A Role for Basic Transcription Element-binding Protein 1 (BTEB1) in the Autoinduction of Thyroid Hormone Receptor β†

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Thyroid hormone (T3) induces gene regulation programs necessary for tadpole metamorphosis. Among the earliest responses to T3 are the up-regulation of T3 receptor β (TRβ; autoinduction) and BTEB1 (basic transcription element-binding protein 1). BTEB1 is a member of the Krüppel family of transcription factors that bind to GC-rich regions in gene promoters. The proximal promoter of the Xenopus laevis TrβA gene has seven GC-rich sequences, which led us to hypothesize that BTEB1 binds to and regulates TrβA. In tadpoles and the frog fibroblast-derived cell line XTC-2, T3 up-regulated Bteb1 mRNA with faster kinetics than TrβA, and Bteb1 mRNA correlated with increased BTEB1 protein expression. BTEB1 bound to GC-rich sequences in the proximal TrβA promoter in vitro. By using chromatin immunoprecipitation assay, we show that BTEB1 associates with the TrβA promoter in vivo in a T3 and developmental stage-dependent manner. Induced expression of BTEB1 in XTC-2 cells caused accelerated and enhanced autoinduction of the TrβA gene. This enhancement was lost in N-terminal truncated mutants of BTEB1. However, point mutations in the zinc fingers of BTEB1 that destroyed DNA binding did not alter the activity of the protein on TrβA autoinduction, suggesting that BTEB1 can function in this regard through protein-protein interactions. Our findings support the hypothesis that BTEB1 associates with the TrβA promoter in vivo and enhances autoinduction, but this action does not depend on its DNA binding activity. Cooperation among the protein products of immediate early genes may be a common mechanism for driving developmental signaling pathways.

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Autoinduction of nuclear hormone receptors is a common but poorly understood phenomenon in animal development (1). The autoinduction of thyroid hormone (T3) receptor genes (Tr) during amphibian metamorphosis is a dramatic example of this form of gene regulation (1). All vertebrates possess two Tr genes designated Tra and Trβ (also known as NR1A1 and NR1A2, respectively); Xenopus laevis has two Tra and two Trβ genes each designated A or B because of its pseudotetraploidy (2). Thyroid hormone is the primary morphogen controlling tadpole metamorphosis, and TRs are ligand-dependent transcription factors. One of the earliest gene regulation events during amphibian metamorphosis is the up-regulation of Trβ genes by T3 (3). This regulation depends on TRs binding to thyroid hormone-response elements (TREs) present in the Trβ promoters (receptor autoinduction; see Refs. 2, 4). It is hypothesized that autoinduction of Trβ genes is essential for metamorphosis (1). The gene regulation programs induced by the T3TR complex that lead to tissue morphogenesis have been characterized in several tadpole tissues (5–12).

Basic transcription element-binding protein 1 (Bteb1) is an immediate early gene induced by T3 in most tadpole tissues during metamorphosis (there are two Bteb1 genes in X. laevis designated “a” and “b”; see Refs. 6, 13, 14). The direct regulation of the X. laevis Bteb1 genes by T3 is explained by one or more TREs located upstream of the transcription initiation sites (6, 14). BTEB1 is a member of the Krüppel family of transcription factors (KLF; also known as KLF-9 (15)) and first isolated in a screen for proteins that bind to a GC-rich (GC box) sequence in the promoter of the rat cytochrome P-450IA1 gene (designated the basic transcription element or BTE (16, 17)). BTEB1 possesses a DNA binding domain (DBD) consisting of three Cys2-His, zinc finger domains (18, 19). Krüppel-like proteins are distantly related to the specificity protein (Sp) family members, including Sp1 (18, 19). The BTEB1 DBD shares 72% sequence similarity with rat Sp1 (17), and the two proteins bind with similar affinity to the BTE sequence (20). Although Sp1 and BTEB1 have very similar DNA binding domains, and they bind to similar or identical consensus DNA sequences, the two pro-

4 The abbreviations used are: T3, 3,5,3'-triiodothyronine; TRβα, T3 receptor β; TRE, thyroid hormone-response element; UTR, untranslated region; RT, reverse transcription; ChIP, chromatin immunoprecipitation; EMSA, electrophoretic mobility shift assay; DBD, DNA binding domain; RT2PCR, quantitative real time PCR; BTE, basic transcription element; ANOVA, analysis of variance; NF, Nieuwkoop and Faber.
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proteins are completely different outside of the DBD. In addition to other KLF family members, three proteins designated BTEB2, -3, and -4 have been identified in mammals, although the BTEB2 appears to be more distantly related to BTEB1 than the other two proteins (19, 21–23). As with BTEB1 and Sp1, the BTEB proteins share almost identical DNA binding domains but are divergent in their N-terminal regions that harbor domains necessary for their transactivation and in some cases transcription functions (15).

Basic transcription element-binding protein 1 mRNA and protein is strongly up-regulated by T3 in tadpole tissues (13), but the genes that BTEB1 regulates, and thus its functions in tadpole development, are unknown. It is noteworthy that BTEB1 is the only KLF/Sp1-like family member known to be up-regulated by T3 in tadpole tissues (5–12). Earlier, we and others showed that BTEB1 is also regulated by T3 in developing rodent brain where it promotes neurite outgrowth (24–26).

