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

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Thyroid hormone (T_3) induces gene regulation programs necessary for tadpole metamorphosis. Among the earliest responses to T_3 are the up-regulation of T_3 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, T_3 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 T_3 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.

Autoinduction of nuclear hormone receptors is a common but poorly understood phenomenon in animal development (1). The autoinduction of thyroid hormone (T_3)[4] receptor genes (*Tr*) during amphibian metamorphosis is a dramatic example of this form of gene regulation (1). All vertebrates possess two *Tr* genes designated *Trα* and *Trβ* (also known as NR1A1 and NR1A2, respectively); *Xenopus laevis* has two *Trα* 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 T_3 (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 T_3·TR 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 T_3 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 T_3 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 Cys_2-His_2 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-

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[S] The on-line version of this article (available at http://www.jbc.org) contains supplemental Table 1.

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[4] The abbreviations used are: T_3, 3,5,3′-L-triiodothyronine; TRβ, T_3 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; RTqPCR, quantitative real time PCR; BTE, basic transcription element; ANOVA, analysis of variance; NF, Nieuwkoop and Faber.

teins 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 transrepression functions (15).

Basic transcription element-binding protein 1 mRNA and protein is strongly up-regulated by T_3 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 T_3 in tadpole tissues (5–12). Earlier, we and others showed that BTEB1 is also regulated by T_3 in developing rodent brain where it promotes neurite outgrowth (24–26). BTEB1 is expressed in uterine endometrial cells where it transactivates the uteroferrin 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 transactivation 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 T_3 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 T_3- and developmental 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 T_3. 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 T_3 plays a role in the transcriptional regulation of the *TrβA* gene during tadpole development.

TABLE 1

Oligonucleotides used for semi-quantitative and quantitative real time RT-PCR

Oligonucleotide primers for semi-quantitative RT-PCR and TaqMan assays were designed to span exon/intron boundaries. The primers for semi-quantitative RT-PCR amplified mRNAs from both *Bteb1A* and *Bteb1B* genes.

Semi-quantitative RT-PCR	
Bteb1	
Forward	5′-CGTGGCAAAGTTTATGGG-3′
Reverse	5′-GGATGGAAGTCGGTATGG-3′
TaqMan Assays	
TrβA	
Forward	5′-GGAAGCCACTGGAAACAGAAAA-3′
Reverse	5′-CATTAACTATGGGAGCTTGTCCAA-3′
Probe	FAM-AAAATTTTTGCCAGAGGAC-MGBNFQ
Bteb1b	
Forward	5′-CCAGTCAGGTCAACCAATGAAA-3′
Reverse	5′-AAACTTTGCCACACCCAGTGT-3′
Probe	FAM-AGG CAC AGG TGT CC-MGBNFQ
rpL8	
Forward	5′-TTTGCTGAAAGAAATGGCTACATC-3′
Reverse	5′-CAC GGC CTG GAT CAT GGA-3′
Probe	VIC-AGG GTA TTG TGA AAG ACA-MGBNFQ

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 brittle (Nasco, Fort Atkinson, WI). Developmental stages were assigned according to Nieuwkoop and Faber (NF) (38). Tadpoles were treated with 3,5,3′-L-triiodothyronine (T_3; 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 mM $MgCl_2$, dNTP mix (1.25 mM each), forward and reverse primers for each gene of interest (10 μM), and *Taq*DNA 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 2

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

The Δ indicates deletion of the first 30–120 amino acids. Primers used for point mutations generated a histidine → 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	
Δ30 xBTEB1 For	5′-ATAGGATCCGCCGCCATGGAAGTGGAGCAGCCC-3′
Δ99 xBTEB1 For	5′-ATAGGATCCGCCGCCATGGAGTTAAACAAGTACC-3′
Δ120 xBTEB1 For	5′-ATAGGATCCGCCGCCATGGACAGCGATGTCACCA-3′
xBTEB1 Rev	5′-ATACTCGAGTCAGGTGAATGATGAATTGGAC-3′
Primers used to generate point mutations in the three zinc finger domains of xBTEB1	
xBTEB1 H211A	5′-GTCTTCCCATCTCAAAGCCGCTTACAGAGTCCATACAGGT-3′
xBTEB1 H241A	5′-CCGATGAGTTAACTCGCGCCTACAGAACCCACACAG-3′
xBTEB1 H269A	5′-TGAGAAGCGATCACTTGACCAAAGCTGCACGTCGCCA-3′

TrβA 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. *TrβA* and *Bteb1* mRNAs were normalized to the level of *rpL8* mRNA.

Plasmid Constructs—The pCMV-xBTEB1 expression plasmid was described by Hoopfer *et al.* (13). The *X. laevis TrβA* 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-xBTEB1Δ30 has a deletion of the first 30 amino acids that includes transactivation domain A; pCS2-xBTEB1Δ99 has both transactivation domains A and B removed; and pCS2-xBTEB1Δ120 represents only the DNA binding domain.

The plasmid construct pCS2-xBTEB1 C2AH harbors histidine to alanine substitutions (H211A, H241A, and H296A) in the first histidine residues of each of the three zinc fingers of BTEB1. This construct was generated using the QuikChange multisite-directed mutagenesis kit (Stratagene) with pCS2-xBTEB1 as template and three primers shown in Table 2.

Electrophoretic Mobility Shift Assay—We conducted electrophoretic mobility shift assay (EMSA) as described by Hoopfer *et al.* (13) with minor modifications. The BTE and mutated BTE probes used were as described by Yanagida *et al.* (16). Bacterial cell lysate containing the fusion protein GST-xBTEB1[DBD] was prepared as described by Hoopfer *et al.* (13). Recombinant wild type BTEB1 or BTEB1 C_2AH 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(dI-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.

