The Transcription Factor Basic Transcription Element-Binding Protein 1 Is a Direct Thyroid Hormone Response Gene in the Frog *Xenopus laevis*

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Several genes have been identified that are activated or repressed by thyroid hormone in tadpole tissues during metamorphosis of the frog *Xenopus laevis*. One rapidly and strongly induced gene encodes the *Xenopus* homolog of basic transcription element-binding protein 1 (xBTEB1), an SP1-related transcription factor. xBTEB1 has similar DNA-binding activity and transcriptional activation properties as mammalian BTEB1. The thyroid hormone-dependent regulation of xBTEB1 was investigated using a modified electrophoretic mobility shift assay to scan genomic DNA for receptor-binding sites. Due to the tetraploid *X. laevis* genome, xBTEB1 is duplicated, and thyroid hormone regulates both copies. A consensus thyroid hormone response element (TRE) lies far upstream of the transcriptional start site of both genes. The TRE is nested within a 200-bp region of high sequence conservation between these two genes that duplicated millions of years ago. The TRE acts as a strong response element in transfection assays using a heterologous promoter or its native context. Thus, one of the earliest thyroid hormone-induced genes in tadpoles is a transcription factor regulated through an evolutionarily conserved TRE. xBTEB1 is predicted to play an important role in downstream gene regulation leading to the growth and remodeling of tissues at metamorphosis. (Endocrinology 143: 3295–3305, 2002)

RISING LEVELS of thyroid hormone (TH) from the developing thyroid gland control metamorphosis in amphibians, including the frog *Xenopus laevis* (1). This developmental switch involves the loss of larval tissues such as the tail, the remodeling of tissues such as the intestine and brain, and the growth of adult structures such as the limbs. These morphogenetic processes are precisely coordinated; for example, low levels of TH induce limb growth, which is completed before the highest levels of TH at climax induce tail resorption (1). These changes can be induced precociously by adding appropriate levels of TH to the rearing water of competent tadpoles. Many processes, including tail resorption and limb outgrowth, can be reproduced in isolation in organ culture, implying that each tissue is preset to respond directly to the hormone in a tissue-autonomous and TH concentration-dependent way (2). TH induces metamorphosis by initiating and maintaining gene expression changes (3). TH regulates target gene transcription by binding to a pair of evolutionarily conserved TH receptors (TRs) that function as ligand-regulated transcription factors (4, 5). The TRs repress transcription in the absence of hormone and derepress and then further activate transcription in the presence of hormone (4). Transcriptional regulation is controlled partly by reorganizing the chromatin structure of the response gene promoter (6). We predicted that the most rapidly induced genes regulated by TH will encode transcription factors (3), and these genes should contain discrete DNA sequences specifically bound by TRs, known as thyroid hormone response elements (TRES). Generally, TRES are comprised of a repeat of the half-site sequence AGGTCA (4). However, reported TRES vary widely in their sequence and arrangement of half-sites, and often require overexpression of exogenous TRs to observe a measurable response. The variety of reported TRs might reflect the fact that the genes from which these sequences are derived vary in the speed and magnitude of the response to TH and the tissue where they are induced. In addition, many TH response genes that have been studied in mammalian systems are weakly induced with sluggish kinetics. Very few TRES have been identified from amphibian TH response genes, where induction is typically much more robust (7, 8).

Amphibian metamorphosis presents an excellent opportunity to study TH regulation of gene expression cascades. One advantage to studying TH control of transcription in *Xenopus* is the large collection of well characterized TH response genes that are activated with varying kinetics, including a set of clear direct response genes. These response genes were isolated using a gene expression screen (9) to isolate cDNAs from genes regulated by TH in a number of metamorphosing tissues (10–14). In this paper we provide evidence that a direct and rapid TH response gene is the *Xenopus* homolog of rat and human basic transcription element-binding protein 1 (BTEB1), a zinc finger transcription factor that is related to SP1. BTEB1 was originally identified as a factor regulating the expression of cytochrome P450 genes in the rat liver (15). Interestingly, TH regulates BTEB1 expression during mammalian brain development (16). In addition, BTEB1 appears to work in concert with the progesterone receptor in progesterone regulation of the porcine
uteroferrin gene (17, 18). Here, we demonstrate that Xenopus BTEB1 (xBTEB1) has similar biochemical properties to mammalian BTEB1. xBTEB1 is duplicated in the Xenopus genome, and both copies are similarly up-regulated by TH. We used a modified EMSA to screen large amounts of genomic DNA for thyroid hormone response elements. We found a potent consensus TRE in both duplicated xBTEB1 genes. In the case of amphibian metamorphosis, direct transcriptional up-regulation of rapidly responding genes such as the transcription factor xBTEB1 through a strong TRE probably results in the regulation of downstream genes and the resultant dramatic tissue-specific morphological changes.

