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Developmental Biology

Cultured cells as a model for amphibian metamorphosis

(thyroid hormone/gene regulation/subtractive hybridization/*Xenopus laevis*)

AKIRA KANAMORI AND DONALD D. BROWN

Department of Embryology, Carnegie Institution of Washington, 115 West University Parkway, Baltimore, MD 21210

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ABSTRACT **Gene expression screens have been applied to a cultured cell line of *Xenopus laevis*, XL-177, to isolate genes that are up- and down-regulated in the first 8 h after thyroid hormone (TH) induction. At least 14 up-regulated genes were isolated from TH-induced cells grown in the presence or absence of cycloheximide, an inhibitor of protein synthesis. These genes respond directly to TH as demonstrated by the resistance of up-regulation to protein synthesis inhibition in the cultured cells or in tadpoles. Kinetics of mRNA accumulation after TH induction is similar for these genes, including those that are superinduced by cycloheximide. Their mRNAs start to be up-regulated several hours after TH treatment and reach maximum levels between 8 and 16 h. These genes show up-regulation in one or more tadpole organs in response to exogenous TH. Only a few minimally down-regulated genes were identified. Fourteen of the 20 genes that were found to be up-regulated by TH in tadpole tail are also up-regulated in XL-177 cells. Their up-regulation falls into the same two kinetic patterns in the cultured cells as it does in tadpole tail. Another cell line of *X. laevis*, XLA, is greatly reduced in its ability to up-regulate the same genes isolated from XL-177 cells and tadpole tails in response to TH. Thus these cell lines make up a model system to examine the interactions of gene expression triggered by TH during amphibian metamorphosis.**

We have been studying amphibian metamorphosis as a complex program that results from changes in gene expression. The program is initiated by thyroid hormone (TH) (1, 2) presumably through its interaction with thyroid receptors (TRs) that are known to act as transcription factors (3, 4). Our approach to understanding this program began by cloning *Xenopus laevis* TRα and TRβ (5), correlating their expression with metamorphosis (6), and then characterizing the earliest part of the TH-induced response. This required the development of a sensitive PCR-based subtractive hybridization method that can isolate cDNA fragments derived from TH-induced up- and down-regulated genes (7).

The subtractive hybridization method, termed a "gene expression screen," has been applied to the early TH responses of hind limb (8), tail (7, 9), and intestine (Y.-B. Shi and D.D.B., unpublished data) of *X. laevis* tadpoles. Many TH-regulated genes have been isolated and some have been identified by their sequence relatedness to known gene(s). This paper addresses the next phase of the project, a model system that can be used to analyze the functional interactions of genes during cascades of gene expression triggered by TH. Currently, to our knowledge, there are no reliable methods to study gene function in tadpoles, so we asked whether cultured cells might serve as a simplified model system where familiar gene transfer methods could be used. The gene expression screen has been applied to a *X. laevis* cultured cell line (XL-177) that had been shown to express TRα mRNA constitutively and to respond to TH by up-regulating TRβ mRNA (11). We report here that the XL-177 cell line up-regulates many of the same genes that respond to TH in tadpoles including a subset of the genes with a delayed response. Another cell line of *X. laevis* (XLA) expresses TRα mRNA constitutively but fails to up-regulate TRβ mRNA. This cell line is greatly reduced or defective in its ability to up-regulate many of the genes isolated in the present study or those induced in metamorphosing tadpoles. The combination of these two cell lines, one that responds to TH similarly to tadpoles and the other that is defective in this response, can serve as a model system for the interactions of these genes and their products during amphibian metamorphosis.

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MATERIALS AND METHODS

Animals. *Xenopus laevis* tadpoles were raised in dechlorinated slowly recirculating tap water and fed nettle powder and salmon brittle. Developmental stages were determined according to Nieuwkoop and Faber (12). Ten tadpoles were treated in 300 ml of water to which 5 nM 3,5,3′-L-triiodothyronine (T_3) and/or protein synthesis inhibitors were added.