TABLE 3

Oligonucleotide primers used for the analysis of the *X. laevis TrβA* promoter

For each pair of oligonucleotides the top sequence is the forward primer, and the bottom sequence is the reverse primer. Capital letters in parentheses correspond to promoter regions given in Fig. 2 and supplemental Table 1. qPCR indicates primer sets that were used for quantitative real time PCR using SYBR Green. All other primer sets were used for standard PCR with radiolabeled precursor.

***TrβA* promoter region**	
−1138/−823 (A)	5′-CAG TGG AGT AAC TAC CAG-3′
	5′-GTA CAC ATG CCT GCA CTA-3′
−841/−604 (B)	5′-TAG TGC AGG CAT GTG TAC-3′
	5′-GAG CAG GTG CAG CAT CTA-3′
−622/−414 (C)	5′-TAG ATG CTG CAC CTG CTC-3′
	5′-ACT ATG GCA TGT TAC AGC-3′
−432/−266 (D)	5′-GCT GTA ACA TGC CAT AGT-3′
	5′-GCC TGA GTG AAG ACC CAT-3′
−283/−92 (E)	5′-ATG GGT CTT CAC TCA GGC-3′
	5′-GTC ATG AAA CTC CTC GGT-3′
−109/+182 (F)	5′-ACC GAG GAG TTT CAT GAC-3′
	5′-TAT AGA CAC AGG CAG CTT A-3′
+164/+366 (G)	5′-TAA GCT GCC TGT GTC TAT A-3′
	5′-TGA CAG TCA GAG GAA CTG A-3′
Exon 3/exon 4	5′-CAG AAA CCT GAA CCC ACA CAA-3′
	5 ′-CAC TTT TCC ACC CTC GGG CGC ATT-3′
qPCR −885/−752	5′-TTG TGC CTG CTT GCT TGC TA-3′
	5′-ACT ATA ATA GGC GGG CCA AGC TGA-3′
qPCR +165/+322	5′-AGC TGC CTG TGT CTA TAC TGA TGG-3′
	5′-ACA GGG AGA TCT ACA GCT GAT CGT-3′
qPCR exon 5	5′-CCC CGA AAG TGA AAC TCT AAC GT-3′
	5′-AAA CCA CTC CAA GTC CTC CAT TTT-3′
Ef1α promoter	5′-TGC ACA GTT GGC GCA GTG-3′
	5′-TGA GGA AGA GAG CGA ACC-3′
Ifabp promoter	5′-ATA GCA GCA GGT GGT TGC G-3′
	5′-GGC CAC AAG ATC TAC TCG-3′

The ability of regions of the proximal *X. laevis TrβA* 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 trβA* 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 *TrβA* promoter fragments that demonstrated competitive binding to GST-xBTEB1[DBD] (see above). Oligonucleotides (24 bp) were syn-

TABLE 4

Short oligonucleotides corresponding to GC-rich regions (bold, underlined) of the *X. laevis* *Trβ*A promoter used as probes and competitors in EMSA

For each pair of oligonucleotides the top sequence is the forward primer, the bottom sequence is the reverse primer.

GC box number[a]	Region of *Trβ*A promoter	Oligonucleotide sequence
1,2	−1032/−1013	5′-gatcGGG**GGCGGG**GGG**CCCGCC**CT-3′ 3′-CCCCGCCCCCCGGGCGGGActag-5′
3	−978/−957	5′-gatcAGATA**GGCGGG**GGGGTGGTG-3′ 3′-TCTATCCGCCCCCCCACCACctag-5′
4	−770/−751	5′-gatcTGG**CCCGCC**TATTATAGTTT-3′ 3′-ACCGGGCGGATAATATCAAActag-5′
5	−723/−704	5′-gatcTCT**GGCGGG**GCCCTGTTATC-3′ 3′-AGACCGCCCCGGGACAATAGctag-5′
6	−545/−526	5′-gatcGGATGCG**GGCGGG**CGCGGGC-3′ 3′-CCTACGCCCGCCCGCGCCCGctag-5′
7	+228/+247	5′-gatcCTC**CCCGCC**CCCCCTATCCT-3′ 3′-GAGGGGCGGGGGGGATAGGActag-5′

[a] Numbering is based on position within the *Trβ*A gene as depicted in Fig. 2.

thesized, each of which encompassed one or two GC boxes within each *TrβA* promoter region (see Fig. 2 and Table 4). Each GC-box containing oligonucleotide was labeled with [^{32}P]dCTP and used as a probe in EMSA.

Cell Culture and Transfection Assays—We plated XTC-2 cells at a density of 2×10^5 cells per well in 6-well plates for gene expression and transfection assays. For ChIP assays, we plated cells at a density of 1×10^6 cells per 100-mm plate. Before transfections or hormone treatments, cells were cultured overnight in a humidified atmosphere of 5% CO_2 at 25 °C. Cells were cultured in Leibovitz-15 medium (L-15; diluted 1:1.5 for amphibian cells; Invitrogen) supplemented with sodium bicarbonate (2.47 g/liter), penicillin G sodium (100 units/ml), streptomycin sulfate (100 μg/ml), and 10% fetal bovine serum that had been stripped of thyroid hormone following the method of Samuels *et al.* (42). For gene expression, transfection, and ChIP assays, cells were treated for different times with or without 5 nM T_3 before harvest.