Materials and Methods

Animals and cell culture

X. laevis tadpoles were raised in the laboratory or purchased from Nasco, Inc. (Fort Atkinson, WI), and maintained in continuously running dechlorinated tap water. All animals were treated in accordance with an approved institutional animal use and care protocol. Tadpoles were staged according to Nieuwkoop and Faber (19). Tadpoles and frogs were anesthetized in ice water before dissection. All tissue samples were frozen in liquid nitrogen and stored at −80 °C. X. laevis cultured cells were maintained in 70% L-15 medium (Invitrogen, Carlsbad, CA) supplemented with 10% heat-inactivated fetal bovine serum (Invitrogen) at 22–25 °C. Indicated concentrations of t-3 (Sigma, St. Louis, MO) were added to tadpole rearing water or to culture medium containing serum stripped of TH (8). The cell lines used in this study were XLA, a kidney epithelial cell line indistinguishable from the A6 line by a variety of assays, and XTC fibroblast cells (gift from Donald St. Germain, Dartmouth Medical School, Hanover, NH).

Isolation of cDNA and genomic clones

A random primed and an oligo(dethymidinyl)-primed cDNA library (3) were screened using a short cDNA fragment encoding xBTEB1 that was isolated from the original subtractive hybridization of tadpole tail RNA (9). Other cDNA clones were prepared by RT-PCR using specific primers. Both strands of DNA were sequenced to minimize sequence errors using the PRISM 310 Genetic Analyzer (PE Applied Biosystems, Foster City, CA) and dye terminator reagents according to the manufacturer’s instructions. A genomic library was constructed from X. laevis genomic DNA (gift from Keith Joho and Donald Brown, Carnegie Institution of Washington, Baltimore, MD) and screened with a fragment of the full-length xBTEB1 cDNA from the 5′ end to the SacI site at +750 bp as described previously (8).

Northern analyses and primer extension

Total RNA was extracted with guanidinium isothiocyanate and purified by CsCl gradient centrifugation or TRIzol reagent (Invitrogen) and analyzed by Northern blotting as described previously (8). DNA probes were radiolabeled with [α-32P]deoxy-CTP (NEN Life Science Products, Boston, MA) by random priming. For the modified primer extension assay (20), RNA was reverse transcribed with gene-specific primers, then the cDNAs were electrophoresed through a 2.8% formalin agarose gel made of 2.1% NuSieve GTG and 0.7% Seakem GTG agarose (FMC Bio Products) and transferred to nylon membranes. The blots were hybridized with a double-stranded DNA–radiolabeled probe synthesized by PCR including [α-32P]deoxy-CTP (NEN Life Science Products), using xBTEB1A primers at the 5′ end of the longest cDNA and the RT primer.

EMSAs

Full-length synthetic xBTEB1, xTRA, xTRβ, xRXRa, or xRXRβ mRNAs were translated in vitro as described previously (8). For reverse gel shift assays, [35S]methionine (Amersham Biotech, Piscataway, NJ) was included in the translation mix for xTRA and xTRβ. A GST fusion protein containing the full-length xBTEB1 was made in Escherichia coli and purified by binding to glutathione beads (Sigma). Tail nuclear extracts were prepared by homogenizing tails (from tadpoles treated with or without 100 nM T3, for 48 h) in 10 mM Tris-Cl, 1 mM dithiothreitol, and a protease inhibitor cocktail (Roche Molecular Biochemicals, Indianapolis, IN) with a Tekmar Tissumizer (Tekmar-Dohrmann, Mason, OH). Homogenates were centrifuged at 500 × g, and the pellets were resuspended in 0.2 μl in homogenization buffer with a Dounce homogenizer ( Kontes Co., Vineland, NJ). An equal volume of homogenization buffer containing 0.8 mM KCl was added gradually with additional homogenization. The extract was centrifuged at 14,000 × g for 15 min at 4 °C. Brain whole-cell extracts were prepared as described above but without the Tissumizer homogenization and low speed centrifugation steps. Extracts were snap-frozen in liquid nitrogen and stored at −80 °C before use.