Cell Culture. Cell line XL-177 was derived from tadpole epithelium of *X. laevis* (13) and was the gift of Leo Miller (University of Illinois at Chicago). Cell line XLA is a *X. laevis* epithelial cell line of uncertain origin that resembles in morphology and may be identical to the A6 cell line derived from adult kidney (14). Cultured cells were maintained in 70% (vol/vol) Leibovitz medium containing 10% (vol/vol) fetal bovine serum (GIBCO/BRL), gentamycin sulfate (100 μg/ml), and 10 mM Hepes (pH 7.5) at 25°C. Cells were fed twice a week and passaged before they became confluent. Semiconfluent cells were cultured for 24 h prior to use in medium supplemented with serum that had been passed through the resin AG1-X8 (Bio-Rad) to deplete TH (15). XL-177 cells cultured in the absence of TH for more than a week leads to their detachment from cultured dishes. Readdition of TH rescues this detachment. TH has no effect on the morphology of XLA cells (A.K. and D.D.B., unpublished data).

RNA Extraction and Blot Analysis. Total RNA was extracted from cells and tadpoles by using guanidinium thiocyanate and cesium chloride centrifugation (16), electrophoresed, blotted, and hybridized as described (11). The autoradiograms used preflashed films and were exposed for various times at −80°C with an intensifying screen so that the resulting bands could be quantified by densitometry (Molecular Dynamics, Sunnyvale, CA). Probes for *X. laevis* TRα, TRβ, and actin have been described (11).

PCR-Based Quantification of TRβ Transcripts. The transcripts of TRβ were quantified as described (11). Aliquots of total RNA were reverse-transcribed with complementary RNA internal standards by using an antisense primer complementary to the coding region of TRβ. The resulting DNA was amplified by PCR with pairs of primers located either in

Abbreviations: TH, thyroid hormone; TR, TH receptor; T_3, 3,5,3′-L-triiodothyronine; CHX, cycloheximide.

exon a or exon b, the two 5′ most exons of the TRβ transcription units.

PCR-Based Library Subtraction. Total RNA was extracted from XL-177 cells treated for 8 h with or without 5 nM T_3 and/or cycloheximide (CHX, 10 μg/ml). Poly(A)$^+$ RNA was prepared with oligo(dT) columns (Pharmacia, type 7) (17) and converted to double-stranded cDNA with oligo(dT) priming using a Copy Kit (Invitrogen, San Diego). Four subtractive libraries were constructed according to Wang and Brown (7) with minor modifications. All subtractive hybridizations were scaled down to half the volume. The enriched tracer cDNA was amplified by PCR without purification (5 μl of enriched tracer per 100 μl of reaction mixture). The original PCR-amplified cDNAs (prior to hybridization) were used as drivers in short hybridizations throughout the procedure. After three rounds of subtractive hybridizations, enriched cDNA fragments were amplified by PCR, digested with *Eco*RI, cloned into plasmid vector pBluescript KS(−) (Stratagene), and transformed into DH5α cells.

Plasmids containing cDNA fragments from TH up-regulated genes were sequenced by dideoxynucleotide chain-termination using deoxyadenosine 5′-[α-[^{35}S]thio]triphosphate, 7-deaza-dGTP, and Sequenase Version 2 (United States Biochemical) or by an automated system using fluorescent dye terminators (Applied Biosystems). The DNA sequences and predicted peptide sequences were searched for homology to known sequences in the data bases using the FASTA program (18).

Southern Blot Analysis of PCR-Amplified cDNAs. PCR-amplified cDNA samples from cells and tadpole organs were separated with 1.5% agarose and transferred to Nytran filters (Schleicher & Schuell) with 0.5 M NaOH/0.6 M NaCl. After overnight transfer, filters were neutralized with 5× SSC (1× SSC is 15 mM sodium citrate/150 mM NaCl, pH 7.0) for 10 min, UV-irradiated to fix the DNA, and hybridized as for RNA blot analysis.

RESULTS

The Screen for TH-Regulated Genes in Cultured Cells. To isolate TH-regulated genes from cultured cells, four populations of double-stranded cDNAs were prepared from control and TH-treated XL-177 cultured cells in the presence or absence of CHX, a protein synthesis inhibitor. The reciprocal hybridization enrichment procedure paired the control (−) cDNA and TH-induced (+) cDNA. Hybridization of excess (+) cDNA (driver) against (−) cDNA (tracer) enriches for down-regulated genes (library A). The opposite procedure enriches for up-regulated genes (library B). The cDNA prepared from CHX-treated cultures was paired with that from cells treated with both CHX and TH. These two reciprocal enrichments yield down-regulated (library C) and up-regulated (library D) genes. The purpose of the latter two screens was to search for "immediate-early" genes that respond directly to TH, especially those that are superinduced by inhibition of protein synthesis, a phenomenon that has been seen for immediate-early direct-response genes in other programs of gene expression (19, 20). To maximize the chance of finding the earliest-response genes, cells were collected after 8 h of TH induction. This time was chosen because the kinetics of TRβ up-regulation has a lag of ≈4 h in XL-177 cells after TH induction (11). Cells treated with CHX for >8 h show nonspecific reduction of RNA synthesis (data not shown).