For the luciferase reporter assay experiments, we transfected cells using the polyethyleneimine (Sigma) method (43). The total amount of DNA per well was normalized by adding empty vector (pCMVneo). All cells were cotransfected with the pRenilla-luciferase plasmid for normalization of cell transfection by dual reporter luciferase assay following the manufacturer's instructions (Promega, Madison, WI). Just prior to transfection, the cells were washed twice with serum-free L-15, and the polyethyleneimine/DNA solution was added directly to the wells. After 1 h the transfection medium was replaced with growth medium, and the cells were incubated overnight. Cells were then treated with or without T_3 for different times before harvest and analysis of luciferase activity. Luciferase activity was quantified (measured as relative light units) using a luminometer (femtometer FB 12; Zylux Corp., Maryville, TN). Each transfection experiment was conducted three times with 4–5 wells per treatment.

For analysis of the effects of forced expression of wild type or mutant BTEB1 on endogenous *TrβA* mRNA, we used the pCS2-based expression vectors described above and transfected XTC-2 cells using FuGENE 6 transfection reagent (Roche Applied Science). Each well of a 6-well plate received 1 μg of plasmid DNA, and the total amount of DNA per well was normalized by adding empty vector (pCS2). Forty eight hours after transfection, cells were treated with or without T_3 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 pCS2-xBTEB1 or pCS2 and extracts of XTC-2 cells treated ±T_3. 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 T_3 (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-xBTEB (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 (*Ef1α* 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 PCRs for ChIP on tadpole brain or tail included [^{32}P]dCTP (1 μCi/reaction), and 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-

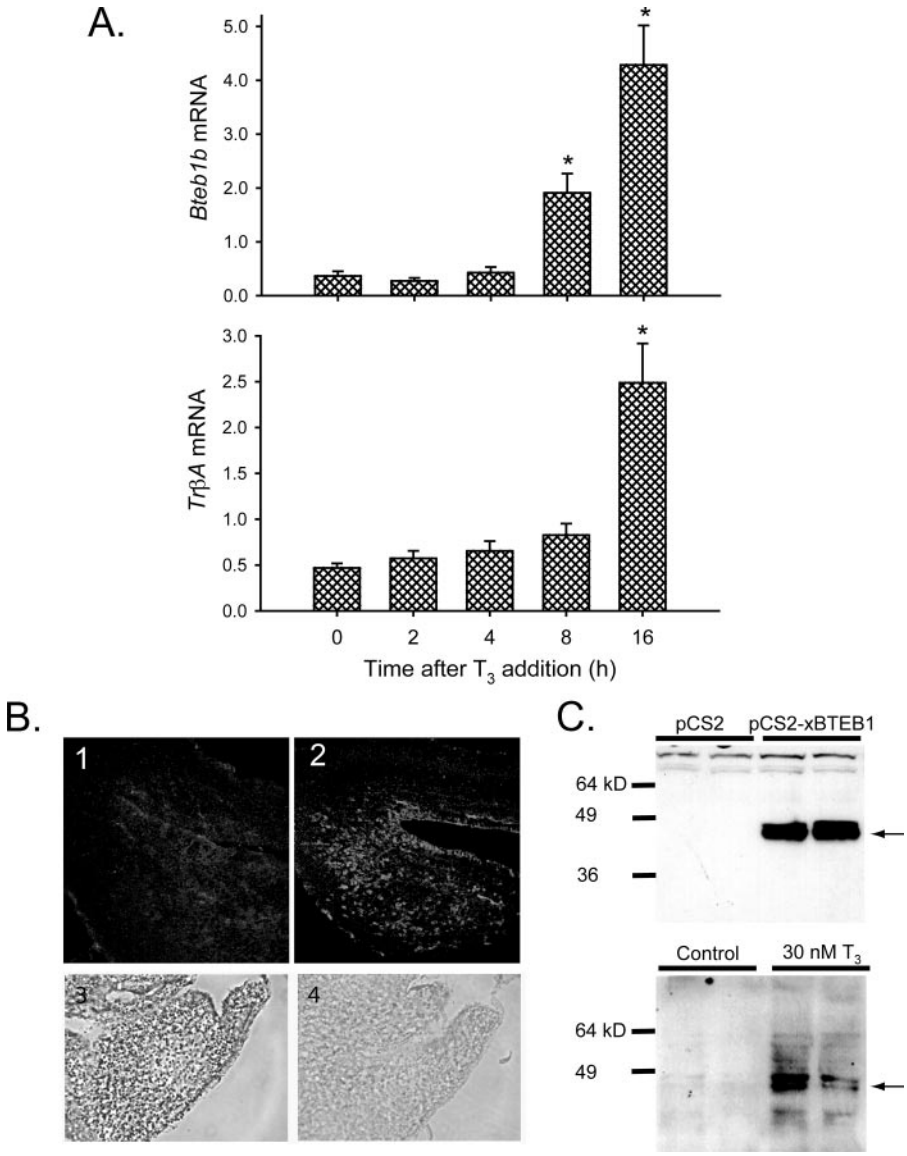

FIGURE 1. **Thyroid hormone up-regulates *Bteb1* mRNA in tadpole brain with faster kinetics than *TrβA* mRNA.** Increased *Bteb1* mRNA correlates with elevated BTEB1 protein. *A,* up-regulation of *Bteb1* (*top*) and *TrβA* (*bottom*) mRNAs in premetamorphic *X. laevis* tadpole brain (NF stage 52) following exposure to T_3 (10 nM) added to the aquarium water ($n = 4$/time point). Gene expression was analyzed by RTqPCR. *Asterisks* designate significant differences from the zero time point ($p < 0.0001$; Scheffe's test). *B,* treatment with T_3 increases BTEB1 protein expression in *X. laevis* tadpole brain. *Panel 1,* BTEB1 protein is expressed at a very low level in NF stage 52 tadpole brain (optic tectum shown) but is increased dramatically by T_3 treatment (*panel 2*; 10 nM in aquarium water for 24 h). *Panel 3,* representative sagittal brain section (hypothalamic region) from an NF stage 52 tadpole treated with T_3 in the aquarium water (10 nM; 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 T_3 (30 nM) for 24 h (*lower panel*). This dose of T_3 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.

facturer's protocol with annealing temperatures adjusted for each primer set. Oligonucleotide PCR primers used for ChIP analyses are shown in Table 3.

