

Basic transcription element binding protein is a thyroid hormone-regulated transcription factor expressed during metamorphosis in *Xenopus laevis*

Eric D. Hooper, Liyue Huang and Robert J. Denver*

3065C Natural Science Building, Department of Molecular, Cellular and Developmental Biology, The University of Michigan, Ann Arbor, MI 48109-1048, USA.

Basic transcription element binding protein (BTEB) is a member of the Krüppel family of zinc finger transcription factors. It has been shown that BTEB plays a role in promoting neuronal process formation during post-embryonic development. In the present study, the biochemical properties, transactivation function, and the developmental and hormone-regulated expression of BTEB in *Xenopus laevis* (xBTEB) are described. xBTEB binds the GC-rich basic transcription element (BTE) with high affinity and functions as a transcriptional activator on promoters containing multiple or single GC boxes. xBTEB mRNA levels increase in the tadpole brain, intestine and tail during metamorphosis, and are correlated with tissue-specific morphological and biochemical transformations. xBTEB mRNA expression can be induced precociously in premetamorphic tadpole tissues by treatment with thyroid hormone. *In situ* hybridization histochemistry showed that thyroid hormone upregulates xBTEB mRNA throughout the brain of premetamorphic tadpoles, with the highest expression found in the sub-ventricular zones of the telencephalon, diencephalon, optic tectum, cerebellum and spinal cord. xBTEB protein parallels changes in its mRNA, and it was found that xBTEB is not expressed in mitotic cells in the developing brain, but is expressed just distal to the proliferative zone, supporting the hypothesis that this protein plays a role in neural cell differentiation.

Key words: gene expression, metamorphosis, neuronal differentiation, thyroid hormone, *Xenopus*.

Introduction

Thyroid hormone (3,5,3'-triiodothyronine; T₃) plays a critical role in vertebrate development, particularly in the maturation of the central nervous system (Legrand 1983; Porterfield & Hendrich 1993). However, the molecular basis for T₃ action in development remains poorly understood. In amphibian larvae, T₃ is the primary morphogen controlling metamorphosis. The hormone acts by binding to nuclear receptors that function as ligand-activated transcription factors, and thus it activates tissue-specific gene regulation programs. The protein products of these hormone-inducible genes provide the basis for the dramatic morphological and biochemical transformations that are characteristic of metamorphosis. The immediate, early gene regulation programs have been partially characterized in the tail (Wang & Brown 1993; Brown *et al.* 1996), hindlimb (Buckbinder & Brown 1992), intestine (Shi & Brown 1993) and brain (Denver *et al.* 1997). However, the biological functions of the protein

products of these genes have been characterized in only a few cases (Shi 2000).

The basic transcription element binding protein (BTEB) gene was isolated as a direct T₃ response gene in gene expression screens of premetamorphic tadpole tail (Wang & Brown 1993; Brown *et al.* 1996) and brain (Denver *et al.* 1997). This gene is rapidly and strongly induced (>10-fold) by exogenous T₃ in these tissues. We showed that BTEB expression is also regulated by the thyroid in the developing rodent brain (Denver *et al.* 1999; Cayrou *et al.* 2002). BTEB was first isolated from rat (Imataka *et al.* 1992) in a screen for proteins that bind to a GC-rich (GC box) sequence in the promoter region of the cytochrome P-450A1 gene (Yanagida *et al.* 1990). This *cis*-acting DNA element was shown to be necessary for basal transcription of P-450A1 and it is required for maximal inducible expression of this gene by xenobiotics; it was thus designated the basic transcription element (BTE; Yanagida *et al.* 1990). Subsequent to the initial cloning of rat BTEB, other closely related genes were identified in mammals. The rat BTEB gene first isolated by Imataka *et al.* (1992) is now designated BTEB1, while two other paralogous members of this family are designated BTEB2 (Sogawa *et al.* 1993a) and BTEB3 (Martin *et al.* 2000). BTEB2 and BTEB3,

*Author to whom all correspondence should be addressed.