The enrichment process was followed by Southern blot analysis of the cDNA fragments (Fig. 1). In each case, the enriched radioactive cDNA mixture itself was used as a probe. The hybridization profile shows that those fragments enriched in B cDNA are also enriched, but to a lesser extent, in D cDNA and vice versa. Down-regulated genes are not

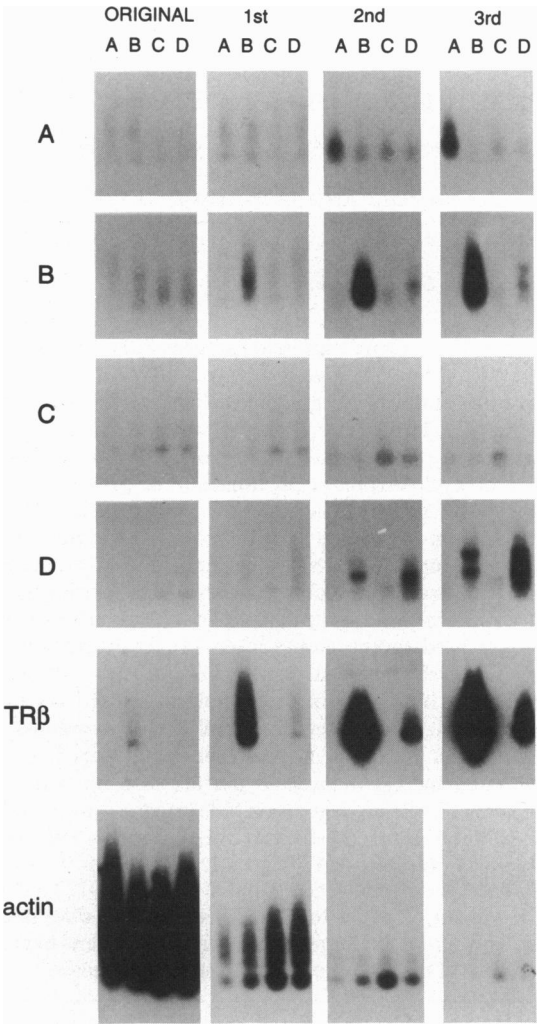

FIG. 1. Southern blot analyses of cDNA fragments that remain in the tracer after each of three subtractive hybridization enrichment steps. PCR-amplified cDNA fragments (1 μg), before subtraction (original) and after the first, second, and third rounds of subtraction, were loaded in each lane, separated in 1.5% agarose, blotted on Nytran filter, and then hybridized with one of the final (third round) enriched cDNA mixtures A, B, C, or D, TRβ, or actin.

abundant since A and C cDNAs hybridize weakly even with themselves. The extent of enrichment of a known up-regulated gene (TRβ) was followed. Its enrichment in D cDNA (200-fold) is less than in B (400-fold) because of the reduced up-regulation of TRβ mRNA in the presence of CHX compared to that in control cells (11). The mRNA encoding actin is common to all four mRNA preparations, and the actin cDNA fragments are suppressed two orders of magnitude by the subtraction procedure.

Since no significant cross-hybridization occurred between the partners (A and B or C and D) after three rounds of subtraction (Fig. 1), the four mixtures of enriched cDNA fragments were converted to plasmid libraries, and individual cloned cDNA fragments were assayed for TH-induced regulation by hybridizing with RNA blots (Fig. 2). We identified 12 and 10 non-cross-hybridizing cDNA fragments from cDNA libraries B and D, respectively. Two cDNA fragments were isolated independently from both libraries (42D and 57D in Table 1). This is expected since B and D cDNAs cross-hybridize (Fig. 1).

