAs (up-regulated genes) were cloned into the pBluescript KS– plasmid (Stratagene) at the *Eco*RI site in the polylinker region. During the subtraction, the extinction of abundant, unregulated cDNAs and the enrichment of T_3 -regulated cDNAs were followed by Southern blot analysis using random-primed ³²P-labeled cDNA probes for one unregulated gene (the ribosomal L8 gene; Ref. 26) and one T_3 -regulated (up-regulated) gene (TR β ; Ref. 27; see Fig. 1).

Differential Screening—After colony hybridization (with ³²P-labeled – or + subtracted cDNAs) to identify clones containing enriched cDNA inserts (25), 100 colonies were isolated (50 – or +), plasmids were purified, and cDNA inserts were prepared. Dot blots were pre-

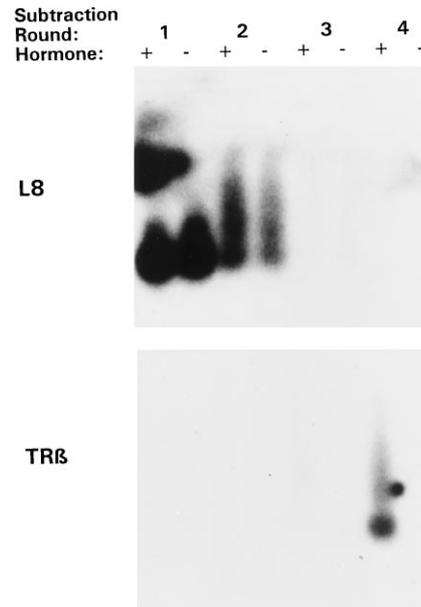


FIG. 1. Southern blots of the PCR-amplified subtractive cDNA libraries prepared from *Xenopus* tadpole diencephalon. The upper blot shows the disappearance of the abundant, unregulated cDNA corresponding to the *Xenopus* ribosomal protein L8 gene (26) through successive rounds of subtraction. The lower blot shows the progressive enrichment of the cDNA corresponding to the T_3 -regulated TR β gene. Tadpoles were treated with or without 5 nM T_3 in the aquarium water (+ or – hormone) before tissues were collected and cDNA libraries prepared.

pared with purified cDNA inserts (28) to screen for cross-hybridizing species. This analysis identified 46 non-cross-hybridizing cDNAs. Initial screening to verify that the isolated cDNAs corresponded to T_3 -regulated genes was done by probing Southern blots made with the starting PCR-amplified cDNA libraries; this assumes that the abundance of any fragment between the starting amplified + and – cDNAs accurately represents its relative abundance in the original mRNA preparations (25). T_3 regulation was later confirmed for each gene by Northern blot hybridization. These analyses reduced the number of unique, non-cross-hybridizing T_3 -regulated genes to 34.

Southern and Northern Blot Analyses—To determine the tissue distribution of mRNAs for isolated genes, Southern blots were prepared from + or – T_3 PCR-amplified starting cDNA libraries (tail (12), hind limb (14), and intestine (15); 5 μ g). Southern blots were also prepared from digested, homozygous diploid *X. laevis* genomic DNA (29) to determine gene copy number (see Ref. 15). DNAs were electrophoretically separated on a 1.5% agarose gel and transferred to nylon membrane (Genescreen, Dupont) by capillary transfer using 0.5 M NaOH, 1.5 M NaCl as transfer buffer (which also served to denature the DNA during transfer; Ref. 28). The genomic DNA was digested (5 μ g/reaction) with *Bam*HI, *Eco*RI, or *Hind*III before electrophoresis.

RNA blots were prepared with total RNA extracted from tadpole diencephalic tissue to analyze the developmental and hormone-regulated expression of each of the genes isolated in the subtraction. For analysis of developmental expression, brain tissue was dissected from tadpoles at different stages of postembryonic development. For the analyses of hormone regulation, stage 52 tadpoles were treated with 5 nM T_3 for various times before isolating brain tissue for analysis. Each experiment was repeated three times; the expression patterns of each of the genes were analyzed at least twice. For RNA blots, total RNA (10–15 μ g) was separated by electrophoresis in a 1% formaldehyde-agarose gel, hydrolyzed in 0.05 M NaOH, 0.01 M NaCl, and transferred to nylon membrane using 20 \times SSC as the transfer buffer.

Northern and Southern blots were prehybridized in Hybrisol I (Oncor) for 2–4 h and hybridized for 16 h at 42 °C with subtracted cDNA fragments labeled with [³²P]dCTP by random priming (Amersham Corp.). Blots were washed with 2 \times SSC, 0.5% SDS at room temperature for 10 min and then with 0.25 \times SSC, 0.1% SDS at 65 °C for 1 h before exposure to x-ray film for 1–14 days.

Small cDNA fragments were sequenced either manually using Sequenase, Version 2.0 (Amersham Corp.) or by cycle sequencing (ABI Prism, Perkin-Elmer) using an automated ABI sequencer. Sequence