BTEB1 is expressed in uterine endometrial cells where it transactivates the uroferrin gene, and it may influence cell proliferation by regulating cell cycle and growth-associated genes (27–29). The actions of BTEB1 in endometrial cells appear to involve direct protein–protein interactions with the progesterone receptor (30). We found that X. laevis BTEB1 is capable of activating synthetic promoter constructs containing multiple or single GC boxes (13). Mammalian BTEB1 also has transcription function on several synthetic and native promoters (17, 27, 31–34). BTEB proteins have been reported to activate or repress transcription depending on the number of GC boxes present in the promoter construct tested, the target gene analyzed, and the cell type (13–15, 27, 30–36). Whether these proteins function as transcriptional activators or repressors may depend on the architecture of the specific promoter and the chromatin environment (15).

Based on the early response kinetics of the Bteb1 and TrβA genes, the observation that the protein products of these genes are expressed in the same cells (13), and the identification of seven GC-rich regions in the proximal X. laevis TrβA promoter, we hypothesized that TrβA may be a target gene for BTEB1. We further hypothesized that the up-regulation of BTEB1 plays a role in the autoinduction of TrβA, perhaps functioning as an accessory transcriptional activator. Here we show that the kinetics of Bteb1 mRNA up-regulation in response to T3 are faster than TrβA and that BTEB1 binds to regions of the proximal TrβA promoter that contain GC boxes. Using chromatin immunoprecipitation (ChIP) assay, we show that BTEB1 associates with the TrβA promoter in vivo in a T3-dependent stage-dependent manner. Forced expression of BTEB1 in the X. laevis fibroblast cell line XTC-2 (37) accelerates the activation of the TrβA promoter and expression of endogenous TrβA mRNA in response to T3. This action depends on the first 30 amino acids of BTEB1, but not on its DNA binding capacity, because point mutations in the zinc fingers did not alter the activity. Taken together, our findings support the hypothesis that the up-regulation of BTEB1 by T3 plays a role in the transcriptional regulation of the TrβA gene during tadpole development.

### EXPERIMENTAL PROCEDURES

**Animals and Hormone Treatments**—Tadpoles of X. laevis were reared in dechlorinated tap water (water temperature, 20–22°C) and fed pulverized frog bristle (Nasco, Fort Atkinson, WI). Developmental stages were assigned according to Nieuwkoop and Faber (NF) (38). Tadpoles were treated with 3,5′,3′-triiodothyronine (T3; sodium salt; Sigma) by adding it to the aquarium water to a final concentration of 10 nM for various times; water was changed and hormone replenished daily over the treatment period. Tadpoles were then euthanized by immersion in 0.01% benzocaine (Sigma), and whole brains and tails were collected for RNA or ChIP analyses (see below). Animal care was in accordance with institutional guidelines.

**RNA Extraction and Reverse Transcription (RT)-PCR Analysis**—Total RNA was isolated from tadpole brains or XTC-2 cells using the TRIZol reagent (Invitrogen) following the manufacturer’s instructions. The RNA was treated with DNase I (Roche Applied Science) prior to reverse transcription to remove genomic DNA contamination following the methods of Manzon and Denver (39). The DNase-treated RNA was reverse-transcribed using SuperScript II (0.5 μl, 200 units/μl; Invitrogen), and 0.2 to 2 μl of the resulting cDNA was used for PCR.

**Semi-quantitative RT-PCR**—Standard PCRs were initiated in 25 μl containing 10× PCR buffer, 1.5 μM MgCl2, dNTP mix (1.25 μM each), forward and reverse primers for each gene of interest (10 μM), and TaqDNA polymerase (1.25 units; Promega, Madison, WI). Each thermal cycle consisted of 94°C for 1 min, 55°C for 1 min, and 72°C for 2 min. The number of cycles for each gene was determined empirically by constructing linear amplification curves. We used 32 cycles for Bteb1, 36 for TrβA, and 28 for ribosomal protein L8 (rpl8; a housekeeping gene used to normalize for RNA loading and cDNA synthesis). Oligonucleotide primer sequences for Bteb1 are given in Table 1. Primer sequences used for rpl8 and TrβA were as described by Manzon and Denver (39). PCR products were electrophoresed on 1% agarose gels, stained with ethidium bromide, and densitometry was conducted using Scion Image Software (version 3.0, Scion Corp.). The band densities of Bteb1 and

### TABLE 1

| Oligonucleotides used for semi-quantitative and quantitative real time RT-PCR |
|-----------------------------------------------|
| **Bteb1**                                      |
| Forward: 5′-CCTGGCGAAATTTATAGGG-3′           |
| Reverse: 5′-GGATAGAAGTGCTGATAGG-3′           |
| **TaqMan Assays**                             |
| TrβA: Forward: 5′-GGAAACCCCATGGAACAGAAA-3′  |
| Reverse: 5′-CAATTTATAGGGCCACAGAAA-3′        |
| Probe: FAM-AAAAAATTTGCCACAGGG-MGBNFQ         |
| Bteb1b: Forward: 5′-CCAGTCATGCAAAATGAAA-3′  |
| Reverse: 5′-AAACCTTGGGAAACCAGTCC-3′         |
| Probe: FAM-AGG CAC AGG TCT CC-MGBNFQ         |
| rpl8: Forward: 5′-TTCTCTAAGAAGGTTGACTCATC-3′|
| Reverse: 5′-CAC GCC CGT GAT CHP GGA-3′       |
| Probe: VIC-AGG GTA TGG TGA AAG ACA-MGBNFQ    |
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**TABLE 2**