The gene expression screen is applied to short cDNA fragments prepared by restriction digestion of full-length cDNAs. Therefore, independently isolated fragments that do

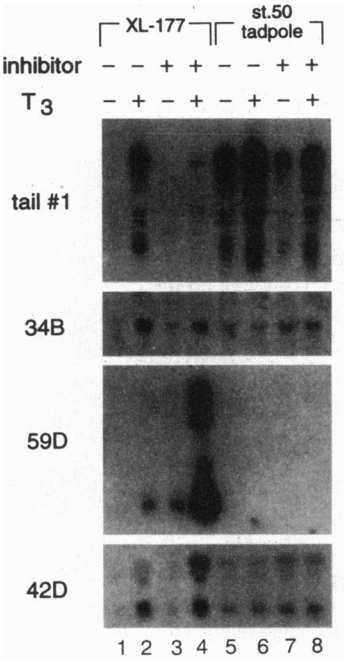

FIG. 2. TH up-regulates genes cloned from libraries B (tail 1 and 34B) and D (59D and 42D). XL-177 cells (lanes 1–4) or stage 50 tadpoles (lanes 5–8) were treated for 8 h with various combinations of 5 nM T_3 and protein synthesis inhibitors (CHX at 10 μg/ml for cells and CHX at 20 μg/ml plus 100 μM anisomysin for tadpoles). Total RNA (10 μg) was loaded in each lane and analyzed by RNA blot hybridization.

not hybridize with each other can be derived from the same original full-length mRNA. As the search for fragments identifies these multiple fragments from the same gene, the extent of complexity of the mixture can be assessed (7). Two independent cDNA fragments were found that are derived from a TRβB genomic clone. Two cDNA fragments were mapped to tail 1 gene, which had been cloned independently from the tadpole tail screen (7). Suggestive, but less conclusive, evidence that two cDNA fragments originate from the same mRNA comes from the similarity of their RNA blots (i.e., the size of the mRNA, the kinetics of TH induction, and developmental and organ specificity profiles in metamorphosing tadpoles and adults; data not shown). From these experiments, we conclude that the 20 cDNA fragments represent a maximum of 18 and a minimum of 14 up-regulated genes (Table 1). Those fragments that might originate from the same gene, by these criteria, are designated in Table 1.

Table 1. TH up-regulated genes in XL-177 cells

Gene	Feature	RNA, kb	Tadpole organ B	HL	T	I	XLA
TRβB		11	+	+	+	+	
Tail 1	Zinc finger	12,10,1.8	+	+	+	+	
Tail 3		11	+	+	+	+	
81B	Fragment A	11	+	+	+	+	
49B		11		+	+	+	
34B		3		−	+	+	
26B		2.8	−	−	−	−	−
28B		13	+	+	+		
42D*		5,1.7	−	−	+	−	+
57D*	Fragment B	13			+		+
85D*	Fragment B	13			+		+
33D*	Fragment B	13			+		+
59D*	Fragment B	13–1.3			+		+
62D		13				+	+
61D		11	+	+	+	+	−
46D		11	+	+	+	+	
52D*		12			+		
58D*	Cadherin	11	+	+	+	−	−

Genes TRβB through 28B were cloned from library B. Genes 42D and 57D were cloned independently from both libraries B and D. The remaining genes were cloned from library D. Genes with an asterisk are superinduced in XL-177 cells in the presence of T_3 and CHX. Fragment A, cDNA fragment that may be derived from tail 1 or tail 3; fragment B, four cDNA fragments that may be derived from a single gene. B, brain; HL, hind limb; T, tail; I, intestine; +, up-regulated by T_3 in 18–24 h; −, not regulated by T_3 but a baseline of gene expression is detectable; blank, no signal detected. XLA cells were treated for 8 hr with or without 5 nM T_3. +, Up-regulated by T_3; −, not regulated by T_3 but a baseline of gene expression is detectable; blank, no signal detected.

A similar search for down-regulated genes in the enriched cDNA mixtures A and C yielded four fragments that by Northern blot are down-regulated only 2-fold in 8 h. This rarity of down-regulated compared to up-regulated genes agrees with the gene expression screens carried out with various tadpole organs (refs. 8 and 9 and Y.-B. Shi and D.D.B., unpublished data). All further experiments have concentrated on the up-regulated genes.