TABLE I
 Thyroid hormone-regulated genes, organized by developmental class

Gene	RNA size <i>kb</i>	Gene copy ^a	Up- or down- regulated by 20 h	Developmental class ^b	TH kinetic class ^c	CHX sensitivity ^d	Tissue distribution of TH-regulated genes ^e			
							Diencephalon	Intestine	Hind limb	Tail
Up-regulated Genes										
<i>xh2</i>	1.7, 7	1	3	1	2	R	+	+	0	0
<i>xh3</i>	1.8, 9	2	5	1	2	ND	+	+	+	0
<i>xh4</i>	1, 4, 8	1	>10	1	1	R	+	+	+	+
<i>xh7</i>	2	1	3	1	1	R	+	+	+	+
<i>xh8</i>	6.2, 9.5	2	3	1	2	R	+	0	0	0
<i>xh10</i>	3.7, 6, 6.4	2	3	1	2	R	+	0	0	0
<i>xh11</i>	0.5, 8	2	4	1	1	R	+	0	0	0
<i>xh12</i>	1.8, 4, 6, 9	>2	4	1	2	ND	+	+	+	+
<i>xh13</i>	0.5, 8	>2	3	1	1	ND	+	0	0	0
<i>xh14</i>	.7, 3.5, 6.5	2	2	1	1	ND	+	0	0	0
<i>xh15</i>	1.5, 5.8, 9	2	3	1	1	R	+	+	+	+
<i>xh16</i>	0.5, 2.5	ND	4	1	1	ND	+	0	0	0
<i>xh18</i>	0.5	>2	3	1	1	R	+	0	0	0
<i>xh20</i>	1.7, 7	1	3	1	2	R	+	+	0	0
<i>xh5</i>	2, 4.5, 6	2	>10	2	1	ND	+	+	0	+
<i>xh17</i>	2	2	3	2	2	R	+	+	0	0
<i>xh19</i>	4.5, 8	2	3	2	2	R	+	0	+	+
<i>xh9</i>	0.8	2	3	2	2	ND	+	0	0	0
<i>xh6</i>	1.5, 4.5, 8	1	>10	3	1	R	+	+	+	+
<i>xh1</i>	2	ND	3	ND	2	ND	+	+	+	+
Down-regulated genes ^f										
<i>xh25</i>	0.5, 4, 6.4	ND	5	1	3	ND	-	ND	0	0
<i>xh26</i>	.7, 3.5, 6.5	1	3	1	3	R	-	0	0	0
<i>xh29</i>	2, 4	ND	3	1	ND	ND	-	-	-	0
<i>xh30</i>	3	2	3	1	ND	ND	-	+	0	0
<i>xh32</i>	.7, 3.5, 6.5	1	5	1	3	R	-	0	0	0
<i>xh33</i>	2	ND	3	1	3	ND	-	ND	ND	ND
<i>xh34</i>	2	1	3	1	3	ND	-	0	0	-
<i>xh21</i>	1.5, 4.4, 8	1	3	2	ND	ND	-	0	0	0
<i>xh22</i>	.5, 2.8, 10	1	5	2	3	NR	-	-	-	0
<i>xh23</i>	0.5	1	5	2	3	R	-	0	0	0
<i>xh27</i>	1.8	ND	3	2	3	ND	-	0	-	0
<i>xh28</i>	1.5, 4.6, 9	1	3	2	3	R	-	0	-	0
<i>xh31</i>	2	1	3	2	3	-	-	0	0	0
<i>xh24</i>	0.2, 5, 8	>2	5	2	3	R	-	ND	0	0
Genes identified by hybridization ^g :										
GR	4, 6, 8	1	5	1	3	ND	-	-	ND	-
CRH	1.8	2	3	1	3	ND	-	ND	ND	ND
TRH	1, 3.5, 7	1	5	1	2	R	+	ND	0	0
TR β	>10	2	10	2	1	R	+	+	+	+
bZIP	5.5	1	>10	3	1	R	+	+	+	+

^a As determined by Southern blot of homozygous diploid genomic DNA (see text). ND, not determinable.

^b 1, mRNA level rises during prometamorphosis and climax and remains high in adult; 2, mRNA level rises during prometamorphosis and climax and drops in adult; 3, mRNA level increases at stage 62 and then drops.

^c 1, mRNA level rises by 4–12 h and remains high throughout week; 2, mRNA level rises by 4 h, drops by 8–12 h, and then rises; 3, mRNA level rises by 4 h, drops by 8–24 h, and then rises.

^d R, upregulation resistant to cycloheximide; NR, not resistant to cycloheximide.

^e Tissue-specific gene expression in control and T₃-treated stage 52 tadpoles was measured 20 h after exposure to the hormone (5 nM in the aquarium water). +, upregulated; -, downregulated; 0, not regulated.

^f Many of these genes exhibit complex (*i.e.* biphasic) regulation rather than simple down-regulation. They were isolated from the library representing genes whose mRNA level is reduced at 20 h following continuous T₃ treatment; however, Northern blot analysis shows that many of these genes are actually upregulated at 4 and 48 h (see Fig. 2 and text).

^g Clones isolated by traditional cloning (TR β (27), glucocorticoid receptor (GR) (47), corticotropin-releasing hormone (CRH) (50), thyrotropin-releasing hormone (TRH) (51)) or by subtractive hybridization (bZip (12)) were used as probes to screen the subtractive cDNA libraries produced from diencephalon. cDNAs corresponding to these genes were found to be enriched in these libraries.

data were analyzed using the National Center for Biotechnology Information programs FASTA and BLAST.