Both reverse and standard gel shift assays were performed as described previously (8). The sequences for the BTEB1 binding experiments of one strand (5′ to 3′) of oligonucleotides are as follows: GAGAAGGAGCCTGCGCAAC (20 mer) for BTE, ATCGGGGCGGCCITC (16 mer) for Sp1, and CAGTGGAGCTACCGCGGAA (21 mer) for the random oligonucleotide. Bold letters indicate the putative BTEB1-binding sites. The oligonucleotides used for the TR gel shift and transient transfection experiments are as follows: ACCTACGGGAGGTCATCTAGGTGTCACCTTA for the DR +4 TRE, ACCTACGGGAGGTCATCTAGGTGTCACCTTA for the xBTEB1 TRE. ACCTACGGGAGGTCATCTAGGTGTCACCTTA for the xBTEB1 mTRP (the nucleotides changed from wild-type are underlined), ACCTACGGGAGGTCATCTAGGTGTCACCTTA for the xBTEB1 TRE (GGT spacer), ACCTACGGGAGGTCATCTAGGTGTCACCTTA for the xBTEB1 TRE (GGG spacer), ACCTACGGGAGGTCATCTAGGTGTCACCTTA for the xBTEB1 TRE (GGC spacer), ACCTACGGGAGGTCATCTAGGTGTCACCTTA for the xBTEB1 TRE (GGC spacer).

Transient transfection assays

Wild-type or mutated TRE oligonucleotides were cloned into the unique HindIII site in the ΔMTV-luciferase vector (8). The orientation and number of inserts were verified by sequencing. xBTEB1 genomic clones were prepared by PCR using Pfu polymerase (Stratagene, La Jolla, CA) and were subcloned into the multiple cloning site of the pGL-2 Basic vector (Promega Corp., Madison, WI). Plasmids for transfection were purified using QIAGEN (Chatsworth, CA) midiprep kits. For transient transfection assays, 0.1 μg each of the reporter, the indicated amount of expression plasmids, and 0.1 μg pCS2-β-galactosidase vector (gift from David Turner, Fred Hutchinson Cancer Research Center, Seattle, WA) were cotransfected with 4 μl Lipopectamine reagent (Invitrogen) for cells in six-well culture plates (35-mm diameter) as described previously (8). Cells were replenished with culture medium after 1 d, cultured for 2 d with or without 100 nM T3, then assayed for luciferase and β-galactosidase activities (8).

Results

xBTEB1 is rapidly induced by TH in multiple X. laevis tadpole tissues

Two cDNA fragments were isolated in a subtractive hybridization screen for genes up-regulated by TH in resorbing tadpole tails (10). The sequences of these gene fragments showed a high degree of similarity to the rat BTEB1 cDNA (10). Using one of these partial xBTEB1 cDNAs as a probe, we found that this gene was up-regulated by TH in all tadpole organs and cell lines tested to date in addition to the tail. There were multiple mRNA species encoded by this gene, and the major bands at 12 and 10 kb comprised about 95% of the signal (Fig. 1A). Many of these bands were detectable
in polyadenylated (polyA+) tail RNA, including one as small as 1.7 kb. The xBTEB1 gene is constitutively expressed at varying levels in many frog organs and tissues, with the most abundant expression in spleen, skin, muscle, and fat body (data not shown). We examined the kinetics of xBTEB1 induction in three tissues: tail, hindlimb, and brain (Fig. 1B). xBTEB1 is up-regulated within 2–4 h in each tissue, making it one of the most rapid TH response genes in the tadpole. Previous studies have additionally shown that TH induction of xBTEB1 in the tail is mostly resistant to cycloheximide treatment (10). Cycloheximide resistance suggests that all factors required for at least the initial transcriptional response, including TRs and RXRs, exist before TH addition.

**Cloning of full-length xBTEB1 cDNAs**

To obtain the sequence of the complete open reading frame of X. laevis BTEB1, multiple full-length cDNAs encoding xBTEB1 were isolated by screening a tail cDNA library with the above short fragments isolated in the PCR-based subtractive screen (9). The longest cDNA includes a long 5'-untranslated region (5'UTR) of more than 500 bases with 9 AUG and stop codons in phase, an open reading frame encoding 292 amino acids, and a downstream consensus polyadenylation signal (Fig. 2A). This cDNA probably corresponds to the approximately 1.7-kb message detected on Northern blots of polyA+ RNA (Fig. 1A). Some of the cDNA clones isolated showed significant differences from the originally isolated cDNA (2% at the nucleotide level). Just four of these differences change amino acids (Fig. 2A), suggesting the presence of two similar genes for xBTEB1, as is the case for many X. laevis genes. This conclusion was later confirmed by isolating two distinct genomic clones with sequences identical to the two closely related cDNAs (called xBTEB1A and xBTEB1B). Only xBTEB1A cDNAs were used in the subsequent functional experiments.