Oligonucleotide primers used to generate truncated mutants and point mutations in the three zinc fingers of *X. laevis* BTEB1

The $\Delta$ indicates deletion of the first 30–120 amino acids. Primers used for point mutations generated a histidine $\rightarrow$ alanine substitution in each of the three zinc fingers. For indicates forwards, and Rev indicates reverse.

| Primers used to generate N-terminal truncated xBTEB1 mutants | 5'-ATAGGATCCGCGAGTTAAGGGACGACCC-3' |
|-------------------------------------------------------------|-----------------------------------|
| $\Delta$30 xBTEB1 For                                      | 5'-ATAGGATCCGCGAGTTAAGGGACGACCC-3' |
| $\Delta$9 xBTEB1 For                                       | 5'-ATAGGATCCGCGAGTTAAGGGACGACCC-3' |
| $\Delta$120 xBTEB1 For                                     | 5'-ATAGGATCCGCGAGTTAAGGGACGACCC-3' |
| xBTEB1 Rev                                                 | 5'-ATAGGATCCGCGAGTTAAGGGACGACCC-3' |

| Primers used to generate point mutations in the three zinc finger domains of xBTEB1 | 5'-GGCAGGAGATTTAGTTGACGCAAGACCCAGCC-3' |
|-----------------------------------------------------------------------------------|----------------------------------------|
| xBTEB1 H211A                                                                       | 5'-GGCAGGAGATTTAGTTGACGCAAGACCCAGCC-3' |
| xBTEB1 H241A                                                                       | 5'-GGCAGGAGATTTAGTTGACGCAAGACCCAGCC-3' |
| xBTEB1 H269A                                                                      | 5'-GGCAGGAGATTTAGTTGACGCAAGACCCAGCC-3' |

$Tr{\beta}A$ amplicons for each sample were normalized to the densities of the $rpl8$ bands.

**Quantitative Real Time PCR (qPCR)—** For quantitative RT-PCR (RTqPCR), we developed TaqMan assays and analyzed samples on an ABI 7500 fast real time PCR machine using TaqMan Universal PCR Master Mix (Applied Biosystems, Inc., Foster City, CA). The primer/probe sets used are given in Table 1 and were designed to span exon/intron boundaries. Standard curves were generated using cDNAs from the time point that exhibited the highest expression level for each gene to provide for a relative quantitation. $Tr{\beta}A$ and $Bteb1$ mRNAs were normalized to the level of $rpl8$ mRNA.

**Plasmid Constructs—** The pCMV-xBTEB1 expression plasmid was described by Hooper et al. (13). The *X. laevis* $Tr{\beta}A$ promoter-luciferase plasmid (40) was a generous gift of Dr. Yun-Bo Shi. Full-length and N-terminal truncated mutants of BTEB1 were generated by PCR (primers in Table 2), and cDNA fragments were directionally cloned into the pCS2 vector. The choice of deletions was based on the location of two putative transactivation domains (A and B) located in rat BTEB1 (41) that are highly conserved in Xenopus BTEB1 (13). The plasmid pCS2-xBTEB1$\Delta$30 has a deletion of the first 30 amino acids that includes transactivation domain A; pCS2-xBTEB1$\Delta$99 has both transactivation domains A and B removed; and pCS2-xBTEB1$\Delta$120 represents only the DNA binding domain.

The plasmid construct pCS2-xBTEB1 C2AH harbors histidine to alanine substitutions (H211A, H241A, and H296A) in addition to alanine substitutions (H211A, H241A, and H296A) in the N-terminal region of the protein (see below). The reaction continued at room temperature for 40 min before fractionation by nondenaturing 6% PAGE in 0.25× Tris borate/EDTA (TBE). Gels were fixed in 30% methanol, 10% acetic acid, dried, and processed for autoradiography.

**Electrophoretic Mobility Shift Assay—** We conducted electrophoretic mobility shift assay (EMSA) as described by Hooper et al. (13) with minor modifications. The BTE and mutated BTE probes were used as described by Yanagida et al. (16). Bacterial cell lysate containing the fusion protein GST-xBTEB1[DBD] was prepared as described by Hooper et al. (13). Recombinant wild type BTEB1 or BTEB1 C2AH mutant were produced in vitro using the Tnt SP6 Quick Coupled Translation System (Promega). For EMSA, 1 µl of a 1:512 dilution of GST-xBTEB1[DBD] lysates or varying volumes of the *in vitro* translated proteins were incubated in a volume of 35 µl with 20,000 cpm of $^{32}$P-BTE and 1.4 µg of double-stranded poly(dl-dC) in buffer containing 20 mM HEPES (pH 7.8), 1 mM dithiothreitol, 0.1% Nonidet P-40, 50 mM KCl, and 20% glycerol. For antibody supershifts, proteins were preincubated for 20 min prior to the addition of $^{32}$P-BTE with 1 µg of normal rabbit serum IgG or affinity-purified anti-xBTEB1 IgG that recognizes only the N-terminal region of the protein (see below). The reaction continued at room temperature for 40 min before fractionation by nondenaturing 6% PAGE in 0.25× Tris borate/EDTA (TBE). Gels were fixed in 30% methanol, 10% acetic acid, dried, and processed for autoradiography.