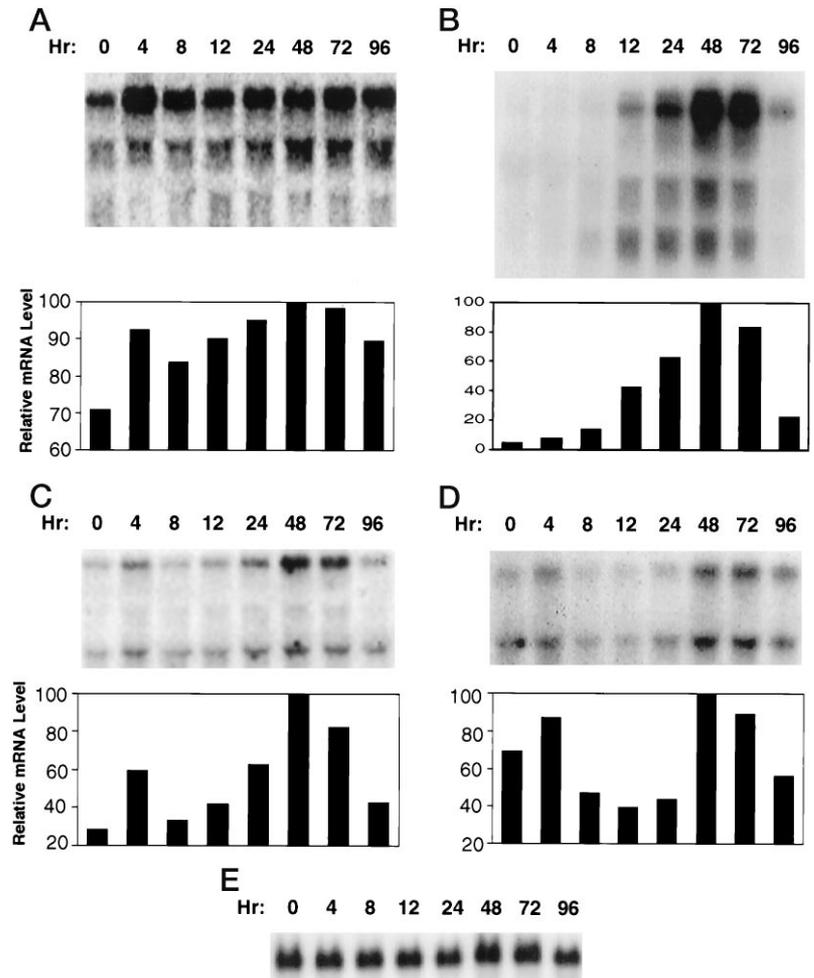
RESULTS

Isolation of Early T₃-responsive Genes from Tadpole Diencephalon—Differential screening of the subtracted cDNA libraries identified 34 non-cross-hybridizing cDNA fragments that correspond to T₃-regulated mRNAs in the premetamorphic *X. laevis* tadpole diencephalon (see Table I). Twenty of the cDNA fragments were from the up-regulated and fourteen from the down-regulated (see below) libraries. It is expected that several of these small cDNA fragments could be derived from the same mRNA if they hybridize to same-sized bands on a

Northern blot. Comparisons of mRNA sizes and expression patterns strongly support three instances in which more than one cDNA fragment could be derived from the same mRNA (up-regulated: *xh2* and *xh20*, *xh11* and *xh13*; down-regulated: *xh26* and *xh32*). Thus, 31 of the cDNAs could correspond to individual mRNAs for unique genes. Examination of full-length cDNA clones will be required to verify this estimate.

Tissue Specificity of T₃-regulated Gene Expression—To determine the tissue distribution and T₃ regulation of genes isolated from the tadpole diencephalon, each cDNA fragment was used to probe Southern blots prepared from the starting PCR-amplified + and - cDNA libraries (used in the subtrac-

FIG. 2. Kinetic profiles of T₃ response for four representative genes isolated by subtractive hybridization from *Xenopus* tadpole diencephalon. Stage 52 tadpoles were treated with 5 nM T₃ in the aquarium water and tissues were collected at various times afterward and analyzed by Northern blot. *A*, a class 1 gene (*xh14*; see Table I legend for class designations) whose mRNA was increased by 4 h and remained elevated through 96 h. *B*, a class 1 gene (*xh4*; BTEB protein; see Table II) that exhibited a lag in its response to T₃. The mRNA for this gene was increased by 8 h following T₃ treatment and declined at 96 h. *C*, a class 2 gene (*xh2*; xPDI-LP) that showed a biphasic expression pattern. The mRNA for this gene was increased at 4 h, dropped at 8–12 h, and then rose again at 24 h. *D*, a class 3 gene (*xh28*) that showed a biphasic expression pattern. The mRNA for this gene increased at 4 h, dropped 8–24, and then rose again at 48 h.



tive hybridization) produced from stage 52–54 tadpole tail, intestine, and hind limb (12, 14, 15). Wang and Brown (25) showed that the relative abundance of the individual cDNAs in these libraries reflects the original mRNA abundance in the tissues from which they are derived. Thirteen of the isolated cDNAs correspond to mRNAs that are regulated by T₃ only in brain (this number may be 12 if *xh11* and *xh13* correspond to the same gene): 8 of the up-regulated and 5 of the so-called down-regulated genes (see below). One gene is expressed exclusively in the brain (*xh33*: neural-specific β tubulin; see below). Four of the genes are regulated by T₃ only in the brain and the intestine (three if *xh2* and *xh20* correspond to the same gene), which could possibly reflect neuroendocrine tissue specificity. One of the genes exhibits opposite regulation in different tissues (*xh30*); however, this could be due to slightly different kinetics of the biphasic response in brain and intestine (see below).

T₃ Response Kinetics and Resistance of Up- or Down-regulation to Protein Synthesis Inhibition—We determined which of the isolated cDNA fragments correspond to immediate, early response genes and which to delayed, late genes by doing time course analyses of mRNA accumulation. We analyzed brain mRNA levels for each gene at various times during 96 h of continuous exposure of tadpoles to 5 nM T₃. We also determined whether gene regulation by T₃ was resistant to inhibition of protein synthesis. If a gene exhibited early response kinetics (e.g. up- or down-regulation within the first 4–12 h) and its regulation was resistant to inhibition of protein synthesis, we suggest that it is a direct T₃-response gene. However, to confirm direct *versus* indirect regulation, either nuclear run-on or

transient transfection assays and/or demonstration of a TRE in the upstream sequence of the target gene will be necessary.

As for the tail (12), intestine (15), and cultured *Xenopus* cells (23), most of genes isolated from neural tissue in this study exhibit response kinetics that can be characterized as early; i.e. the up-regulated genes exhibit a 4–8-h lag time followed by accumulation of mRNA up to 72–96 h (e.g. Fig. 2; Table I). Furthermore, of the genes that could be successfully evaluated, most exhibited resistance to protein synthesis inhibition in their up- or down-regulation.