**Relatedness of Xenopus and mammalian BTEB1 genes**

The nucleotide and derived amino acid sequences from the open reading frame of what we now designate xBTEB1 are most closely related to those of the rat and human BTEB1 genes (Fig. 2A). BTEB1 was cloned by its ability to bind to a GC-rich DNA element called basic transcription element (BTE) (15). Human BTEB1 was cloned subsequently and has an amino acid sequence almost identical to that of rat BTEB1 (23) (Fig. 2A). The three zinc fingers at the carboxyl termini of the mammalian and Xenopus proteins are virtually identical. This region is similar to a number of zinc finger proteins, including the transcription factor Sp1 (71% identity in the DNA binding domain). The amino termini of the mammalian and Xenopus proteins are also similar despite several insertions and deletions. In contrast to Sp1, xBTEB1 and mammalian BTEB1 do not have an obvious transcription activation domain. However, a sequence near the amino terminus that interacts with the corepressor Sin3A is completely conserved in xBTEB1 (24).

Both in vitro translated xBTEB1 (33 kDa) and a purified GST-xBTEB1 fusion protein (59 kDa) expressed in bacteria bind to the BTE sequence in gel-shift assays (Fig. 2B). The protein binds with highest affinity to the BTE element, with lower affinity to the related Sp1 element, and not at all to an unrelated element. We next tested the ability of xBTEB1 to regulate transcription of a BTE-containing reporter gene (Fig. 2C). We constructed luciferase reporter plasmids with one or five BTE sites inserted into a minimal promoter. The BTE elements function as a strong enhancer in Xenopus kidney (XLA) cells in a dose-dependent manner. Cotransfection of an expression plasmid containing xBTEB1 downstream of the human cytomegalovirus promoter strongly inhibits luciferase expression. This apparent repressor activity by
**Fig. 2.** *Xenopus* BTEB1 is structurally and functionally related to mammalian BTEB1. A, Schematic of the comparison of *Xenopus* BTEB1A and -B with rat and human BTEB1 (rBTEB1 and hBTEB1, respectively). The original full-length cDNA is shown for xBTEB1A, with amino acid sequence numbers given above the open reading frame (boxed) and nucleotide sequences given below. 5′- and 3′UTRs. Only coding sequences are shown for xBTEB1B and rat and human BTEB1. The exon/intron junction is present just after the first zinc finger in both xBTEB1 genes, as indicated by the black vertical arrow. The DNA-binding domains are shaded, and the three zinc fingers are indicated as horizontal.

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**B**

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**C**

<table>
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<tr>
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<td>600 ng CMX-BTEB</td>
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**C**

Reporter Construct

- Control vector
- 200 ng CMX-BTEB
- 600 ng CMX-BTEB
xBTEB1 was also observed in other Xenopus cell lines: XTC, XL-177, XL-58, and XLF (data not shown).

Genomic organization of the duplicated xBTEB1 genes

An xBTEB1 cDNA probe was hybridized against homozygous diploid genomic DNA from a clonal X. laevis line. The line was made by fertilization with irradiated sperm and suppression of the first mitotic cleavage (25). The use of homozygous diploid genomic DNA eliminated the possibility of extra bands resulting from restriction site polymorphisms that may exist in out-bred, wild-type genomic DNA. The band patterns (Fig. 3A) confirmed that there are two copies of each gene per haploid complement of DNA. Genomic DNA corresponding to each xBTEB1 duplicate gene was cloned from a homozygous diploid X. laevis genomic library. Two overlapping clones from one gene (xBTEB1A) and a single clone representing the duplicated gene (xBTEB1B) were identified by sequencing their exons and restriction mapping (Fig. 3B). Exon 1 contains the entire 5′-UTR and coding sequence through the first zinc finger. Exon 2 encodes the final two zinc fingers, the rest of the coding sequence, and at least some of the 3′-UTR including consensus A2UA3 polyadenylation signals. The 5′ and 3′ splice junctions fit the consensus sequences for eukaryotic genes (data not shown).

To demonstrate that both duplicated xBTEB1 genes are up-regulated by TH, we used a modified primer extension assay with gene-specific primers selected from a region in the 5′-UTR of the xBTEB1 genes. The specificity of these primers was confirmed using in vitro transcribed cRNA from xBTEB1A or xBTEB1B (data not shown). When applied to RNA from control and TH-treated tadpoles we found that both genes were up-regulated by TH (Fig. 3C). There are two main bands from both gene transcripts; the upper one is about 0.26 kb long, which is the expected length if the transcription start site is at the end of the original cDNA clone.

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However, the lower band is about 3 times more abundant than the upper one and is about 0.17 kb long (Fig. 3C). Identical results were obtained from XL-177 cultured cells (data not shown). Further confirmation came from the sequences of 12 independent cDNAs isolated from a random primed cDNA library using a 5'UTR probe. Four of the clones corresponded to xBTEB1A and eight to xBTEB1B. The 5' ends of all but one of these clones are located in a cluster about 100 bp downstream from the end of the longest original cDNA shown in Fig. 2A (data not shown). Taken together these data demonstrate that the major start sites in transcription for both xBTEB1 genes are located about 100 bp downstream of the end of the original cDNA we obtained for xBTEB1A.