RESULTS

Bteb1 mRNA Is Up-regulated by T_3 in Premetamorphic Tadpole Brain with Faster Kinetics than TrβA mRNA—Exposure of premetamorphic (NF stage 52) tadpoles to T_3 (10 nM in the aquarium water) resulted in significant time-dependent increases in brain *Bteb1b* ($F = 222.35$, $p < 0.0001$; ANOVA) and *TrβA* ($F = 74.03$, $p < 0.001$) mRNA levels (Fig. 1). The earliest time point at which a significant increase in *Bteb1b* 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 T_3 (10 nM for 24 h; representative brain sections shown in Fig. 1*B*, *panels 1* and *2*). The strong nuclear staining for BTEB1 was completely abolished by preabsorption with GST-xBTEB (Fig. 1*B*, *panels 3* and *4*).

Western blot analysis with affinity-purified anti-xBTEB1 IgG on protein extracts of transfected XTC-2 cells showed that the antiserum detected the overexpressed BTEB1 protein but did not cross-react with endogenous cellular proteins (Fig. 1*C, upper panel*). Native BTEB1 protein was increased in untransfected XTC-2 cells by 24 h of treatment with T_3 (Fig. 1*C, 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

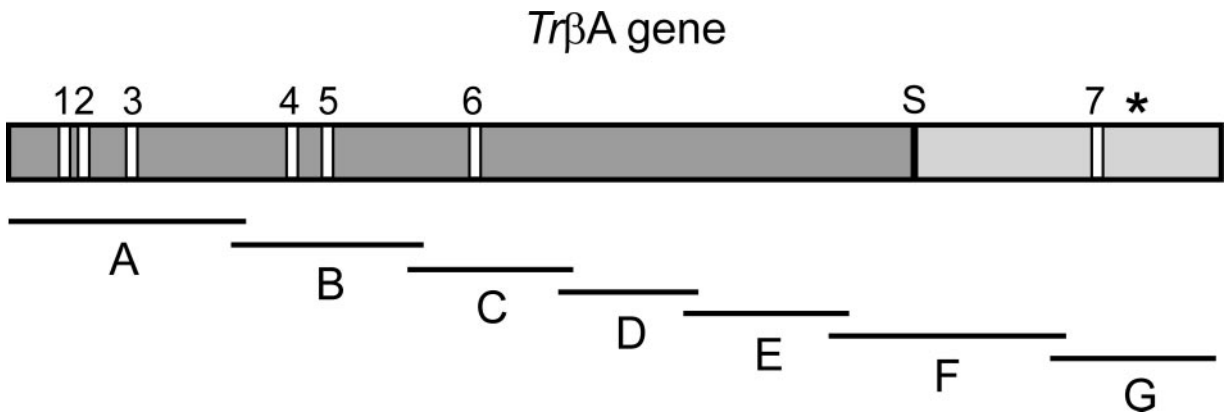

FIGURE 2. **Schematic representation of the *X. laevis TrβA* gene with locations of GC boxes and regions analyzed by EMSA and ChIP assay.** The *bars* with *letters below* indicate the general regions of the *TrβA* gene targeted for analysis by EMSA and in the ChIP assay and correspond to the specific sequences given in supplemental Table 1. The numbering of the seven GC boxes corresponds to that given in Table 4. The *dark gray filled box* represents the upstream region, the *S* indicates the transcription start site, the *light gray filled box* represents the 5′-UTR, and the *asterisk* indicates a TRE that has been characterized and proposed to mediate T_3-dependent transactivation (40, 58).

Table 1, and Table 3), and we used them as competitors in EMSA for binding of bacterially expressed GST-xBTEB1[DBD] (13) to a ^{32}P-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. 3*A*).

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. 3*B*). 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 T_3 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 T_3 for 48 h before sacrifice. We found BTEB1 associated with the proximal *TrβA* promoter *in vivo*, and the signal was increased in a T_3-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