Most of the genes identified in this screen have complex patterns of T₃-regulated expression. They exhibit a biphasic response with immediate and late up-regulation but intermediate down-regulation (see Fig. 2; Table I). The level of mRNA for these biphasic response genes increases by 4 h, drops between 8 and 48 h, and rises again between 24 and 48 h. The length of this down-regulated period (i.e. whether the mRNA level increased by 24 or 48 h) most likely determined whether the gene was isolated as an up- or down-regulated gene (because the present screen was done after 20 h of hormone treatment). Thus, the mRNA level for the so-called down-regulated genes is only transiently reduced, with the sustained effect of the hormone being up-regulation.

Developmental Expression of T₃-Regulated Genes—Although showing that the isolated genes are regulated by T₃ on Northern blots suggests that they can be induced or repressed by the endogenous hormone during spontaneous metamorphosis, showing that gene expression is correlated with rising titers of plasma T₃ (and, by extension, with metamorphic changes) is necessary to suggest that the gene is involved in morphogene-

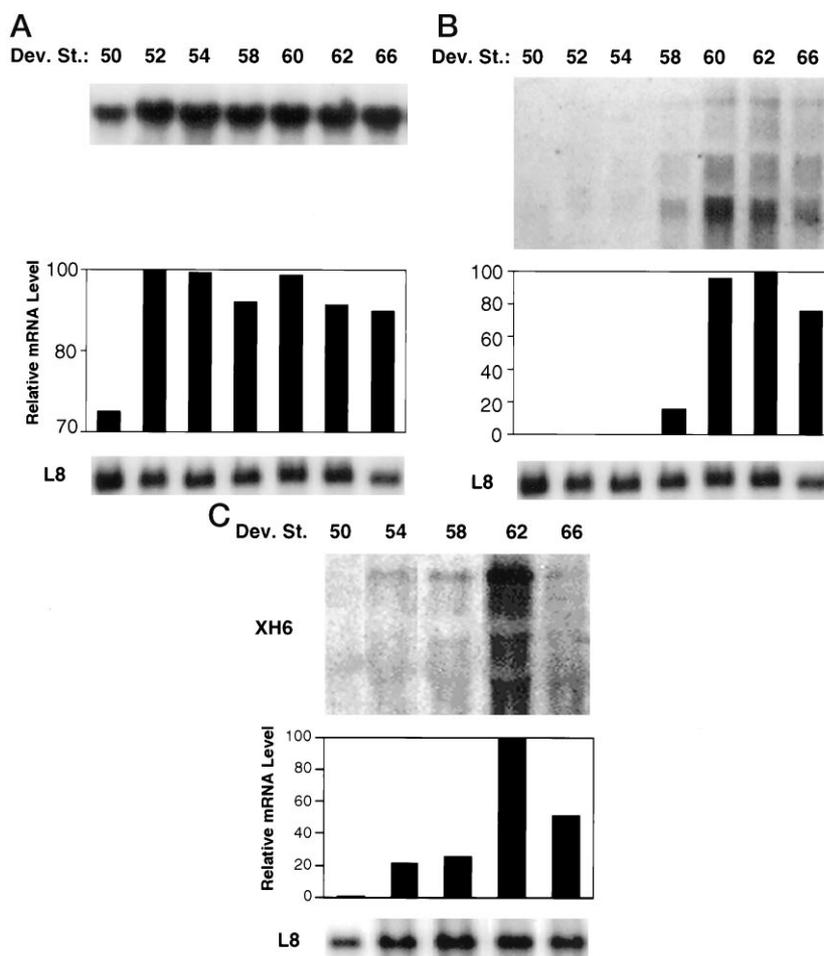


FIG. 3. Patterns of developmental expression of three representative genes isolated by subtractive hybridization from *Xenopus* tadpole diencephalon. Brain tissue was isolated at various developmental stages and analyzed by Northern blot. *A*, a class 1 gene (*xh7*; see Table I legend) whose mRNA level is low in premetamorphosis and increased during prometamorphosis and remained high in the adult. *B*, a class 2 gene (*xh4*; BTEB protein; see Table II) whose mRNA level is low in premetamorphosis and early prometamorphosis rose during late prometamorphosis and climax and dropped in adult. *C*, a class 3 gene (*xh6*) whose mRNA level is low in pre- and prometamorphosis increased abruptly at climax and dropped in adult. *Dev. St.*, developmental stage.

sis. The embryonic period of *Xenopus* takes about 72 h, after which time the eggs hatch (stage 35–36), and the tadpole begins to feed shortly thereafter (stage 45). During the premetamorphic period (stages 46–52), the animal grows but little morphogenesis occurs, although the tadpole's tissues are competent to respond to T₃ (*i.e.* if given exogenously) during this time (63). The first histological evidence of thyroid gland development in *X. laevis* is at stage 49–50 (20), although thyroid hormone does not become measurable in the blood plasma until around stage 53–54 (22); however, a biologically significant level of T₃ could be present in the blood earlier than this stage (*e.g.* see below). The prometamorphic period, the first external morphological evidence for which is the appearance and development of the hind limb bud, extends from stage 53 to stage 60, when morphogenesis begins to accelerate and plasma T₃ levels rise. The most rapid phase of morphogenesis is metamorphic climax (stage 60–66), when plasma T₃ levels are maximum.

Northern blot analysis was done on diencephalic tissue isolated from tadpoles at various stages of development to assess developmental patterns of gene expression (due to the small size of the tissue of early stage tadpoles (*i.e.* stage 49), the entire midbrain region was analyzed). This analysis showed three basic patterns of gene expression (Fig. 3). The first group of genes (Class 1 in Table I; Fig. 3) exhibits low but detectable expression during premetamorphosis, which rises when endogenous T₃ levels begin to increase and remains elevated at a constant level throughout prometamorphosis and climax and in the adult. The second group of genes (Class 2 in Table I; Fig. 3) either are absent or are expressed at a very low level during premetamorphosis, rise during prometamorphosis, peak at climax, and then are expressed in the adult, although at a slightly

reduced level. The third pattern of gene expression is exhibited by only one gene, *xh6* (Class 3 in Table I; Fig. 3). This gene peaks in expression at stage 62, the most active period of morphological change.