Isolation of high affinity TR-binding sites in xBTEB1 genes

To investigate TH-induced regulation of xBTEB1 genes, we used a reverse gel shift to map high affinity genomic binding sites in both duplicated genes (8, 26). Gel-shift assays using Rsal-digested xBTEB1 genomic clones mixed with [35S]xTRα with and without xRXRα are shown in Fig. 4. In the absence of added xRXRα at 50 mM KCl, multiple faint bands were observed in all lanes. However, when cold xRXRα was added to the reaction, only one or two strong bands were detected, which were specific to the genomic inserts. A single band was seen in all clones that corresponded to a cryptic TR/RXR-binding site in λ DNA. A further increase in specificity was observed when the reactions were carried out under more stringent DNA binding conditions using 150 mM KCl (Fig. 4A, right panels). Under these conditions there was no detectable binding with xTRα alone, and there was a significant reduction in the amount of the shifted band derived from the λ vector. The upper band (site 2) in the xBTEB1 genomic clones was reduced, but site 1 was not. This was best demonstrated in the λ clone xBTEB1 A-1 at high KCl and limiting TR concentrations, where site 1 competed for binding of both the λ site and site 2 in the same reaction.

The lower affinity site 2 mapped to a large subclone centered around exon 1, but it did not map to the same region.

![Fig. 4](image-url)

**Fig. 4.** Identification of TR-binding sites in xBTEB1 genomic DNA. A, Reverse gel shift assay of xBTEB1 genomic clones. Genomic clones (A1, A2, and B1; see Fig. 3B) and the λ vector were digested with Rsal. The DNA fragments were mixed with [35S]labeled, *in vitro* translated xTRα with or without cold, *in vitro* translated xRXRα. The binding reactions were carried out with either 50 mM KCl (left panels) or 150 mM KCl (right panels) in the binding buffer. Free and DNA-bound TRα were separated on 4% polyacrylamide gels. Only the results for xBTEB1 clone A1 and λ DNA are shown in the −RXRα reactions at 150 mM KCl, because the other digests were identical (i.e. no specific complexes detected). B, Further mapping of the xTRα/RXRα-binding sites in xBTEB1 genomic subclones was performed by three rounds of subcloning into plasmid vectors, digestion with Rsal, and analysis as in A at 150 mM KCl. The results for site 1 from the BTEB1 A1 clone are shown. Site 1 was finally localized to a 110-bp Psal fragment (subclone 10).
in xBTEB1 A and B. Site 2 was localized to a 2-kb fragment in intron 1, and a 5'-flanking region adjacent to the start site of transcription in xBTEB1A and -B, respectively (data not shown). No further analysis was carried out on this lower affinity site. Site 1 localized to a 3-kb EcoRI fragment (subclone 2; Fig. 4B) in the 5'-flanking region of xBTEB1 A1. It was subcloned again using HindIII (subclone 5). Site 1 localized to a 110-bp PsiI fragment (subclone 10) of subclone 5. By the same method, site 1 mapped to the same fragment in the xBTEB1B gene as in xBTEB1A, approximately 6.5 kb upstream of the transcription start site.

**Sequence conservation of regulatory elements in the two xBTEB1 genes**

Two copies of genes such as xBTEB1A and -B that were regulated identically should have conserved regulatory elements surrounded by diverged DNA. Subclone 10, containing the highest affinity TR/RXR-binding site, lies within a 250-bp region of almost complete sequence conservation, which then diverges significantly on either side (Fig. 5A). A DNA fragment of subclone 10 that lacks 35 bp at its 3' end does not bind to the TR/RXR heterodimer (data not shown). This region contains a DR+4+4 that is very close to the known TRE consensus binding sequences (Fig. 5B). The proximal promoter region of the xBTEB1 genes is also strongly conserved (Fig. 5A). The two promoters are about 90% identical for approximately 200 bp upstream of the cluster of start sites.

**Fig. 5. Localization and sequence of the xBTEB1 TRE.** A. Sequence comparison of genomic DNA surrounding the TR-binding site in xBTEB1 A and B. The conservation of the nucleotide sequences of the two regions of the duplicate xBTEB1 genes is plotted. Location of subclone 10 (see Fig. 4B) containing the TRE is within a conserved region about 6.5 kb upstream from the transcription start site of both xBTEB1 genes. The solid black portion is the 5' UTR adjacent to the shaded coding region representing the first exon. B. Comparison of xBTEB1 TRE with an optimized TRE sequence and other Xenopus and mammalian TREs. The xBTEB1 TRE is aligned with an optimized TRE sequence (top), X. laevis xTRβ and TH/bZIP TREs, and the most closely related mammalian TREs from the MoMLV LTR and the rat Hr gene. Shaded nucleotides indicate identity with the consensus AGGTCA half-site (under the arrows). Nucleotides in the spacer that are identical to the xBTEB1 TRE are underlined. Distance relative to each gene's transcription start site is given to the right. N in the optimized sequence represents any nucleotide.