The ability of regions of the proximal *X. laevis* $Tr{\beta}A$ promoter (GenBank™ accession number U04675) to displace GST-xBTEB1[DBD] binding to the $^{32}$P-BTE was tested by competitive EMSA (1.89 µM for each competitor DNA). The *X. laevis tr{\beta}A* promoter fragments were generated by PCR and gel-purified using QIAEX II (Qiagen, Valencia, CA). The regions of the promoter that we analyzed are shown in Fig. 2 and supplemental Table 1, and the oligonucleotides used to amplify the sequences by PCR are given in Table 3.

We then synthesized short oligonucleotide probes for EMSA containing predicted GC boxes in the *Tr{\beta}A* promoter fragments that demonstrated competitive binding to GST-xBTEB1[DBD] (see above). Oligonucleotides (24 bp) were syn-
were then treated with or without T3 for different times before harvest and RNA extraction. Each transfection experiment was done two to three times with six replicates per treatment.

**Western Blotting and Immunocytochemistry**—We prepared Western blots following the methods of Ranjan et al. (40) with protein extracts of XTC-2 cells transfected with pcDNA-xBTEB1 or pcDNA and extracts of XTC-2 cells treated ± T3. Forty micrograms of total protein for each sample were separated by electrophoresis on 10% denaturing SDS-polyacrylamide gels. Proteins were transferred to nitrocellulose membranes and probed with an affinity-purified antiserum to *X. laevis* BTEB1 (13). The antiserum was generated in a rabbit against the full-length *X. laevis* BTEB1 protein and affinity-purified such that the IgGs recognize only the N-terminal region of the frog BTEB1 protein (13) (0.2 μg of purified IgG/ml). These antibodies do not recognize the DBD of xBTEB1 (13), which is critical to the specificity of the reagent given the high degree of conservation of the DBDs among Krüppel and Sp1-like family members. This purified antiserum was also used for ChIP assays (described below).

We conducted immunocytochemistry for BTEB1 protein following the methods that we described previously (13). Briefly, NF stage 52 tadpoles were treated with or without T3 (10 nM) for 24 h before sacrifice. Brains were fixed for 24 h at 4 °C in 4% paraformaldehyde and then saturated in 30% sucrose for 24 h. Tissues were embedded in M-1 embedding matrix (Shandon Lipshaw Inc., Pittsburgh, PA), frozen, and cryosectioned sagittally at 20 μm. We used five brains per treatment in the analyses. Cryosections were blocked, incubated with anti-*X. laevis* BTEB1 IgG, and immune complexes detected with either a goat anti-rabbit horseradish peroxidase secondary antibody (Vectastain Elite ABC and Vector VIP kits; Vector Laboratories Inc., Burlingame, CA) or with a goat anti-rabbit Cy3-conjugated fluorescence secondary antibody (Jackson ImmunoResearch Laboratories, West Grove, PA). To test for the specificity of the immunohistochemical reaction, we preabsorbed the antibody with *Escherichia coli*-expressed GST-xBTEB1 (10 μg/ml) (13). Tissue sections were analyzed using an Olympus IX81 inverted fluorescence microscope.

**Chromatin Immunoprecipitation Assay**—We conducted ChIP assays as described previously for tadpole tissues (44). We used the ChIP assay kit from Upstate Biotechnology, Inc. (Lake Placid, NY), following the manufacturer’s instructions. The negative controls included no primary antibody, replacement of the primary antibody with normal rabbit serum, and the analysis of regions outside of the proximal TrβA promoter (Efα promoter, TrβA exon 3/4, TrβA exon 5, intestinal fatty acid-binding protein (fabp) promoter). For ChIP we used affinity-purified IgGs against *X. laevis* BTEB1 (4 μg of purified IgG/reaction). The PCR products were analyzed on 6% polyacrylamide gels followed by autoradiography, or using an Agilent Technologies 2100 Bioanalyzer (Agilent Technologies, Inc., Santa Clara, CA). ChIP assays on XTC-2 cells were analyzed using quantitative, real time PCR using the iCycler iQ real time PCR detection system from Bio-Rad. We used iQ Syber Green Supermix (Bio-Rad) following the manu-
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FIGURE 1. Thyroid hormone up-regulates Bteb1 mRNA in tadpole brain with faster kinetics than TrβA mRNA. Increased Bteb1 mRNA correlates with elevated BTEB1 protein. A, up-regulation of Bteb1 (top) and TrβA (bottom) mRNAs in premetamorphic X. laevis tadpole brain (NF stage 52) following exposure to T₃ (10 nM) for 24 h; representative brain sections (hypothalamic region) from an NF stage 52 tadpole brain (optic tectum shown) but is increased dramatically by T₃ treatment (panel 2; 10 nM in aquarium water for 24 h). Strong BTEB1 staining was restricted to cell nuclei. Panel 4, immunostaining for BTEB1 was eliminated by preabsorption with GST-xBTEB. BTEB1 immunoreactivity was detected by Cy3 immunofluorescence (panels 1 and 2) or by horseradish peroxidase staining (panels 3 and 4). C, Western blot analysis of xBTEB1 in protein extracts from pCS2 or pCS2-xBTEB1-transfected XTC-2 cells (upper panel); endogenous BTEB1 in protein extracts of XTC-2 cells were treated with or without T₃ (30 nM) for 24 h (lower panel). This dose of T₃ causes a maximal response in TrβA and Bteb1 mRNA (data not shown). Immunoblotting was conducted using affinity-purified IgG that recognizes the N-terminal region of xBTEB1 (see “Experimental Procedures”). Arrows point to the two BTEB1 bands.