Email: rdenver@umich.edu

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however, are more distantly related to BTEB1 than some of the other members of this family of transcription factors (Krüppel-like family; Dang *et al.* 2000). The frog (*Xenopus laevis*) BTEB (xBTEB) shares greatest sequence similarity with mammalian BTEB1 proteins (Fig. 1).

The GC box sequence is commonly found in promoters of cellular and viral genes, and was originally shown to be a recognition site for Sp1 (Dyanan *et al.* 1985; Courey & Tjian 1988; Mitchell & Tjian 1989). BTEB proteins are members of the Krüppel-like family of proteins (Dang *et al.* 2000). Krüppel is a protein product of a segmentation gene first identified in *Drosophila melanogaster* (Schuh *et al.* 1986). Members of this family possess three Cys₂-His₂ zinc fingers that form the DNA-binding domain (DBD) of the protein. Krüppel-like proteins are distantly related to Sp1 and other Sp family members, and share a high degree of similarity in the DBD; for example, the DBD of rat BTEB1 shares 72% sequence similarity with rat Sp1 (Imataka *et al.* 1992). However, the proteins are highly divergent outside of the DBD; for example, the size of rat BTEB is 244 amino acids while rat Sp1 is 788 amino acids. Thus, one can predict that these proteins present very different interfaces for protein-protein interactions that govern which promoters are regulated and the nature of the regulation.

In an effort to understand the role that BTEB plays in amphibian metamorphosis and in vertebrate

development generally, we analyzed the molecular and biochemical characteristics, and the expression pattern of xBTEB mRNA and protein in *X. laevis*. We show that xBTEB binds DNA with high affinity and is capable of activating transcription from GC box-containing promoters. xBTEB mRNA is strongly upregulated by treatment with T₃ in the brain, intestine and tail, and shows a developmental pattern of expression that parallels the morphological and biochemical transformations that occur in each tissue. In addition, we verified that xBTEB protein is expressed during metamorphosis and is upregulated by T₃. The protein is not expressed at detectable levels in cells induced to proliferate by T₃ treatment, but is strongly upregulated in adjacent cells. This finding supports previous findings in mammalian cells which show that BTEB plays a role in cellular differentiation but not in proliferation (Denver *et al.* 1999; Cayrou *et al.* 2002).

Materials and Methods

Animal care, hormone and drug treatments, and hormone analyses

Xenopus laevis tadpoles were raised in dechlorinated tap water (water temperature 20–22°C) and fed pulverized rabbit chow ad libitum. Developmental stages were assigned according to the Nieuwkoop and Faber (NF) method (Nieuwkoop & Faber 1956).