**X. laevis TRs bind directly to the xBTEB1 TRE**

To verify that TRs can bind to the xBTEB1 TRE directly, standard gel-shift assays were performed using cold in vitro translation products or endogenous TRs from tissue extracts. Using in vitro translated receptors, a specific shifted band was observed only when xTRs and xRXRα were both added to the reaction (Fig. 6A). No binding was observed to a mutated version of the TRE that contains a 1-bp change in each half-site. Quantitative competitive binding assays with the individual TREs were used to assess the relative binding affinities of various TRE sequences (Fig. 6B). The strongest binding TRE was a perfect direct repeat of AGGTCA separated by four nucleotides (using the xBTEB1 spacer sequence). The binding affinity of the wild-type xBTEB1 TRE was similar to the perfect repeat (3-fold reduced), whereas the combined TH/bZIP TRE1 and TRE2 oligonucleotide was 8-fold reduced from the xBTEB1 element. No differences were found using xTRα or xTRβ or using either xRXRα or xRXRβ as the heterodimer partner (data not shown).

The xBTEB1 TRE was also bound specifically by TRs from premetamorphic tadpole tail and brain extracts in gel-shift assays (Fig. 6C). A strongly shifted band was apparent in the T3-treated tail extracts, with a much weaker band apparent in the untreated tail extracts. These bands were not competed away with a mutated xBTEB1 TRE, but were completely competed with cold excess xBTEB1 TRE. As xTRs, like mammalian TRs, do not require T3 to bind to DNA (data not shown), the increase in specific binding to the xBTEB1 TRE is probably due to an increase in xTR synthesis. xTRβ transcription and resulting translation were strongly induced by T3 in the tail (22), and an antiserum that was specific for xTRα did not alter the gel shift pattern. Instead, an antiserum that recognizes both xTRβ and xTRα supershifted the complex from T3-treated tails, identifying xTRβ as the major compo-
ent in the T₃-induced complex. By contrast, extracts from control and T₃-treated tadpole brains bound xBTEB1 TRE almost equally. No specific binding to the mutated TRE was detected. This time, most of the specific complex was super-shifted in both treated and untreated extracts with the xTR/H9251-specific antisera. Both treated and untreated complexes were again shifted by the pan-isotype-specific xTR antiserum. These results demonstrate that endogenous TRs bind tightly and specifically to the xBTEB1 TRE. In addition, we show that xTRβ is the predominant TR isotype in extracts from T₃-treated tails. xTRα is constitutively expressed in the brain, and although xTRβ induced by T₃, it is to an apparently lesser extent than in the tail.

Behavior of the xBTEB1 TRE in transient transfection assays

We next tested whether xBTEB1 TRE could confer TH responsiveness on the transcription of a reporter gene as assayed by transient transfection in cultured Xenopus cells. The X. laevis cell line XLA up-regulates a subset of the tadpole tail TH response genes including xTRβ and xBTEB1 in response to 100 nM T₃ (data not shown). Various natural and synthetic TREs were cloned into a minimal promoter driving the expression of the firefly luciferase gene (Fig. 7A). TH weakly induced the parent vector; however, the single xBTEB1 TRE permitted repression without TH and strong TH.
induction of this reporter. Mutation of 1 bp in each half-site of the xBTEB1 TRE (xBTEB1 Mut) completely prevented induction of the reporter. Activation of the native X. laevis xBTEB1 TRE was greater than that of other TRE sequences, including a synthetic DR4/H11001 perfect repeat, a TRE with the same half-sites as the xBTEB1 element but with the spacer changed to GGGT, and an inverted palindromic TRE (TRE-pal; Fig. 7A). Cotransfection of xTRα or xTRβ expression vectors in XTC cells had a modest effect on the fold activation of the xBTEB1 TRE and the coupled Xenopus TH/bZIP TREs, causing further repression in the absence of hormone and stronger activation in the presence of hormone (Fig. 7B).