RESULTS

Bteb1 mRNA Is Up-regulated by T₃ in Premetamorphic Tadpole Brain with Faster Kinetics than TrβA mRNA—Exposure of premetamorphic (NF stage 52) tadpoles to T₃ (10 nM in the aquarium water) resulted in significant time-dependent increases in brain Bteb1 (F = 222.35, p < 0.0001; ANOVA) and TrβA (F = 74.03, p < 0.001) mRNA levels (Fig. 1). The earliest time point at which a significant increase in Bteb1 mRNA was detected was 8 h (p < 0.0001; Scheffe’s test), and the mRNA level continued to increase up to 16 h. By contrast, a significant increase in TrβA mRNA expression was not detected until 16 h.

Thyroid Hormone Up-regulates BTEB1 Protein in Premetamorphic Tadpole Brain—Similar to results that we reported earlier (13), we observed a strong increase of BTEB1 protein expression in premetamorphic tadpole brain (NF stage 52) following treatment with T₃ (10 nM for 24 h; representative brain sections shown in Fig. 1B, panels 1 and 2). The strong nuclear staining for BTEB1 was completely abolished by preabsorption with GST-xBTEB (Fig. 1B, panels 3 and 4).

Western blot analysis with affinity-purified anti-xBTEB1 IgG on protein extracts of transfected XTC-2 cells showed that the antisera detected the overexpressed BTEB1 protein but did not cross-react with endogenous cellular proteins (Fig. 1C, upper panel). Native BTEB1 protein was increased in untransfected XTC-2 cells by 24 h of treatment with T₃ (Fig. 1C, lower panel). We routinely detected two bands by Western blot that corresponded to the BTEB1 protein. The basis for BTEB1 protein heterogeneity is currently unknown, but likely reflects post-translational modifications (X. laevis BTEB1 is predicted to have up to four phosphorylation and two N-linked glycosylation sites (13)).

BTEB1 Binds to the Proximal TrβA Promoter in Vitro—Computer analysis of the proximal X. laevis TrβA promoter sequence showed the presence of seven GC-rich regions commonly characterized as Sp1-binding sites (based on 40). The approximate locations of these GC-rich regions are shown in Fig. 2, and the precise locations are given in supplemental Table 1. We used EMSA to determine whether BTEB1 can bind to the TrβA promoter in vitro. We generated ~200–300-bp fragments of the TrβA promoter by PCR (Fig. 2, supplemental
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Table 1, and Table 3), and we used them as competitors in EMSA for binding of bacterially expressed GST-xBTEB1[DBD] (13) to a ³²P-labeled probe consisting of the BTE sequence of the rat cyp1a1 gene (17). Each of the TrβA gene promoter fragments with GC-rich sequences competed for binding in the EMSA, and the degree of competition correlated with the number of GC boxes contained within the fragment. By contrast, promoter fragments that did not possess GC boxes exhibited no competition in the EMSA (Fig. 3A).

We next synthesized short oligonucleotide probes (24 bp) encompassing one or two GC boxes within the proximal TrβA promoter region (to include all seven predicted GC boxes; Fig. 2 and Table 4) and tested for BTEB1 binding to these DNA elements by EMSA. Radioinert oligonucleotides were used as competitors to verify the specificity of binding. This experiment showed that BTEB1 bound to all but one of these GC box sequences and that the binding could be competed with unlabeled probe (Fig. 3B). We observed no binding with probe 5, which contains one GC box of identical sequence to the GC box elements found in other regions. As a positive control for the quality of the oligonucleotide probes, we conducted EMSAs with nuclear extracts from X. laevis tadpole brain, which has abundant GC box binding activity (13). This showed that nuclear proteins formed complexes to an equal extent with each of the radiolabeled DNAs (including probe 5; data not shown).

BTEB1 Associates with the Proximal TrβA Promoter in Vivo in a T₃ and Developmental Stage-dependent Manner—To determine whether BTEB1 associates with the proximal TrβA promoter in vivo, we conducted ChIP assays on the brain and tail of premetamorphic X. laevis tadpoles that had been treated with or without T₃ for 48 h before sacrifice. We found BTEB1 associated with the proximal TrβA promoter in vivo, and the signal was increased in a T₃-dependent manner in both brain and tail in most regions (not region G in brain or tail, nor region B in tail; Fig. 4). As controls for the ChIP assays we included the elimination of the primary antibody or the replacement of the primary antibody with normal rabbit serum. In each case the ChIP signal was below or at the limit of detection in the assay (data not shown). Another important control was the analysis of regions outside of the proximal TrβA promoter (Ef1α promoter, TrβA exon 3/4, TrβA exon 5, and Ifabp promoter), which showed little or no association of BTEB1. It should be noted that although we analyzed the promoter by targeting relatively small regions for PCR (~200–300 bp), the nature of the ChIP assay, in which genomic fragments ranging from 500 to 1000 bp are produced by sonication, does not allow us to determine with precision where within the promoter BTEB1 is associating. Nevertheless, our data show that BTEB1 associates with the proximal TrβA promoter in vivo and that the signal is increased following T₃ treatment.
We found no BTEB1 associated with control DNA sequences that included an intronic region of the TrβA gene that is at least 30 kb downstream from the start site (TrβA exon 3/exon 4), the Efa1α, or the Ifabp promoters (an indirect T3 response gene that is down-regulated by T3 (45)).