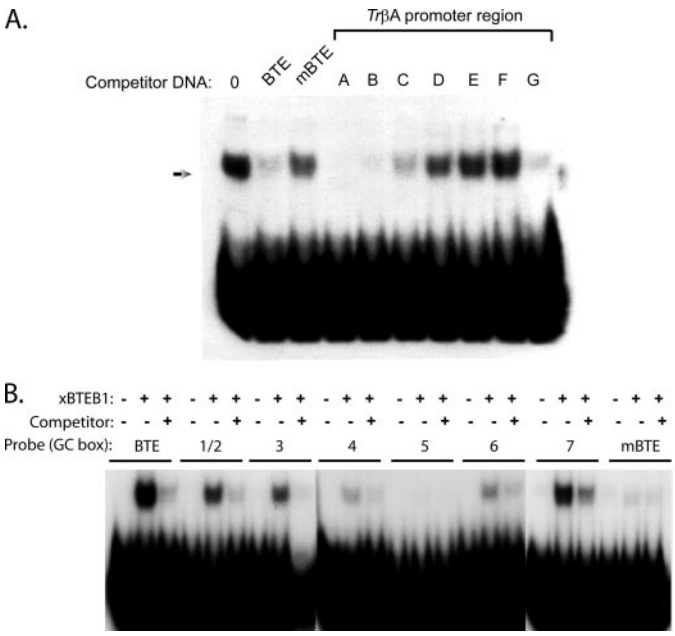

FIGURE 3. *A,* binding of GST-xBTEB1[DBD] to regions of the proximal *X. laevis TrβA* promoter *in vitro*. We used EMSA to test the ability of radioinert DNA fragments (1.89 μM/reaction) corresponding to different regions of the proximal *TrβA* promoter (generated by PCR; see Fig. 2 and supplemental Table 1) to displace GST-xBTEB1[DBD] binding to the ^{32}P-BTE probe. *mBTE*, mutated BTE. *B,* binding of GST-xBTEB1[DBD] to GC-rich regions of the proximal *TrβA* promoter. We used EMSA to test whether GST-xBTEB1[DBD] could bind to short ^{32}P-labeled oligonucleotides encompassing one or two GC boxes in the *TrβA* promoter. The numbering of the GC boxes included in each oligonucleotide probe is based on that given in Fig. 2 and Table 4. In each case homologous, radioinert competitors (1.89 μM) were used to displace binding.

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 T_3 treatment.

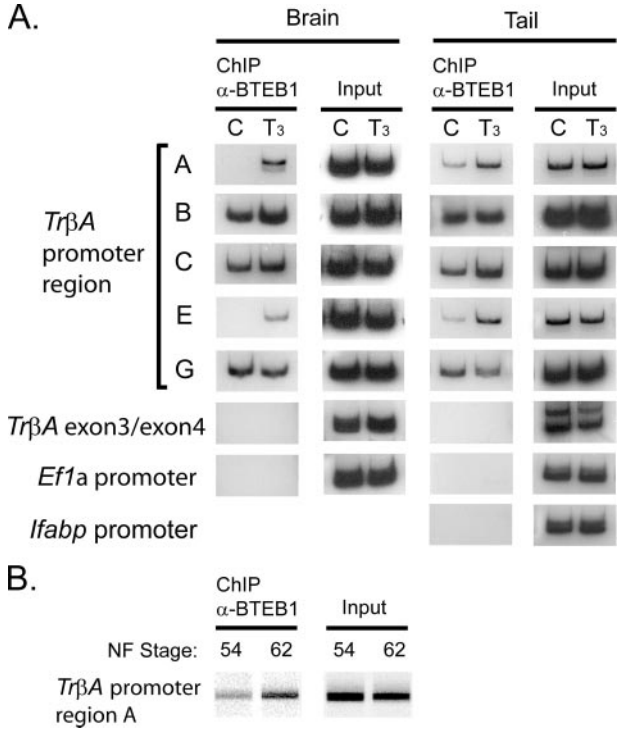

FIGURE 4. **BTEB1 associates with the proximal *TrβA* promoter *in vivo* in a T_3 and developmental stage-dependent manner.** ChIP assay was conducted using an affinity-purified IgG directed against the N-terminal region of *X. laevis* BTEB1. *A,* T_3-dependent association of BTEB1 with the proximal *TrβA* promoter in tadpole brain and tail. Premetamorphic (NF stage 52) *X. laevis* tadpoles were treated with 10 nM T_3 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 *Efα1* 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 T_3-dependent association of BTEB1 was targeted for ChIP analysis in this experiment. Each of the ChIP experiments was repeated three times with similar results.