Dependence of T₃-induced Gene Expression on the Continued Presence of T₃—Showing that the gene of interest is expressed at the appropriate developmental stage and is correlated with rising titers of endogenous hormone is necessary but not sufficient to prove that the gene is normally regulated by the hormone during this time. We determined if the expression of one of the genes, *xh2* (the identity of which is the *Xenopus* basic transcription element binding (BTEB) protein; see below), requires the continued presence of T₃ throughout the metamorphic period. Treatment with the goitrogen methimazole at various stages of metamorphosis effectively stopped morphogenesis up to the period just before metamorphic climax, when the initiation of metamorphic changes were no longer reversible. Northern blot analysis showed that sustained expression of the BTEB protein depended on the continued presence of T₃ (see Fig. 4).

Identification of T₃-regulated Neural Genes—Sequence similarity searches of the genetic data bases identified four of the cDNA fragments as previously cloned *Xenopus* genes (see Table II). A 254-bp cDNA fragment (*xh4*) isolated from the tadpole brain in this study covers nucleotides 963–1216 of the *Xenopus* basic transcription element binding (BTEB) protein. The cDNA for the BTEB protein was first cloned from rat (30) and later from tadpole tail (12, 13). BTEB protein is a member of the Sp1 family of proteins, which are constitutive-acting transcription factors (31–34). As in the tail, the up-regulation of BTEB protein mRNA in brain by 12 h is resistant to inhibition of protein

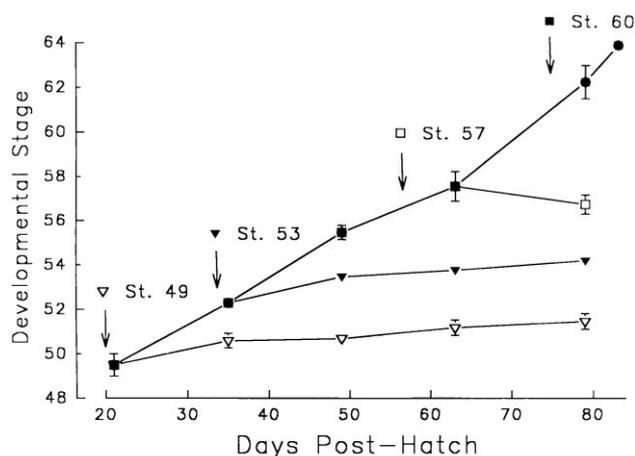


FIG. 4. Inhibition of morphogenesis and T₃-dependent gene expression by treatment of tadpoles with the antithyroid compound methimazole. Tadpoles were treated with 1 mM methimazole in the aquarium water as described in under "Experimental Procedures." Treatment was initiated at different developmental stages indicated by the arrows on the graph. The Northern blot shows expression of the *xh4* gene (BTEB protein) in stage 58 tadpoles treated without (lane 1) or with (lane 2) methimazole (for 2 weeks before tissue isolation).

synthesis, suggesting that this may be a direct T₃-response gene (Table I). Another early T₃-response gene is encoded by the 227-bp cDNA fragment *xh7*, which corresponds to the *Xenopus* type III 5-monodeiodinase (5-D) gene first isolated from tail (35). The partial cDNA isolated from brain covers the very 5' end of the mRNA, spanning positions -26 to 202. The primary function of the type III 5-D is to degrade T₃ (35).

Xenopus T₃-regulated genes that were not identified in screens of other tadpole tissues include the cDNA clones *xh1* and *xh33*. Partial sequencing of *xh1* (91 bp) demonstrated 100% similarity over nucleotide positions 8370-8455 of the *Xenopus* mitochondrial genome; this fragment corresponds to amino acid positions 326-352 of the *Xenopus* mitochondrial enzyme cytochrome *c* oxidase subunit I (36). Cytochrome *c* oxidase is an important mitochondrial proton-pumping respiratory protein, catalyzing the transfer of electrons from cytochrome *c* to O₂ (37). The *xh33* cDNA clone corresponds to the *Xenopus* nervous system-specific, class II β tubulin isotype (38). Partial sequencing (124 bp) of this clone showed that it covers the very 5' end of the mRNA, spanning nucleotide positions -11 to 113. Tubulins are highly expressed in developing brain (39) and are required for normal axonal development and synapse formation (4).

Three of the other cDNA fragments exhibited significant sequence similarity to known genes of other species. The 300-bp *xh20* cDNA fragment exhibits 68.5% identity to human

TABLE II
Identification of T₃-regulated genes in hypothalamus

Gene	Homolog/Product	Reference
<i>xh1</i>	<i>Xenopus laevis</i> Cytochrome C oxidase subunit I	36
<i>xh4</i>	<i>Xenopus laevis</i> BTEB	13
<i>xh7</i>	<i>Xenopus laevis</i> Type III monodeiodinase	35
<i>xh20</i>	<i>Homo sapiens</i> Protein disulfide isomerase	40
<i>xh27</i>	<i>Gallus domesticus</i> Creatine kinase B	44
<i>xh29</i>	<i>Rattus norvegicus</i> HMG-box protein	46
<i>xh33</i>	<i>Xenopus laevis</i> Neural-specific β tubulin	38
T ₃ -regulated genes identified by hybridization:		
	<i>Xenopus laevis</i> Thyroid hormone receptor β	27
	<i>Xenopus laevis</i> bZip protein	13
	<i>Xenopus laevis</i> Glucocorticoid receptor	47
	<i>Xenopus laevis</i> Corticotropin-releasing hormone	50
	<i>Xenopus laevis</i> Thyrotropin-releasing hormone	51