Earlier we showed that Bteb1 mRNA and protein exhibit dramatic increases in tadpole brain during spontaneous or T3-induced metamorphosis (13). We therefore tested whether the increased BTEB1 protein expression in brain during spontaneous metamorphosis resulted in increased association of BTEB1 with the proximal TrβA promoter. As predicted, we found that the amount of BTEB1 associated with the TrβA promoter (regions A/B were analyzed) was increased in animals at metamorphic climax (NF stage 62) when T3 production and BTEB1 protein are the highest (13) compared with premetamorphic tadpoles (NF stage 54) (Fig. 4). Bteb1 and TrβA mRNAs Are Coordinately Up-regulated and BTEB1 Associates with the Proximal TrβA Promoter in XTC-2 Cells—We found statistically significant, time-dependent effects of T3 on Bteb1 (F = 11.255, p < 0.0001; ANOVA) and TrβA (F = 36.936, p < 0.0001) mRNA expression in XTC-2 cells (Fig. 5A). Significant up-regulation of Bteb1 mRNA occurred by 3 h (p = 0.009; Scheffe’s test), which was the maximum level of induction observed, and was maintained through 48 h of treatment. By contrast, TrβA mRNA was not significantly increased until 6 h (p = 0.001) and then reached a maximum by 12 h that was maintained through 48 h.

Using ChIP assay on XTC cells that had been treated with T3 for 24 h, we observed association of BTEB1 with two regions of the proximal TrβA promoter. Real time PCR analysis of the ChIP assay showed significantly greater association of BTEB1 with the TrβA gene which has no GC boxes. Earlier we showed that Bteb1 mRNA was maximally induced at 3 h (p = 0.009; Scheffe’s test) and maintained through 48 h of treatment. TrβA mRNA was significantly induced at 6 h (p = 0.001), reached a maximum by 12 h, and was maintained through 48 h. Bars represent the mean ± S.E. (n = 6 wells/time point), and letters above the means indicate significant differences among time points (i.e. means with the same letter are not significantly different; p < 0.05; Scheffe’s test). BTEB1 associates with the proximal TrβA promoter in vivo in a T3 and developmental stage-dependent manner. ChIP assay was conducted using an affinity-purified IgG directed against the N-terminal region of X. laevis BTEB1. A, T3-dependent association of BTEB1 with the proximal TrβA promoter in tadpole brain and tail. Premetamorphic (NF stage 52) X. laevis tadpoles were treated with 10 nM T3 added to the aquarium water for 48 h prior to tissue collection for ChIP assay (see “Experimental Procedures”). The lettered TrβA promoter regions analyzed correspond to those given in Fig. 2 and supplemental Table 1. The TrβA exon 3/exon 4, and the Efa1α and Ifabp promoters were used as negative controls. B, developmental stage-dependent association of BTEB1 with the proximal TrβA promoter in early prometamorphic (NF stage 54) and climax stage (NF stage 62) X. laevis tadpole brain. Only region A of TrβA promoter, which showed robust T3-dependent association of BTEB1 was targeted for ChIP analysis in this experiment. Each of the ChIP experiments was repeated three times with similar results.
different from background (i.e. ChIP with normal rabbit serum; data not shown; see Fig. 5B). We observed a small but statistically significant (p = 0.043; t test) T3-dependent increase in BTEB1 association with the upstream region of the TrβA promoter (region A/B) in XTC-2 cells (Fig. 5C). Note that the level of BTEB1 induction by T3 was lower in XTC-2 cells (~2.5-fold) compared with the brain in vivo (~10.5-fold).

**Induced Expression of BTEB1 in XTC-2 Cells Accelerates Autoinduction of the TrβA Gene**—We used XTC-2 cell transfection and promoter-reporter assays to test the hypothesis that BTEB1 enhances autoinduction of the TrβA gene. Treatment with T3 caused significant time-dependent increases in luciferase activity in cells transfected with empty vector (ANOVA; F = 55.564, p < 0.0001) and pCMV-xBTEB1 (F = 601.043, p < 0.0001; luciferase activity was significantly elevated by 2 h in both treatments; p < 0.05; Scheffe’s test; Fig. 6A). Forced expression of BTEB1 had no effect on basal promoter activity but resulted in a significant acceleration of TrβA promoter autoinduction. Luciferase activity in pCMV-xBTEB1-transfected cells was significantly greater than empty vector controls at 2 and 6 h of T3 treatment (p < 0.0001 for both; unpaired t test; Fig. 6A).

We also used XTC-2 cells to determine whether forced expression of BTEB1 could alter the autoinduction of the endogenous TrβA gene. Treatment with T3 caused a time-dependent increase in endogenous TrβA mRNA in cells transfected with empty vector (pCS2; ANOVA; F = 86.02, p < 0.0001) and pCS2-xBTEB1 (ANOVA; F = 215.2, p < 0.0001; Fig. 6B). At all time points measured, TrβA mRNA was significantly greater in pCS2-xBTEB1-transfected cells compared with empty vector controls (p < 0.05 for 0 h; p < 0.01 for 2, 4, and 6 h; unpaired t test). Furthermore, the increase in TrβA mRNA caused by forced BTEB1 expression occurred in a dose-dependent manner, with 0.3 and 1 μg of pCS2-xBTEB1 plasmid increasing TrβA mRNA 1.2- and 1.4-fold, respectively, over empty vector controls (data not shown).