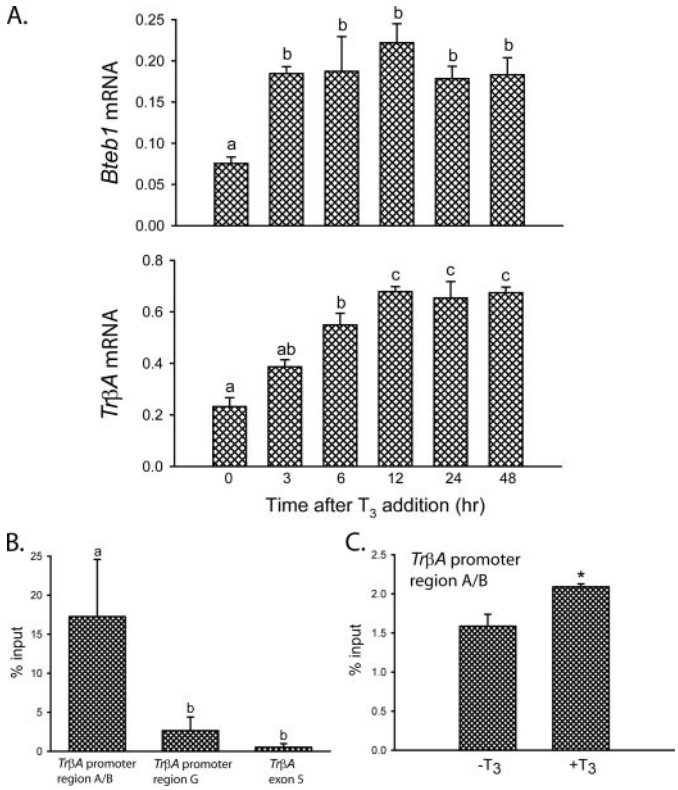

FIGURE 5. **Bteb1 and *TrβA* mRNAs are up-regulated by T_3, and BTEB1 associates with the proximal *TrβA* promoter in XTC-2 cells.** *A,* T_3 up-regulates *Bteb1* mRNA in XTC-2 cells with faster kinetics than TRβ. XTC-2 cells were treated with T_3 (5 nM) for various times before harvest for RNA isolation and semi-quantitative RT-PCR analysis of *Bteb1* and *TrβA* mRNA expression. Gene expression was normalized to the level of *rpL8* expression (a housekeeping gene). *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). *B,* BTEB1 associates with the proximal *TrβA* promoter in XTC-2 cells. XTC-2 cells were treated with T_3 (5 nM) for 24 h, and we used ChIP assay coupled with quantitative real time PCR to detect BTEB1 association with the *TrβA* gene. We found significantly greater association of BTEB1 at an upstream region of the promoter (overlapping with *regions A* and *B* shown in Fig. 2; −885 to −752), which contains multiple GC boxes compared with a region in the 5′-UTR (region G; +166 to +322) that has only one GC box, or the exon 5 of the *TrβA* gene which has no GC boxes. *Letters* indicate significant differences among gene regions (*i.e.* means with the same letter are not significantly different; $p < 0.05$; Scheffe's test). *C,* treatment of XTC-2 cells with T_3 (5 nM, 24 h) increases BTEB1 association with the upstream TRβA promoter (*region A/B*) as analyzed by ChIP assay (*, $p = 0.043$; *t* test).

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 *Ef1α*, or the *Ifabp* promoters (an indirect T_3 response gene that is down-regulated by T_3 (45).

Earlier we showed that *Bteb1* mRNA and protein exhibit dramatic increases in tadpole brain during spontaneous or T_3-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 T_3 production and BTEB1 protein are the highest (13) compared with premetamorphic tadpoles (NF stage 54) when T_3 and BTEB1 are low (Fig. 4*B*).

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 T_3 on *Bteb1* ($F = 11.255$, $p < 0.0001$; ANOVA) and *TrβA* ($F = 36.936$, $p < 0.0001$) mRNA expression in XTC-2 cells (Fig. 5*A*). 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 T_3 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 upstream region of the *TrβA* promoter (*region A/B*; see Fig. 2 and 3), compared with the region located in the 5′-UTR (*region G*; Fig. 5*B*; $F = 12.957$, $p < 0.0001$; ANOVA). The BTEB1 signal at exon 5 of the *TrβA* gene (which is far downstream from the transcription start site) was not significantly

different from background (*i.e.* ChIP with normal rabbit serum; data not shown; see Fig. 5*B*). We observed a small but statistically significant ($p = 0.043$; *t* test) T_3-dependent increase in BTEB1 association with the upstream region of the *TrβA* promoter (*region A/B*) in XTC-2 cells (Fig. 5*C*). Note that the level of BTEB1 induction by T_3 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 Trβ*A *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 T_3 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. 6*A*). 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 T_3 treatment ($p < 0.0001$ for both; unpaired *t* test; Fig. 6*A*).

We also used XTC-2 cells to determine whether forced expression of BTEB1 could alter the autoinduction of the endogenous *TrβA* gene. Treatment with T_3 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. 6*B*). 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 Trβ*A *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-xBTEB1Δ30) or both domains A and B (pCS2-xBTEB1Δ99 or pCS2-xBTEB1Δ120) abolished the activity of BTEB1 on *TrβA* autoinduction (compare with cells transfected with pCS2-xBTEB1; Fig. 7*A*; $p < 0.001$). Deletion of only transactivation domain A (pCS2-xBTEB1Δ30) resulted in apparent dominant negative activity, for it also reduced the T_3-induced *TrβA* mRNA as compared with the empty vector control ($p < 0.001$; Fig. 7*A*).

*DNA Binding Capacity of BTEB1 Is Not Required for Trβ*A *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 Cys_2-His_2 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-xBTEB C_2AH) retained full activity on *TrβA* autoinduction compared with wild type BTEB1 (Fig. 7*B*). The loss of DNA binding capacity in the BTEB1 C_2AH mutant was confirmed by EMSA (Fig. 7*C*). Similar amounts of wild type BTEB1 and BTEB1 C_2AH mutant were used in the EMSA as verified by Western blotting (data not shown).