Identification of Up-Regulated Genes. All 20 cDNA fragments ranging in size from 206 to 663 bp were sequenced. Six of these had long open reading frames, and two out of these 6 fragments had sequences similar to known proteins in the data bases. The fragment 58D encodes a member of the cadherin family; tail 1 encodes a zinc-finger protein. As mentioned, two fragments of TRβB were identified. All 20 fragments were tested for their ability to hybridize to the collection of large λ cDNA clones that had been isolated in other gene expression screens as encoding TH-induced genes (8, 9). Two fragments hybridized to tail 1 as mentioned, and one fragment hybridized to tail 3.

The Up-Regulated Genes Respond Directly to TH. The response of each gene to TH was tested for its resistance to an inhibitor of protein synthesis (CHX) in XL-177 cells. The results, after 8 h of TH induction in the presence and absence of the inhibitor, are compared with the same response in a stage 50 tadpole (Fig. 2). The tadpoles were treated with anisomysin plus CHX to inhibit protein synthesis by 99% (11). Some of these genes respond differently in cultured cells than in tadpoles, a phenomenon also described for the up-regulation of TRβ (11). Although the kinetics of TRβ up-regulation is the same in cultured cells as in tadpoles, the response is ≈85% inhibited by CHX in cultured cells but mainly resistant to protein synthesis inhibition in tadpoles. The first four genes in Table 1 from library B show this same difference as exemplified by tail 1 in Fig. 2. To explain this paradox, we suggest that some key component of the TH-induced response is labile and less abundant in cultured cells than in tadpole tissues. However, the factors necessary for up-regulation are present from the start in both cultured cells and tadpoles, making these genes directly responsive to the hormone. The other genes from library B are 35–100% resistant to CHX in XL-177 cells as illustrated by gene 34B in Fig. 2. The genes identified from library D are either resistant to or superinduced by CHX (Table 1 and Fig. 2) in the cultured cells. We conclude that these up-regulated genes respond directly to the hormone.

Kinetics of Up-Regulation. An analysis of the kinetics of mRNA accumulation was carried out for each of the up-regulated genes after addition of TH. The earliest increase of mRNA is detectable at 2 h for several genes including tail 1 (Fig. 3). The kinetics for genes superinduced with CHX (Fig. 3*B*) are similar to those that are not superinduced (Fig. 3*A*). In fact, the kinetics of up-regulation of all of the genes identified in this screen are similar (Fig. 3 and data not shown). There is a lag of several hours preceding the onset of up-regulation. The maximum level of mRNA is reached

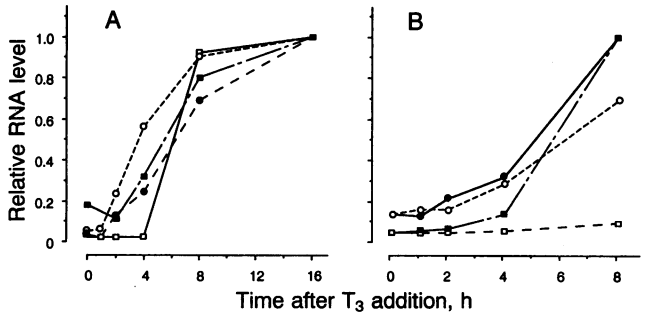

FIG. 3. Kinetics of TH-induced up-regulation in XL-177 cells. Total RNA (25 μg) was analyzed by RNA blot hybridization at various times after the addition of 5 nM T_3. (*A*) Probed with TRβ (□), tail 1 (○), 61D (■), and 34B (●). The amount of mRNA at 16 h was set arbitrarily at 1. (*B*) The mRNA levels of 59D in the presence (■) or absence (□) of CHX at 10 mg/ml. The mRNA levels of 42D in the presence (●) or absence (○) of CHX at 10 mg/ml. The amount of mRNA at 8 h in the presence of CHX was set arbitrarily at 1.

between 8 and 16 h. No transiently up-regulated immediate-early genes were found.