**BTEB1 Transactivation Domain Is Required for TrβA Autoinduction**—Two N-terminal transactivation domains in rodent BTEB1 that were identified by mutagenesis are highly conserved with the frog proteins (13, 41). We constructed truncated xBTEB1 mutants in which one or both of these transactivation domains were removed to determine whether they are necessary for the action of BTEB1 on TrβA autoinduction in XTC-2 cells. Removal of transactivation domain A (pCS2-xBTEB1A30) or both domains A and B (pCS2-xBTEB1A99 or pCS2-xBTEB1A120) abolished the activity of BTEB1 on TrβA autoinduction (compare with cells transfected with pCS2-xBTEB1; Fig. 7A; p < 0.001). Deletion of only transactivation domain A (pCS2-xBTEB1A30) resulted in apparent dominant negative activity, for it also reduced the T3-induced mRNA as compared with the empty vector control (p < 0.001; Fig. 7A).

**DNA Binding Capacity of BTEB1 Is Not Required for TrβA Autoinduction**—We introduced point mutations into the zinc fingers of BTEB1 to disrupt its DNA binding capacity. Histidine to alanine substitutions of the first histidine residue in each of the three Cys2-His2 zinc fingers were generated by site-directed mutagenesis. The histidine to alanine substitution was shown previously to eliminate the DNA binding capacity of KLF1 (46) and another zinc finger protein JAZ (47). The mutant BTEB1 (pCS2-xBTEB1C2AH) retained full activity on TrβA autoinduction compared with wild type BTEB1 (Fig. 7B). The loss of DNA binding capacity in the BTEB1 C2AH mutant was confirmed by EMSA (Fig. 7C). Similar amounts of wild type BTEB1 and BTEB1 C2AH mutant were used in the EMSA as verified by Western blotting (data not shown).

**DISCUSSION**

Thyroid hormone initiates programs of gene expression in diverse tadpole tissues that underlie the dramatic transformation that occurs during amphibian metamorphosis (48). Several of the early T3 response genes that were identified through gene expression screens code for transcription factors (6–10, 49).
These proteins are hypothesized to regulate a secondary response program of genes necessary for adult phenotypic expression (50). The transcription factor BTEB1, whose hormone-dependent expression depends on one or more TREs located upstream of the transcription start site, is the earliest responding gene thus far identified in the tadpole (9, 13, 14, 51). The Trβ gene is strongly up-regulated by T3, which requires direct binding of the T3/Tr complex to the Trβ promoter (a phenomenon referred to as autoinduction; see Ref. 4). Here we show that BTEB1 associates with the promoter region of the Trβ gene and can enhance T3-dependent transcription. Our findings support the hypothesis that the early up-regulation of BTEB1 during tadpole metamorphosis plays a role in the auto-

induction of Trβ genes, which is hypothesized to be essential for metamorphosis (50). Therefore, the protein products of two primary response genes regulate each other’s promoter. Cross-regulation among primary response transcription factors is likely to be an important means for developmental gene regulation causing robust gene expression responses necessary for driving tissue morphogenesis.

The autoinduction of Trβ genes was originally thought to be the earliest molecular response to T3 in tadpole tissues (1, 3). Previous studies that relied on Northern blotting suggested that the up-regulation of TrβA and Bteb1 mRNAs by T3 was, by and large, coordinate (14, 52). However, several lines of evidence support the view that the Bteb1 genes are the most rapidly responding genes yet identified in tadpole tissues (14, 51). By using RT-PCR we clearly show that BTEB1 is induced by T3 with faster kinetics than Trβ, both in the tadpole in vivo and in the X. laevis fibroblast-derived cell line XTC-2. We found detectable accumulation, and maximal induction of BTEB1 transcripts several hours earlier than Trβ (the precise timing depends on whether tissues or cultured cells are analyzed; see also Refs. 13, 53). Also, BTEB1 protein is up-regulated during spontaneous metamorphosis or by exogenous T3 in tadpoles (see Fig. 18; 13), and BTEB1 and Trβ are expressed in the same cells (13). These findings are consistent with the hypothesis that BTEB1 is present within the cell, either commensurate with or prior to the up-regu-

lation of Trβ and could thus influence transcription of the TrβA gene.

The presence of GC-rich sequences in the proximal TrβA promoter (commonly referred to as Sp1 sites; see Ref. 40), and the early and robust T3 response kinetics of BTEB1 led us to hypothesize that this protein binds to and regulates the TrβA gene. We used EMSA to test whether regions of the proximal TrβA promoter possess binding sites for BTEB1. We found that BTEB1 could directly bind to the GC boxes located in the TrβA promoter. However, although the binding of protein to DNA in an EMSA is suggestive of the presence of a transcription factor binding site, it does not determine whether the DNA-binding protein actually associates with the gene of interest in vivo.
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We test this for BTEB1 and TrβA, we used ChIP assay that depended on a specific affinity-purified antiserum directed against the unique N-terminal region of the frog TBE1 protein. Our ChIP experiments clearly show that BTEB1 associates with the proximal TrβA promoter in vivo in a hormone- and developmental stage-dependent manner. Earlier, we showed that BTEB1 protein is strongly induced by T3 in tadpole brain in vivo and is highly expressed during metamorphic climax (compared with premetamorphosis (13)). The enhanced association of BTEB1 with the TrβA promoter with T3 treatment and at metamorphic climax could be due to the increased expression of BTEB1 and/or an active T3-dependent recruitment of BTEB1 to the promoter.