Native genomic xBTEB1A sequences were then tested for regulation by TH in XLA cells. We cloned the following genomic sequences upstream of luciferase: the conserved proximal promoter from -200 to +200 bp relative to the farthest upstream start site, the 200-bp conserved upstream region that includes the TRE fused to the proximal promoter, and the same construct in which the TRE was deleted. As shown in Fig. 8, the predicted proximal promoter supports basal transcription but very little regulation by TH. Inclusion of the upstream TRE-containing site confers repression in the absence of hormone which is then relieved by T3 addition. This repression and activation depends on the presence of the intact TRE. The degree of regulation (~2-fold) was less when using endogenous sequences compared with the xBTEB1 TRE fused to a minimal promoter. The xTRβ wild-type promoter with its strong TRE was also similarly 2-fold regulated in a transient transfection assays (data not shown) (7, 30). Full regulation of the xBTEB1 promoter may require proper chromatin assembly, as has been demonstrated for the xTRβ promoter (31–34), or additional genomic sequences not included in these constructs. Modestly stronger regulation of the xBTEB1A TRE region and proximal promoter was achieved in XTC cells with either cotransfected xTRα or -β expression vectors (data not shown).

**Discussion**

In this paper we demonstrate that a direct TH response gene in X. laevis encodes a transcription factor that is closely related to the rat protein BTEB1, a member of the Sp1 family of transcription factors. Sequences more related to the DNA-binding domain of BTEB1 than to other Sp1 family members are found in diverse organisms, including Ciona and Drosophila (data not shown). As the similarity between DNA-binding domains between X. laevis and rat BTEB1 predicts, xBTEB1 synthesized either in bacteria or cultured cells binds to the BTE and Sp1 elements (Fig. 2B). In transfection studies, overexpression of xBTEB1 acts as a transcriptional repressor of cotransfected reporter genes. Imataka et al. (15) showed that rat BTEB1 acted as a competitor of Sp1-driven transcription depending on the promoter context. As a major difference between BTEB1 and Sp1 is the absence of an apparent transcription activation domain in the former, high levels of xBTEB1 may be acting in these assays as a negative com-

![Fig. 7. The xBTEB1 TRE confers TH regulation on a heterologous promoter in transiently transfected Xenopus cells. A, XLA cells were transiently transfected with the indicated TRE constructs, and extracts were assayed for luciferase activity 48 h after treatment with (■) or without (□) 100 nM T3. Luciferase activity was normalized to β-galactosidase expression from a constitutive promoter. B, XTC cells were transfected with the xBTEB1 or TH/bZIP TRE construct with the indicated xTR expression vector and treated with (■) or without (□) 100 nM T3. In both A and B, each point was performed in triplicate, and the error bars represent the SEM.](image)

![Fig. 8. xBTEB1 genomic sequences including the TRE support TH-regulated transcription in transiently transfected XLA cells. xBTEB1A genomic sequences were cloned upstream of the luciferase gene, transfected into Xenopus XLA cells, and treated with (■) or without (□) 100 nM T3. Arrows indicate the approximate transcription start site. Numbers above the constructs indicate the approximate distance from the transcription start sites. The X indicates that the TRE was specifically deleted in that construct. Each point was determined in triplicate, and the error bars represent the SEM.](image)
petitor of Sp1. Therefore, even though our studies confirm the ability of xBTEB1 to bind DNA and repress transcription in a similar fashion as mammalian BTEB1, they may not reveal the function of endogenous xBTEB1 in vivo. Indeed, BTEB1 has been shown to act as a transcriptional activator in certain cell types and target gene contexts (17, 35–37).

In situ hybridization analysis showed that xBTEB1 is highly expressed in the developing epidermis, as well as satellite cells between muscle, spinal cord, and proliferating ventricles of the brain (38, 39). Given the particularly high level of expression in adult skin and muscle as well (data not shown), xBTEB1 may function to initiate and/or maintain postembryonic skin and muscle differentiation. A key common regulator of keratin gene expression in both mammals and amphibians is the transcription factor activating protein-2 (AP-2). Mammalian BTEB1, in turn, trans-activates the AP-2 gene (40). AP-2 is up-regulated by TH in Xenopus skin (41) and binds to an adult keratin promoter; however, the Xenopus AP-2 promoter has not yet been cloned. In terms of BTEB1 as a transcriptional repressor, TH down-regulates four coordinately expressed larval epidermal genes (42).

These genes were the only down-regulated genes isolated in a subtractive hybridization screen for TH-regulated genes in the tail (9); therefore, these genes are attractive candidates as BTEB1 targets. Finally, BTEB1 is also up-regulated by TH around the time of birth in the mammalian brain and has been suggested to play a role in neurite outgrowth (16). A common function for xBTEB1 in Xenopus and mammalian brain development remains to be investigated.