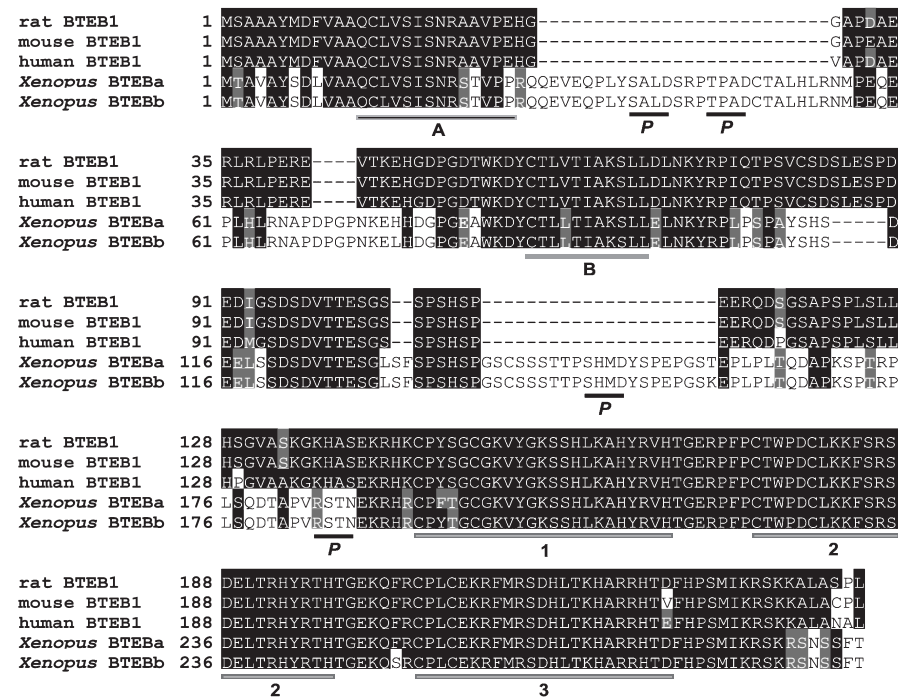


Fig. 1. Comparison of amino acid sequences for mammalian basic transcription element binding protein (BTEB)1 and *Xenopus laevis* BTEB proteins. The DNA-binding domain of BTEB, containing the three Cys₂-His₂ zinc fingers (residues 145–167, 175–197 and 205–227 of rat, and residues 193–215, 223–245 and 253–275 of frog), is highly conserved between mammals and amphibians. Similarly, the putative transcriptional activation domains are highly conserved (region A, residues 13–26 of both rat and frog; region B, residues 33–50 of rat and residues 88–98 of frog). GenBank accession numbers for amino acid sequences are as follows: rat (S25288), mouse (O35739), human (XP_005584), *Xenopus* 1A (AAC59863) and 1B (AAC59864). Red bars indicate the putative transactivation domains (A and B) and purple bars designate the

three zinc fingers (1, 2 and 3; DNA-binding domain). Four putative phosphorylation sites are designated 'P'. Black shading indicates identical amino acid residues and gray shading indicates conserved substitutions.

Thyroid hormones or drugs were administered by addition to the aquarium water; water was changed and drugs or hormones replenished daily over the treatment period. Tadpoles were treated with T₃ (sodium salt; Sigma-Aldrich, St Louis, MO, USA) at 5, 50 or 200 nM for various times. Adults were treated with 20 nM thyroxine (T₄; Sigma-Aldrich) for 10 days. The goitrogen methimazole (MMI; Sigma-Aldrich) was administered at 1 mM for 10 days.

Thyroxine radioimmunoassay Plasma T₄ concentration was determined by radioimmunoassay (RIA; Mackenzie & Licht 1984; Denver & Licht 1988). Primary antiserum for T₄ was purchased from Dr Viggo Kruse (Denmark). All samples were measured in a single RIA with an intra-assay coefficient of variation of 4.7%.

DNA constructs

The full-length cDNA corresponding to the mRNA of the *X. laevis* BTEBa gene (GenBank accession no. U35408) was isolated by polymerase chain reaction (PCR) from a tadpole brain (diencephalon) cDNA library (Denver *et al.* 1997). Primers designed to specifically amplify *X. laevis* BTEBa but not BTEBb (hereafter referred to as xBTEB) were used: forward primer, 5'-GGAATTCTGATGACAGCTGTCGCTTAC-AGT-3'; reverse primer, 5'-GGGCTCGAGTCAGGTG-AATGATGAATT-3'. A region containing the DBD of xBTEB (amino acid residues 120–292) was PCR amplified with the full-length xBTEB cDNA as template using a forward internal primer (5'-GGGGAATTC-TATCGGACAGCGATGTC-3') and the same reverse primer as above. The cDNAs for the full-length xBTEB and the xBTEB[DBD] were directionally cloned into the pBluescript KS⁻ and pGEX-KG vectors at the *EcoRI* and *XhoI* restriction sites. The pGEX-KG prokaryotic expression vector (Guan & Dixon 1991) was used to express xBTEB and xBTEB[DBD] as fusion proteins with a glutathione-S-transferase (GST) tag. Both cDNAs were also subcloned into the pCMV-Neo eukaryotic expression vector (a gift of Michael Uhler, The University of Michigan, Ann Arbor, MI, USA) at the *EcoRV* and *XhoI* sites to produce pCMV-xBTEB and pCMV-xBTEB[DBD]. The constructs were verified by DNA sequencing using the Rhodamine dye terminator cycle sequencing kit (PE Applied Biosystems, Foster City, CA, USA).