protein disulfide isomerase (PDI; Ref. 40) over a 178-nucleotide span covering the first thioredoxin-like domain of human PDI. Protein disulfide isomerases are multifunctional proteins; a major role is to assist in the folding of proteins containing disulfide bonds, and significantly, PDIs are also cellular T₃ binding proteins (41). Analysis of the translation of the *Xenopus* PDI-like protein (xPDI-LP) cDNA fragment (using BLASTX) shows that at the amino acid level, the xPDI-LP is more similar to several members of the endoplasmic reticulum protein 60 (ERp60) class of PDI-like proteins, which were independently identified as endoplasmic reticulum Ca²⁺-binding proteins with PDI activity (41, 42). The xPDI-LP cDNA fragment corresponds to a portion of the region of human PDI where T₃ binds (*i.e.* the first 300 residues; Ref. 43). Interestingly, the xPDI-LP contains a KDEL sequence that is a tetrapeptide motif located at the C terminus of proteins that are retained in the lumen of the endoplasmic reticulum (however, it is notable that other PDIs can escape the endoplasmic reticulum and proceed to the plasma membrane in highly secretory cells; Ref. 41). The presence of the KDEL motif in the xPDI-LP clone suggests that this molecule is a shorter form of PDI-like protein, perhaps representing a new family of PDI-like protein. Further structural and functional analysis of the xPDI-LP should provide valuable insight into the evolution of this class of proteins and the critical biochemical pathways that they catalyze.

Partial sequencing (125 bp) of the *xh27* cDNA fragment showed that it is 72.8% similar to chicken creatine kinase B (brain-type CK; Ref. 44) over nucleotides 899-1018. Creatine kinases catalyze the transfer of phosphoryl groups from phosphocreatine to ATP (37). The brain-type CK is a major enzyme involved with energy metabolism in nonmuscle cells (45).

Partial sequencing (119 bp) of the *xh29* cDNA fragment showed that it is 70.6% similar at the nucleotide level (nucleotides 89-207) to the rat Hbp1 protein that contains a DNA-binding high mobility group (HMG) box domain (46). The rat Hbp1 is a putative HMG transcription factor that was cloned by its capacity to suppress the K⁺ transport-defective phenotype in yeast. Its expression in several mammalian cell lines is correlated with cell differentiation (46).

Hybridization analysis with known *Xenopus* cDNA clones

identified five other T₃-regulated genes in the tadpole diencephalon (Table I). Three are transcription factors: two hormone receptors, TR β (27) and glucocorticoid receptor (47), and a *Xenopus* bZip protein (tail gene 8; Ref. 13). The bZip protein is most similar to human E4BP4 (13, 48), which functions as a transcriptional repressor (48, 49). Two neuropeptide genes were also found to be regulated by T₃: corticotropin-releasing hormone (50) and thyrotropin-releasing hormone (51). These peptides function in the control of pituitary secretion during metamorphosis (18).

DISCUSSION

Thyroid hormone exerts profound effects on the developing vertebrate central nervous system, but the molecular basis of T₃ action on the brain is poorly understood. We have provided a foundation for understanding this basic developmental process by isolating a large number of T₃-regulated genes from premetamorphic *Xenopus* tadpole brain using the PCR-based gene expression screen (subtractive hybridization) described by Wang and Brown (25). cDNA fragments corresponding to as many as 34 unique T₃-regulated genes were isolated; 7 of the fragments share significant sequence similarity to known genes. This approach has been used successfully to isolate genes regulated by T₃ in several other somatic, nonneural tissues of *Xenopus* tadpoles (tail (12, 13), hind limb (14), and intestine (15)).

Each of the cDNA fragments isolated by subtractive hybridization corresponds to a T₃-regulated gene as verified by Northern blot analysis. Furthermore, the mRNA levels for each of the genes exhibit developmental changes that are correlated with morphogenesis and rising titers of plasma T₃, and sustained expression depends on the continued presence of the hormone. Taken together, these observations support the hypothesis that the genes that we have identified play important roles in metamorphosis of the amphibian brain. We recognize that changes in mRNA level may not accurately predict changes in functional protein, and further analyses of the expression of each of these genes at the protein level is required. Furthermore, functional analyses will be required to define the precise roles that each of the proteins play in neural development. Nevertheless, predictions can be made, based on protein structure and the available information on function, regarding the interactions among the protein products of the cloned genes in development (see below).

Several attempts to isolate T₃-regulated genes in neonatal rodent brain by differential screening methods have met with limited success (52–54). Using subtractive hybridization, Munoz *et al.* (52) identified two cDNAs corresponding to T₃-regulated mRNAs that were decreased only 2-fold in brains from hypothyroid rats; one of these was tentatively identified as myelin basic protein, whereas the other was not identified. Two recent attempts identified several mitochondrial genes as T₃-responsive. Vega-Nunez *et al.* (53) used subtractive hybridization starting with neonatal rat brain mRNA to identify three genes: 12S and 16S rRNAs and cytochrome *c* oxidase subunit III. Igelesias and colleagues (54) used a whole-genome PCR method to identify NADH dehydrogenase subunit 3 as a T₃-regulated gene in neonatal mouse brain. It should be noted that in the first two studies (52, 53) neonates were made hypothyroid by treatment of dams with the goitrogen methimazole and then surgical thyroidectomy just after birth. The induction of hypothyroidism extended over several weeks, from the fetal period to several weeks postnatally, the developmental interval when T₃ is presumably critical for normal brain development in rodents (4). Immediate, early T₃-response genes would not have been isolated in these screens. Thus, these investigators

may have missed the interval when differences in gene expression were most pronounced.