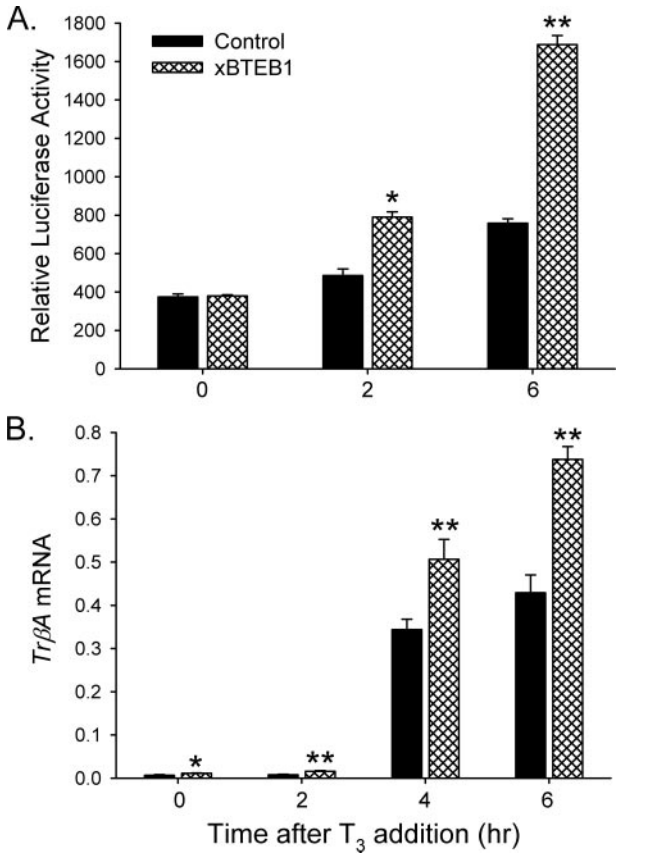

FIGURE 6. **Expression of BTEB1 enhances *TrβA* autoinduction in XTC-2 cells.** *A,* forced expression of BTEB1 accelerates and enhances autoinduction of the *TrβA* promoter. XTC-2 cells were cotransfected with the *X. laevis TrβA* promoter-luciferase plasmid, pCMV-xBTEB1, and pRenilla (to normalize for transfection efficiency using a dual reporter luciferase assay; see "Experimental Procedures"). Cells were treated with 5 nM T_3 for 0, 2, or 6 h before harvest. *Bars* represent the mean ± S.E. The data shown are the means ± S.E. from one transfection experiment ($n = 4$/treatment group), and the experiment was repeated three times with similar results. The T_3-dependent activation of the *TrβA* promoter was not altered by transfection with empty vector (pCMVneo; data not shown.) *Asterisks* denote significant differences from empty vector controls (*, $p < 0.01$; **, $p < 0.001$; Student's unpaired *t* test). *B,* forced expression of BTEB1 increases the expression of endogenous *TrβA* mRNA. XTC-2 cells were cotransfected with 1 μg of pCS2-xBTEB1 or pCS2 empty vector. Forty eight h after transfection the cells were treated with 5 nM T_3 for 0, 2, 4, or 6 h before harvest. Gene expression analysis was done by RTqPCR. Data shown are the means ± S.E. from one transfection experiment ($n = 6$/treatment), and the experiment was repeated four times with similar results. *Asterisks* denote significant differences from empty vector controls (*, $p < 0.05$; **, $p < 0.001$; unpaired *t* test).

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 T_3 response genes that were identified through gene expression screens code for transcription factors (6–10, 49).

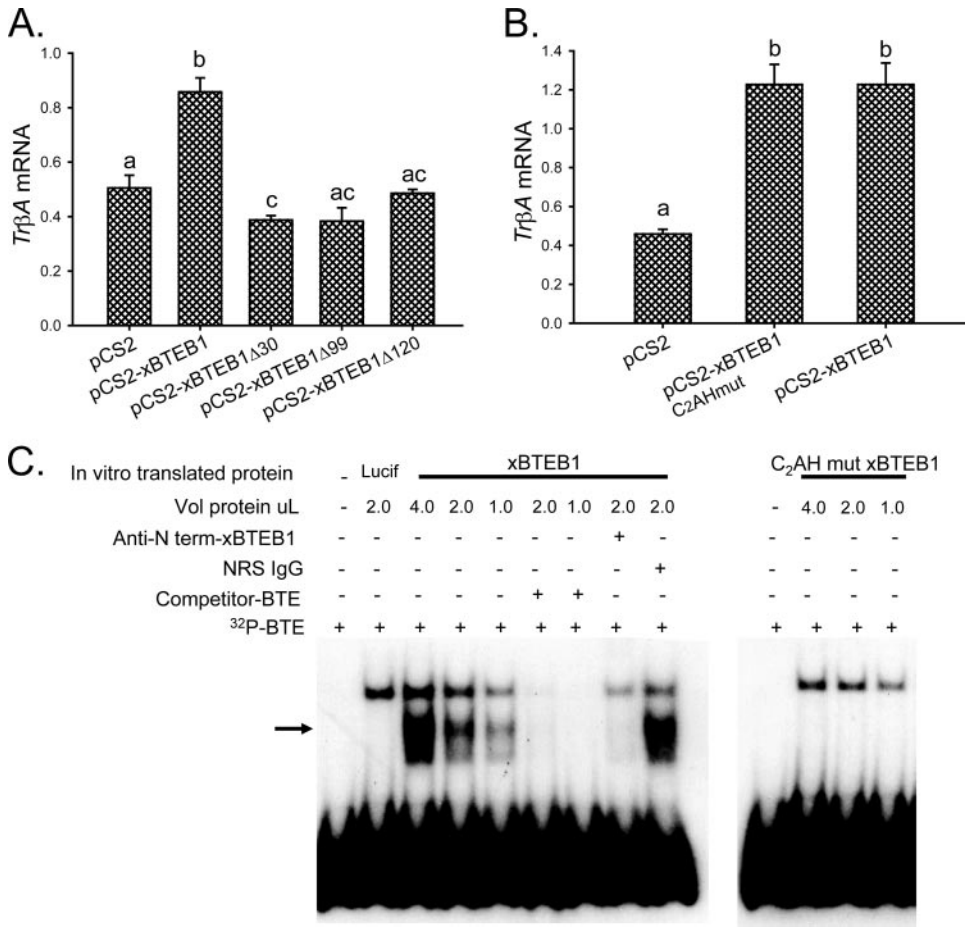

FIGURE 7. **The N-terminal transactivation domains but not the DNA binding capacity of the zinc fingers of BTEB1 are required for *TrβA* autoinduction.** *A,* N-terminal truncated forms of xBTEB1 fail to enhance *TrβA* autoinduction in XTC-2 cells. XTC-2 cells were transfected with the indicated expression vectors, and 48 h later cells were treated with 5 nM T_3 for 6 h. Gene expression analysis was done by RTqPCR. Data shown are the means ± S.E. for the T_3-treated cells only; $n = 6$/treatment. *Letters* indicate significant differences among treatments (*i.e.* means with the same letter are not significantly different; $p < 0.05$; Bonferroni's multiple comparison test). *B,* mutations in the three zinc fingers of BTEB1 do not affect activity on *TrβA* autoinduction. The first histidine residue in each of the Cys_2-His_2 zinc finger DNA binding domain of BTEB1 was mutated to alanine to generate pCS2-xBTEB C_2AH. XTC-2 cells were transfected with the indicated expression vectors, and 48 h later cells were treated with 5 nM T_3 for 6 h. Data shown are the means ± S.E. from one transfection experiment ($n = 6$/treatment) and the experiment was repeated twice with similar results. *Letters* indicate significant differences among treatments (*i.e.* means with the same letter are not significantly different; $p < 0.05$; Bonferroni's multiple comparison test). *C,* electrophoretic mobility shift assay showed that the BTEB1 C_2AH mutant does not bind to DNA. Recombinant wild type BTEB1 and BTEB1 C_2AH mutant proteins were generated by coupled *in vitro* transcription/translation, and varying amounts were tested for their ability to bind to the ^{32}P-BTE probe *in vitro*. Radioinert BTE oligonucleotide was added to some reactions as a competitor, and antibody supershift was used to verify the presence of BTEB1 protein in the protein-DNA complexes formed. Western blot analysis confirmed that equal amounts of wild type and mutant BTEB1 proteins were used in the EMSA (data not shown).