Recombinant fusion protein expression and purification

Expression of recombinant fusion proteins Cultures of *Escherichia coli* transformed with either pGEX-

xBTEB or pGEX-xBTEB[DBD] were grown at 37°C and protein expression induced by addition of 0.4 mM isopropylthio-β-D-galactopyranoside (IPTG). Cells were harvested by centrifugation and then they were resuspended in 150 mM NaCl, 10 mM Na₂HPO₄, 4 mM NaH₂PO₄ (pH 7.3) and 1% Triton X-100 (PBST). The cell lysate was isolated by brief sonication on ice followed by centrifugation at 10 000 *g* for 5 min at 4°C. To determine the solubility of each fusion protein, fractions of the lysate and insoluble pellet (containing inclusion bodies) were analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE). The GST-xBTEB[DBD] was present in the lysate fraction and was isolated under non-denaturing conditions. However, GST-xBTEB was insoluble and required denaturing conditions for purification.

Purification of fusion proteins The soluble GST-xBTEB[DBD] fusion protein was purified as described by Guan & Dixon (1991) using a 50% (v/v) slurry of glutathione-agarose beads (Sigma-Aldrich). Insoluble GST-xBTEB was purified from bacterial inclusion bodies under denaturing conditions. The pellet was boiled in 0.1 M SDS and 5% β-mercaptoethanol for 10 min, fractionated by 10% SDS–PAGE, and fusion protein recovered from gel slices by electroelution. After dialysis to remove SDS, aliquots of both purified recombinant proteins were analyzed by SDS–PAGE to estimate protein purity and concentration (with bovine serum albumin [BSA] standards).

Antibody production and affinity purification

A polyclonal antiserum was generated in rabbit against GST-xBTEB by a commercial laboratory (Lampire Biological Laboratories, Pipersville, PA, USA). The immunoglobulin (IgG) fraction of the anti-xBTEB serum (2 mL) was purified by affinity chromatography on an Affi-Gel protein A column (Bio-Rad Laboratories, Hercules, CA, USA) according to the manufacturer's protocol. This fraction was further purified by affinity column chromatography using a GST-xBTEB[DBD] affinity column. For affinity column purification, the Affi-Gel 10 support (Bio-Rad Laboratories) was chosen based on the isoelectric point of the GST-xBTEB[DBD] (pI = 9.12; calculated using Vector NTI software; Informax, Bethesda, MD, USA). The affinity column was prepared following the manufacturer's protocol by coupling 2 mg GST-xBTEB[DBD] to 1 mL washed support. The column flow-through, which contained antibodies to the N-terminal region of xBTEB, was collected and re-applied to the affinity column twice (i.e. antibodies to the DBD of xBTEB and the GST were 'subtracted'). The specificity of the resultant affinity subtracted IgG

was verified by western blotting. The purified IgG reacted strongly with the full-length xBTEB fusion protein but did not recognize GST-xBTEB[DBD] or GST alone (data not shown).

Electrophoretic mobility shift assay

Electrophoretic mobility shift assay (EMSA) analyses were conducted essentially as described by Wu *et al.* (1998), with minor modifications. The BTE and mutated BTE (mBTE) probes used were as described by Yanagida *et al.* (1990). Recombinant proteins were produced by coupled *in vitro* transcription–translation (TNT system; Promega, Madison, WI, USA; programmed with pBluescript-xBTEB) or by over-expression in *X. laevis* XTC-2 cells (transfected with pCMV-xBTEB). *In vitro* produced xBTEB (5 μ L of a 50 μ L TNT reaction) or extracts from XTC-2 cells (10 μ g total cellular protein) were incubated in a volume of 35 μ L with 20 000–60 000 c.p.m. 32 P-BTE and 1.4 μ g double-stranded poly(dI-dC) in a buffer containing 20 mM HEPES (pH 7.8), 1 mM dithiothreitol (DTT), 0.1% IGEPAL CA-630 (Sigma-Aldrich), 50 mM KCl and 20% glycerol. Unlabeled BTE or mBTE were added as specific or non-specific competitors, respectively. The reaction continued at room temperature for 40 min before fractionation by 6% non-denaturing PAGE. The gel was dried and analyzed by autoradiography. For antibody supershifts, 5 μ L of straight (unpurified) anti-xBTEB, antirat BTEB (a gift of Dr Fujii-Kuriyama, Tohoku University, Sendai, Japan), normal rabbit serum (NRS) or 1 μ L antihuman Sp1 serum (Geneka Biotechnology, Montreal, Québec, Canada) was added to the EMSA reaction and incubated at room temperature for 20 min before adding the probe.