A Common Subset of Genes Are Up-Regulated in XL-177 Cells and in Tadpoles. Four tadpole organs have been screened for TH-induced genes: hind limb (8), tail (9), intestine (Y.-B. Shi and D.D.B., unpublished data), and brain (K. Vernick and D.D.B., unpublished data). The initial PCR-amplified cDNA fragments from control (−) and TH-induced (+) organs resemble the mRNAs from which they were derived (7) and can, therefore, be used as a sensitive but qualitative indication of TH regulation. Southern blots of the original − and + cDNA mixtures from brain, hind limb, tail, and intestine were probed with the cDNA fragments isolated from XL-177 cells. All of these + cDNAs were prepared from mRNA 18–24 h after TH induction, when the accumulation of up-regulated mRNAs is greater than at 8 h. The results are summarized in Table 1. About half of the genes from XL-177 cells are up-regulated in all four organs. Others are regulated in a subset of organs; one gene (26B) is not regulated in any of the four organs. Some of the CHX-superinduced genes isolated from library D (57D, 85D, 33D, and 59D) are expressed at very low levels even in the tail where their up-regulation is observed.

Next, we systematically probed for expression of genes that had been identified by the gene expression screen of the tadpole tail with control and TH-induced cultured cell mRNA. This was of special interest because two kinetic periods had been found in the program of gene expression preceding tail resorption (9). The early period has kinetics that are similar to the genes described here; the later period is delayed by ≈15 h and genes expressed during this period were, therefore, not isolated in the present screen. Many of these genes are also regulated in tadpole organs other than tail (9). Fig. 4 compares the kinetics of up-regulation of two early genes (TRβ and 59D) and three delayed genes identified from tail (tail A, D, and 11). It is clear that both sets of genes are up-regulated in XL-177 cells with kinetics similar to that found in tadpole tail. Altogether, 6 of the 8 early genes identified in the tail are up-regulated as early genes in XL-177 cells (tail 1, 3, 6, 12, 14, and 15). Tail 2 is not detected and tail 16 is expressed constitutively in the cell line. Eight of the 12 delayed genes found in tail are slightly up-regulated in the first day after TH induction, but then increase in mRNA abundance over the next few days (tail A, B, D, 4, 8, 9, 11, and 13). Tail E and 5 are not detected and tail C and 7 are expressed constitutively in XL-177 cells. Therefore, a subset of tadpole gene regulation is faithfully reproduced in cultured cells with the same kinetics of up-regulation.

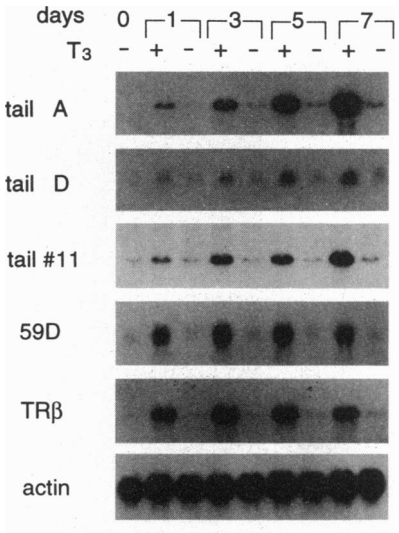

FIG. 4. Two distinct kinetic profiles of up-regulated genes in XL-177 cells. XL-177 cells were treated with or without T_3 (5 nM) for the indicated days with daily medium change. Total RNA (25 μg) was loaded in each lane and analyzed by RNA blot hybridization.

The XLA Cell Line Is Defective in Up-Regulation. Another cell line, XLA, was established many years ago and grows rapidly. XLA cells are constitutive for the expression of TRα mRNA but synthesize no full-length TRβ mRNA detectable by RNA blot analysis in the presence or absence of TH (Fig. 5). A similar observation has been reported for Kr cells, which is a clonal line of A6 and, therefore, may be identical to XLA cells (10). A sensitive PCR quantification (11) was used to detect mRNAs originating from the constitutive (exon a containing) or the inducible (exon b containing) promoters of the TRβ genes. No transcripts are detected from exon a in XLA cells. The transcripts containing exon b are up-regulated in XLA cells minimally (≈3-fold) compared to >20-fold up-regulation in XL-177 cells. In addition, no full-length mRNAs encoded by the TH-induced up-regulated