Similar to our findings in the tadpole in vivo we found that Bteb1 and TrβA mRNAs are up-regulated in XTC-2 cells, and that Bteb1 exhibits faster kinetics than TrβA. We also found that BTEB1 associates with the proximal TrβA promoter in XTC-2 cells by ChIP assay and that the degree of association was higher at an upstream region (region A/B with multiple GC boxes) versus a region in the 5′-UTR (region G with one GC box and versus the TrβA exon 5 where there are no identifiable GC boxes; see Fig. 5B). Furthermore, association of BTEB1 with region A/B in XTC-2 cells was T3-dependent.

Given that BTEB1 and TRs are expressed in the same cells (13) and BTEB1 associates with the proximal TrβA promoter in vitro and in vivo, and frog BTEB1 possesses transactivation function (13), we hypothesized that BTEB1 positively regulates the TrβA gene. In support of this hypothesis we found that induced expression of BTEB1 in XTC-2 cells resulted in faster kinetics and greater absolute magnitude of induction by T3 of the TrβA gene, as determined by promoter-reporter transfection assay and by analysis of the endogenous mRNA expression.

By contrast to the full-length BTEB1, forced expression of N-terminal truncated mutants of BTEB1 in which one or both transactivation domains were removed eliminated activity on TrβA autoinduction. Kobayashi et al. (41) identified two transactivation domains in rat BTEB1 by mutagenesis. Earlier we showed that frog BTEB1 has transactivation activity, and the identified transactivation domains are very similar among the frog and rodent proteins, suggesting conserved functions (13). Our present findings point to an essential role for these regions of BTEB1 for activity on TrβA autoinduction.

Up to this point our results were consistent with BTEB1 binding to GC-rich regions of the frog TrβA gene leading to enhanced autoinduction. We were therefore surprised to discover that this DNA binding capacity was dispensable for BTEB1 action. Substitution of alanines for each of the zinc-chelating histidine residues in the three zinc fingers of BTEB1 destroyed DNA binding but did not alter activity of the protein on TrβA. Thus, although BTEB1 associates with chromatin at the TrβA promoter in vivo, binding to DNA is not required for it to enhance TrβA autoinduction, suggesting that BTEB1 functions in this regard through protein-protein interaction. The GC boxes present in the TrβA promoter could facilitate the targeting of the protein to this genomic region.

Members of the KLF and Sp factor families have been found to synergize with nuclear hormone receptors through protein-protein interactions. For example, Sp1 interacts with the estrogen receptor to regulate several promoters (54–56). BTEB1 was shown to interact with progesterone receptor in the regulation of progesterone receptor target genes in endometrial epithelial cells (30). However, in a preliminary study we found no direct interaction between BTEB1 and TRs using communoprecipitation assays. To our knowledge, other than the PR, BTEB1 interactions with nuclear proteins have not been studied. BTEB1 is a member of a family of proteins (KLF/Sp1-like) that bind to GC- or GT-rich regions in gene promoters (15). It is possible that other KLFs or Sp-like factors regulate the TrβA promoter, and this deserves further study. However, it is noteworthy that BTEB1 is the only KLF identified in several gene expression screens of tadpole tissues that is strongly up-regulated by T3 during metamorphosis (5–12, 49). Also, to our knowledge, BTEB1 is the only KLF/Sp1-like family member found to be regulated by T3 in mammalian cells (24). Thus, if other KLFs participate in Trβ gene regulation they would likely do so as basal or constitutive factors. We propose here that the strong up-regulation of BTEB1 by T3 is critical to the role that BTEB1 plays in regulating the TrβA promoter in vivo.

In conclusion, our results support the hypothesis that the protein product of the immediate early gene Bteb1 associates with the TrβA genomic region in vivo and can enhance autoinduction, i.e. it forms a positive regulatory loop. The surge in plasma T3 that occurs during metamorphic climax in the tadpole is accompanied by a dramatic autoinduction of Trβ genes (57). The autoinduction of Trβ genes is thought to be essential for metamorphosis, especially for later developmental events such as cell differentiation and programmed cell death (e.g. tail resorption; see Ref. 1). Thus, to achieve maximal TR expression to initiate tissue transformation may require that TRs bind to and activate the Trβ promoters (4) and induce the expression of BTEB1, which cooperates with TRs in the autoinduction of their genes. Such cooperativity among the protein products of immediate early genes may be a common phenomenon in animal development.

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REFERENCES

1. Tata, J. R. (2000) Insect Biochem. Mol. Biol. 30, 645–651
2. Yaoita, Y., Shi, Y. B., and Brown, D. D. (1990) Proc. Natl. Acad. Sci. U. S. A. 87, 7090–7094
3. Yaoita, Y., and Brown, D. D. (1990) Genes Dev. 4, 1917–1924
4. Machuca, I., Esslemont, G., Fairclough, L., and Tata, J. R. (1995) Mol. Endocrinol. 9, 96–107
5. Wang, Z., and Brown, D. D. (1991) Proc. Natl. Acad. Sci. U. S. A. 88, 11505–11509
6. Brown, D. D., Wang, Z., Furlow, J. D., Kanamori, A., Schwartzman, R. A., Kholdani, and Jessica Kim for technical assistance.

5 K. L. Howdeshell and R. J. Denver, unpublished data.
Role for BTEB1 in the Autoinduction of T₃ Receptor β