The efficiency of isolating genes by subtractive hybridization is enhanced if the differences between the developmental, hormonal or physiological states are maximized. In the tadpole, there is no T₃ produced until the onset of prometamorphosis (22). Thus, one can choose this threshold stage of development to manipulate thyroid activity and generate a distinct “plus” and “minus” hormone state, a condition that cannot be achieved in mammals due to the presence of T₃ throughout the gestational period from maternal transfer of hormone across the placenta (4).

Postembryonic Development of the Amphibian Brain—Metamorphosis of the amphibian brain involves a coordinated process of cell replacement, cell death, and functional reorganization, all controlled by T₃ (55–57). Certain nervous structures used by the tadpole are eliminated (*e.g.* the Mauthner neurons and motor neurons that innervate tail muscle; Ref. 56), whereas others required for life as an adult tetrapod develop (*e.g.* the major part of the retina and associated visual projections in the diencephalon (58), the cerebellum, the spinal cord segments projecting to the limbs, the mesencephalic V nucleus, etc. (56, 57)). Administration of T₃ to tadpoles results in precocious maturation of the nervous system (56, 57).

Development of the neurosecretory cells located in the tadpole diencephalon is dependent on T₃ (18, 59). The major neurosecretory region of the tadpole brain is the preoptic nucleus, the neurosecretory cells of which develop during premetamorphosis in parallel with the development of the thyroid follicles (18, 60). In addition, the median eminence, the structure that conveys neurohormones from the hypothalamus to the pituitary portal circulation, is dependent on T₃ for its development (18, 61). Thus, T₃ not only stimulates the growth and differentiation of the hypothalamic neurosecretory centers controlling the thyroid axis, but also the structure that conveys the thyrotropin-releasing factor to the anterior pituitary gland.

T₃-regulated Gene Expression during Metamorphosis—Thyroid hormone is thought to drive the transformation of larval into adult tissues by inducing a series of tissue-specific gene expression changes. This hypothesis is supported by studies of gene expression in tadpole tail, hind limb, and intestine (13–15, 25). As for ecdysone-induced insect metamorphosis (62), T₃-regulated gene expression during amphibian metamorphosis can be divided into at least two distinct waves (8, 17). Most of the early genes (primary response genes; many are transcription factors) are probably directly regulated by the hormone and, it is predicted that they induce a second set of genes (secondary response genes). These secondary response genes ultimately lead to the expression of the adult phenotype.

In both insects and amphibians, a single hormonal signal induces very different types of developmental changes in different tissues through direct actions on each of the tissues (*e.g.* in the tadpole, T₃ initiates limb differentiation, intestinal remodeling, and apoptosis in the tail; Refs. 7, 16, and 17). Morphogenesis of the different organs is not synchronous because different tissues develop competence to respond to T₃ at different times (63). As in insects, many of the early response genes are expressed in all tissues (*e.g.* BTEB protein, TR β , bZip protein, type III 5-D), whereas the secondary response genes are tissue-specific (16). Genes that were isolated from more than one tissue in tadpoles include TR β (tail and intestine; Refs. 12, 13, and 15), BTEB protein (tail and brain; Refs. 12 and 13; this study), type III 5-D (tail and brain; Refs. 12, 13, and 35; this study), bZip (tail and intestine; Refs. 12, 13, and 15), and stromelysin 3 (tail and intestine; Refs. 12, 13, and 15). Each of these five genes is ubiquitously up-regulated by T₃.

A general feature of all tissues that have been studied is that the early appearing T_3 -regulated genes belong to different classes. Because these genes are not all transcription factors, a simple gene regulation cascade is not applicable. Instead, T_3 induces a complex series of intra- and extracellular events simultaneously. The cooperation of these processes determines organ-specific transformation.

Integration of the Gene Expression Program—Four general classes of genes were identified in this screen as T_3 -regulated: transcription factors, cellular enzymes, a cytoskeletal element, and secreted signaling molecules. The transcription factors are all early T_3 -response genes that probably function to activate or repress sets of downstream genes. These downstream genes are likely responsible for specifying the adult phenotype.

The functions of some of these transcription factors can be predicted based on their structural similarities to known genes or the known functional roles of some (*e.g.* TR β) in tadpoles. Two genes (TR β and glucocorticoid receptor) are ligand-dependent transcription factors that are members of the steroid receptor superfamily. The first demonstrable change in gene expression in the premetamorphic tadpole exposed to T_3 is the autoinduction of TR β (6, 64). TR β is thought to play a central role in inducing expression of downstream genes (6). The highest concentration of TR mRNA transcripts is found in the tadpole central nervous system (65), which becomes “competent” to respond to T_3 earlier and exhibits a more dramatic response to exogenous T_3 than other tadpole tissues (18, 56, 63, 66). Similar distribution and developmental expression patterns for the TR genes have been reported for rodents and chickens (66–69). Furthermore, autoinduction of TR β has been demonstrated in cultures of chick hypothalamic neurons (70) and rat astrocytes (71). Glucocorticoids are known to exert a number of important actions during vertebrate development, and positive interactions among the thyroid and adrenal corticosteroid axes have been shown in tadpoles (20). This synergy might be explained by cross-regulation of nuclear receptor expression or cooperative interactions at regulatory sites in target genes.

Two transcription factor genes that are regulated by T_3 in neural tissue and that were previously isolated from tadpole tail (12, 13) and intestine (15) code for the BTEB and the bZip proteins. The BTEB protein gene is strongly expressed in brain and other tissues of metamorphosing tadpoles. Given the strong and ubiquitous expression of this gene, the BTEB protein may be central to the induction of secondary, delayed response genes and perhaps the sustained expression of some of the primary response genes. If the *Xenopus* bZip protein is a transcriptional repressor, as has been suggested for its human homolog E4BP4 (48, 49), it could function to repress larval-specific genes or perhaps play a counter-regulatory role on the genes induced by T_3 during metamorphosis. The identity of the putative *Xenopus* HMG-box-containing protein is presently unknown. However, it is interesting that the rat Hbp1, to which the *Xenopus* protein exhibits the greatest similarity, has been implicated in the regulation of cell differentiation pathways (46).