The three known BTEB1 mRNAs (rat, human, and X. laevis) have relatively long 5′ UTRs that contain multiple short open reading frames preceding the authentic start codon. Such 5′ UTRs reduce the rate of translation of BTEB1 (43) as well as mRNAs in general (44). BTEB1 is translated in neural derived cells, but not in HeLa cells (43). A compilation of the UTRs of eukaryotic mRNAs shows that 5′ UTRs greater than 100 bp containing start and stop signals in phase are uncommon (45). However, at least two other X. laevis genes encoding TH-inducible transcription factors as well as TRα and TRβ mRNAs have similar mRNA structures (46). The frequency with which we observed these relatively unusual features in mRNAs up-regulated by TH during metamorphosis suggests that they might share some common transcriptional control mechanism.

xBTEB1 is the most rapidly responding gene identified to date in TH-treated tadpole tissues (10). We demonstrate here that the gene has a single TRE that is 6.5 kb upstream from the transcription start site that is completely conserved in both sequence and position between the two duplicated xBTEB1 genes. This TRE can influence the transcription of a reporter gene in a transient transcription assay that does not require the high level of TR provided by cotransfection of a plasmid that synthesizes excess TR. The distant position of xBTEB1 TRE is not unprecedented. For example, the rat Hr gene has its TRE 9 kb from the start site of transcription (29).

The location of the xBTEB1 TRE far from its promoter, presumably within a conserved enhancer element, is not required for rapid kinetics, because the X. laevis TRβ gene has a consensus TRE 264 bp downstream from the start site of transcription (7, 34). Despite the difference in the genomic positions of their TREs, two genes with the earliest kinetics, xBTEB1 and TRβ, share some common features in addition to their nearly identical TRE sequences. Both have TATA-less promoters and an initiator-like sequence. In addition, there are several SP-1 sites within each promoter as well as an alternating polypurine/polypyrimidine stretch (mostly T/A in TRβ, G/T in xBTEB1). Although the kinetics of up-regulation and the TRE sequences of the xBTEB1 and xTRβ genes are highly similar, differences in their spatial expression patterns exist. For example, in the tail, xTRβ expression is higher than xBTEB1 in cells surrounding the notochord and fibroblasts underneath the skin, where late-responding protease genes are primarily induced (39). Therefore, xBTEB1 may not be a major regulator of these genes. Consistent with this view, stable overexpression of xBTEB1 in a Xenopus cell line does not result in altered regulation of late tail response genes such as the matrix metalloproteinase collagenase-3 or the intracellular protease pepE (data not shown).

Both duplicated xBTEB1 genes have a DR +4 TRE configuration, a result predictable from earlier studies using synthetic TREs. In addition, the 4-base spacer residues between the half-sites are strongly conserved, including T residues at spacer positions 1 and 3 in the xBTEB1, xTRβ, and rat Hr gene TREs. Using in vitro binding site selection to derive a consensus element, an extended octamer with TA residues preceding the AGGTCA hexamer was found to be optimal for TRα binding (28). The 3′ half-site of the TRE that is presumed to bind TR in the TR/RXR heterodimeric complex is also identical in the xBTEB1, TRβ, and rat Hr elements (AG-GACA). We found that changing the spacer region to GGGT negatively influences both repression and activation of TRE. Keeping the spacer the same, but changing the half-sites to a perfect repeat of AGGTCA, increases the binding affinity of the TR/RXR heterodimer, but creates a weaker TRE in transfection assays. We found that genes with more delayed kinetics of mRNA accumulation tend to have lower affinity and more complex TRE half-site arrangements, as exemplified by the TH/bZIP TREs (8). In these cases, lower binding affinity may be due to divergence of the 3′ half-site that binds TR, especially in TH/bZIP TRE2. The sequence comparisons in Fig. 5B lead to the conclusion that both spacer residues and the second TRE half-site play an important role in binding and transcriptional activity, whereas the first, presumably RXR-bound, half-site appears more flexible. The nature of the complex at TREs in vivo in both the presence and absence of hormone remains an important question in the nuclear receptor field. Homodimeric mammalian TRs appear to associate more efficiently with corepressor proteins in vitro (47), yet in our analysis TR homodimers form poorly on the xBTEB1 TRE in vivo under physiological salt concentrations. This observation leads us to speculate that TR/RXR heterodimers are the key constituents of this TRE complex in vivo.

In summary, we have characterized the xBTEB1 expression profile, determined its biochemical relatedness to mammalian BTEB1, and placed it at the top of a TH-induced gene expression cascade due to its strong consensus TRE. These studies represent an important step in our understanding of the evolutionary conservation of the TH signaling pathway. Identification of key regulatory elements in TH-responsive
genes in this important vertebrate model sets the stage for detailed analysis of the protein complexes that assemble on TRES throughout embryogenesis into adulthood. Our results also suggest that BTEB1 is an important player in hormone-induced gene expression cascades in all vertebrates.

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