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 T_3 in tadpole tissues (1, 3). Previous studies that relied on Northern blotting suggested that the up-regulation of *TrβA* and *Bteb1* mRNAs by T_3 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 T_3 with faster kinetics than TR*β*, 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 TR*β* (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 T_3 in tadpoles (see Fig. 1*B*; 13), and BTEB1 and TR*β* 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-regulation of TR*β* and could thus influence transcription of the *TrβA* gene.

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 *TrβA* gene is strongly up-regulated by T_3, which requires direct binding of the T_3·TR complex to the *TrβA* promoter (a phenomenon referred to as autoinduction; see Ref. 4). Here we show that BTEB1 associates with the promoter region of the *TrβA* gene and can enhance T_3-dependent transcription. Our findings support the hypothesis that the early up-regulation of BTEB1 during tadpole metamorphosis plays a role in the auto-

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 T_3 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*. To

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 BTEB1 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 T_3 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 T_3 treatment and at metamorphic climax could be due to the increased expression of BTEB1 and/or an active T_3-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. 5*B*). Furthermore, association of BTEB1 with region A/B in XTC-2 cells was T_3-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 T_3 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 coimmunoprecipitation assays.[5] 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 T_3 during metamorphosis (5–12, 49). Also, to our knowledge, BTEB1 is the only KLF/Sp1-like family member found to be regulated by T_3 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 T_3 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 T_3 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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Supplemental Table 1. The *X. laevis TrβA* promoter divided into seven segments for analysis, and location of GC-rich sequences.

A) -1138/-823 Three GC boxes (-1029/-1024; -1020/-1015; -973/-968)

Cagtggagtaactaccaggggagcaggggtgcgattgggccagggcctgcaccccgtcagggcctcgcgcgccattgact
tctttgtaattctgggcgtacggagggg**GGCGGG**ggg**CCCGCC**ctcctctagttacgttactgtctgcctaggttgca
aagata**GGCGGG**ggggtggtgctgtgcaggtacttaacatttgtctgcctacccctagtctatgctctcttgctcctgctgcct
ctttcctcagttgtgcctgcttgcttgctaccccgtgcacacaacatcatacagtagtgcaggcatgtgtacc

B) -841/-604 Two GC boxes (-767/-762; -720/-715)

tagtgcaggcatgtgtacccacaggggggaggtgaggtggccggccggtttgcctagggtgccaggtcagcttgg**CCCGC**
Ctattatagtttagaaagtgggccctcagcctaaggttttct**GGCGGG**gccctgttatcccagtctgacactgccgacctcg
aactcttcttcgcgggtggcgtcaaatgccggcttctgacttctgggttcgtcttttatagatgctgcacctgctcg

C) -622/-414 One GC box (-538/-533)

Tagatgctgcacctgctcgccccatccctttgtgacttcatcagcggcacaggtctataaaagcaacctggcagtcggatgcg
GGCGGGcgcgggctaaggtaagcccgggttagggtcggggaaataccaacctgcacatcactaccggactggcaatct
gtgggttctggcatatgccagagaggctgctgtaacatgccatagtc

D) -432/-266 No GC box

gctgtaacatgccatagtcattatttagtgggctggtggggagctgagtgggtccttgtgtacttggaatgccagggcctattttga
atcccagtccagacctgcagacacacacaaacccccaatcctgcaactgccccacagcccttatgggtcttcactcaggc

E) -283/-92 No GC box

atgggtcttcactcaggctcttattttcctgcagattctgtttagttgttactgaataagccctgctgcttccaagctgcactctccctgt
gtgactgaagaggcgcaggctggctgagttaatatagtgaataaagtacccccctcttgtaaaatataaggatattataagttaccga
ggagtttcatgacc

F) -109/+182 No GC box

ccgaggagtttcatgaccatataaaaacacgaggccgaaggccgagtgtttttatacaggtcatggaactccgaggtaact
tctaatatcctcgtattttacaactggg**G**gtactttattaattataatacacacatttcagtgagtcatgtgacagaaattacatcacta
ctcaccgtttataactgatgacatcactactcaccgtttataaggatataatttacaggatattcatggcttttgtgtattatatatatgtat
gtatacagtaagctgcctgtgtctata

G) +164/+366 One GC box (+231/+236)

taagctgcctgtgtctatactgatgggatgggaagcagaggctgcccgacccctcctcttgattctcc**CCGCCC**cccctatc
cttgttcgtcctccctaggc**aggtca**tttc**aggaca**gcccagcgccctggtgcacgatcagctgtagatctccctgtctgtgtcgc
tgctgccgctgctacttcagttcctctgactgtcag

GC boxes are indicated in boldtype capitals. TRE half sites are boldtype lowercase underlined. The transcription start site is the capital, boldtype, shaded ‘G’ in fragment F (based on 58). Genbank Accession # U04675.