Another class of genes isolated in this screen code for several types of cellular enzymes. The monodeiodinase that we cloned is identical to a type III 5-deiodinase (converts T_4 to reverse T_3 and T_3 to T_2 , both inactive forms of the hormone) isolated from tadpole tail (12, 13, 35). The primary role of this protein may be to negatively modulate target tissue levels of T_3 .

Although T_3 has no effect on adult brain metabolism in mammals, it does exert a stimulatory action in neonatal animals (72). This action may subservise an adaptive function of providing energy for increased metabolic demands during cell

proliferation and differentiation (72). We isolated two T_3 -regulated genes whose protein products are enzymes that control cellular energy conversions. The increased expression of mitochondrial cytochrome oxidase subunit I in the tadpole may be correlated with changes in brain oxidative phosphorylation. In mammals, neonatal hypothyroidism results in decreased brain oxidative phosphorylation (73) and alterations in mitochondrial morphology (74). Recent reports in rat and mouse show that the expression of several mitochondrial genes is altered by thyroid status in neonatal animals (53, 54, 75). Another energy-converting enzyme identified in this screen is brain-type CK. Thyroid hormone is known to regulate CK activity in muscle cells (76). Brain CK activity increases during development (77), and several lines of evidence suggest that brain-type CK is involved in the energetics of neurotransmitter release, restoration of ion gradients following membrane depolarization and axonal transport (78–82).

A fourth cellular enzyme that we isolated is a protein disulfide isomerase-like protein. Mammalian PDIs are multifunctional proteins that catalyze the isomerization of disulfide bonds and serve as subunits for more complex enzyme systems (41). The isomerase activity is especially important in cells that actively secrete protein (*e.g.* neurosecretory neurons, developing cells producing extracellular matrix; Ref. 41). Mammalian PDIs are members of a class of cytosolic enzymes that bind thyroid hormone (43, 83). The precise role of hormone binding in regulating the activity of these enzymes is not clear. However, in rat neural cells, thyroid hormone exerts rapid effects on actin polymerization and laminin-integrin interactions by a mechanism that is independent of hormone binding to TRs and may involve hormone binding to PDI (83–85). It is also possible that PDI may regulate the bioavailability of thyroid hormone for binding to TRs as has been proposed for other cytosolic thyroid hormone binding proteins (86).

The third and fourth classes of genes identified as T_3 -regulated in tadpole brain include a cytoskeletal element (neural-specific β tubulin) and two neuropeptides (thyrotropin-releasing hormone and corticotropin-releasing hormone), respectively. The expression of components of the cytoskeleton are critical for developmental processes, and T_3 deficiency in mammals results in abnormalities in neuronal outgrowth and synapse formation (4). Levels of tubulin mRNAs have been shown to be T_3 -dependent in neonatal rats (87). The development of the tadpole median eminence (the brain structure that conveys neurohormones to the anterior pituitary gland) is dependent on T_3 (see Ref. 18). This involves axonal extension from neurosecretory cell bodies and contact of modified nerve terminals with a capillary plexus, a process for which increased expression of tubulins and perhaps other cytoskeletal elements may be required. The secreted factors thyrotropin-releasing hormone and corticotropin-releasing hormone are conveyed to the anterior pituitary, where they influence the secretion of polypeptide hormones that control thyroid and adrenal corticosteroid production. These neuropeptides (especially corticotropin-releasing hormone; see Ref. 18) are thought to be critical to the activation of the thyroid axis during metamorphosis. The effects of T_3 on the mRNA levels for these neurohormones probably reflects a dual role for T_3 : differentiation of neurosecretory neurons and negative feedback on neuropeptide gene expression (18).

Conclusions

This study represents the most comprehensive analysis thus far of T_3 -regulated genes in the developing vertebrate brain. Genes isolated in this screen represent several classes of proteins, showing that the program is complex. Future full-length

cloning of the unidentified cDNAs should provide basic information on the gene expression program and may identify new T₃-regulated genes in the vertebrate central nervous system.

Many of the structural and functional changes that occur during the metamorphic transition from larval to adult frog brain are similar or identical to those seen in the mammalian fetus and the chick (e.g. acquisition of adult sensory and motor structures and development of neuroendocrine centers; Ref. 55). Perhaps even more basic and generalizable are the biochemical changes that occur during vertebrate neural development, and preliminary findings show that T₃ regulation of several genes involved in neural cell differentiation are conserved in frogs, birds and mammals. Further analysis of the tadpole gene regulation program will determine whether other genes that have been shown to be T₃-dependent in mammals are also regulated in tadpole brain (e.g. myelin basic protein, myelin-associated glycoprotein, proteolipid protein, neurogranin, neurotrophin-3, and Purkinje cell protein-2; Ref. 88). Conversely, it will be important to determine whether the T₃-dependent genes identified in amphibian brain (e.g. basic transcription element binding protein, bZip protein, protein disulfide isomerase, glucocorticoid receptor, and so forth) are also regulated in neonatal mammal and chick brain. Our studies provide a foundation for determining the functional roles of T₃-response genes in neural development.

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Note Added in Proof—Recently, Iglesias *et al.* (89), using a whole-genome PCR approach, isolated seven clones that correspond to genes that are transcriptionally regulated by T₃ in neonatal rat brain. One of these clones was identified as the neural cell adhesion molecule NCAM and another as α -tubulin. Also, Thompson (90), using subtractive hybridization, identified a novel synaptotagmin and a *hairless* homolog as T₃-regulated genes in neonatal rat cerebellum.

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