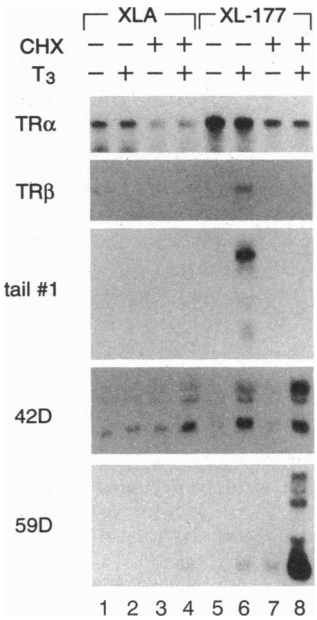

FIG. 5. Expression of TH-induced up-regulated genes in XLA cells. XLA cells (lanes 1–4) and XL-177 cells (lanes 5–8) were treated for 8 h with or without 5 nM T_3 and/or the protein synthesis inhibitor CHX (10 μg/ml). Total RNA (20 μg) was analyzed by RNA blot hybridization.

genes isolated from library B are up-regulated in XLA cells (Fig. 5 and Table 1). Most of the genes that are superinduced by CHX (library D) are superinduced minimally in XLA cells; their mRNAs can be detected at a low level only in the presence of both CHX and TH. None of the delayed tail genes are up-regulated in XLA cells even after 7 days of TH treatment.

DISCUSSION

Using the gene expression screen, we have isolated a minimum of 14 genes that are up-regulated within 8 h after TH induction in the cultured cell line XL-177. Three of these, TRβB, tail 1, and tail 3, were found independently in the screen of TH-up-regulated genes in the tail (7). The identification of multiple cDNA fragments derived from the same original mRNA (TRβB and tail 1) suggests that the number of up-regulated genes in XL-177 cells is limited (for discussion, see ref. 7). In the tail response to TH, we estimated that there are $\approx$35 genes that are up-regulated in the entire 48-h program encompassing two periods of up-regulation (9). In contrast, the limb up-regulates a very large number of genes in the first 24 h after TH induction (8).

All evidence suggests that the XL-177 genes identified in the screen after 8 h of TH induction are the earliest that respond directly to TH. Their up-regulation by TH is resistant to protein synthesis inhibition either in XL-177 cells or tadpoles. Immediate-early genes in some complex programs are up-regulated quickly but their mRNAs are then degraded rapidly (19, 20). Protein synthesis inhibition prolongs their presence, often at greatly elevated levels. We, therefore, screened cells induced with TH in the presence of CHX. A different set of up-regulated genes was detected. This includes genes that are superinduced by the combined treatment of CHX and TH. However, some of their mRNAs are very rare even in tadpole tails where their up-regulation in response to TH is observed. It is possible that their expression is restricted to specific cell types in the tadpole. Most importantly, the kinetics of their up-regulation in the cell line is the same with and without the inhibitor. The induction of these mRNAs has the familiar several-hour lag period that characterizes the early response induced by TH in tadpoles (Fig. 3; refs. 9 and 11 and Y.-B. Shi and D.D.B., unpublished data). We, therefore, conclude that the genes identified by screening the two libraries B and D are the earliest-response genes that require TR and TH for up-regulation.

There are no reliable methods available for analyzing gene function as late in amphibian development as the feeding tadpole stage. The use of *in vitro* cultured cells might provide an alternative solution to this technical obstacle if they reproduce the biological changes that occur in tadpoles during metamorphosis. In fact, TRβ genes, which were the first TH-response genes to be identified in *X. laevis* tadpoles (6), can be up-regulated by exogenous TH in a variety of *X. laevis* cell lines (A.K. and D.D.B., unpublished data), including XL-177 (11) and XTC-2 (10). Y. Yaoita (personal communication) has established a cell line from *X. laevis* tadpole tails in which the cells die upon addition of TH. This observation suggests that at least one of the many morphological changes induced by TH in tadpoles, programmed cell death, can be retained in a cultured cell line. Here we show that more than two-thirds of the genes that are regulated in tadpole tail are controlled by TH in XL-177 cells with the same kinetics. This fact demonstrates that at least part of the gene expression programs triggered by TH in tadpoles can be induced in this cell line. Another cell line (XLA) does not up-regulate TRβ significantly and fails to up-regulate most of the genes so far cloned in XL-177 cells and in tadpoles (Fig. 5 and Table 1). This combination of two cell lines, one that is capable of responding to TH in a manner similar to tadpoles and the other that is not, provides a model system for studying the molecular basis of TH-induced gene regulation by familiar kinds of gene transfer experiments.

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