The DNA-binding affinities of the bacterially expressed GST-xBTEB and GST-xBTEB[DBD] were determined by EMSA competitive binding assays using cold BTE (or mBTE) as competitor, 32 P-BTE as probe and 0.5 ng purified fusion protein (which gave approximately 50% binding in the assay; mBTE did not compete for binding in this assay; data not shown). The resulting autoradiographs were digitized using a flatbed scanner and the mean density of the supershifted bands was measured using Scion Image software (version 3.0; Scion Corporation, Frederick, MD, USA). An inhibition constant (K_i) for each assay was calculated by fitting data to the following regression equation using the computer program Sigmaplot (version 5.0; SPSS Science, Chicago, IL, USA): $y = 1 - (ax) / (b + x)$ (Cortright *et al.* 1995), where y is the relative intensity of the band as determined by optical density; x is the molar concen-

tration of competitor; a is the intensity of the band in the absence of competitor; and b is the K_i . A mean K_i was calculated from data from three independent binding assays for each protein.

Cell culture and transient transfections

Cells were cultured in a humidified atmosphere of 5% CO₂. The incubation temperature was 37°C for mammalian and 25°C for amphibian cells. CV-1 cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Sigma-Aldrich) and XTC-2 cells in Leibovitz-15 medium (L-15; Invitrogen Life Technologies, Carlsbad, CA, USA; diluted 1:1.5 for amphibian cells). All growth media were supplemented with sodium bicarbonate (2.47 g/L), penicillin G sodium (100 U/mL), streptomycin sulfate (100 μ g/mL) and 10% fetal bovine serum. Chloramphenicol acetyl transferase (CAT) content in cell extracts at the end of experiments was analyzed using a CAT-ELISA kit following the manufacturer's instructions (Roche, Mannheim, Germany). Total cellular protein was analyzed by the Bradford method (Pierce, Rockford, IL, USA) and CAT content was normalized to protein content.

Transfection of XTC-2 cells XTC-2 cells were plated at a density of 5×10^5 per 6 cm dish, 24 h before transfection. The polyethylenimine (pEi) method (Meunier-Durmort *et al.* 1997) was used for transient transfection. The total amount of DNA was kept constant by adding pCMV-Neo (empty vector) as a carrier. Cells were washed twice with serum-free L-15, and the pEi/DNA in L-15 was added directly to the cells. After 1 h of incubation, the transfection medium was replaced with growth medium. Cells were incubated for 24 h before harvesting and extraction for EMSA analyses (Ranjan *et al.* 1994).

For reporter assays in XTC-2 cells, cells were co-transfected with a *X. laevis* thyroid receptor β promoter-CAT plasmid (xTR β /CAT; generously provided by Dr J. R. Tata, National Institute of Medical Research, London, UK; Machuca *et al.* 1995) and varying concentrations of pCMV-xBTEB[DBD]. Cells were incubated in the absence or presence of 30 nM T₃ for 24 h before harvesting and analysis of CAT expression (this dose of T₃ produced near maximal induction in a previous experiment; E. D. Hoopfer and R. J. Denver, unpubl. data, 2000). In a separate experiment, plates were harvested for analysis of BTE binding activity by EMSA.

Transfection of CV-1 cells Transient transfection of CV-1 cells by the calcium phosphate precipitation

method (Gorman *et al.* 1982) was carried out under the same conditions used by Imataka *et al.* (1992) for analysis of rat BTEB and Sp1. Cells were co-transfected with 3 μ g promoter/reporter plasmids and 15 μ g of expression plasmids. Promoter/reporter plasmids used were pSV2CAT (Gorman *et al.* 1982), pSV/MC53 (Yanagida *et al.* 1990) or pSV/MC53 + 4GC (Imataka *et al.* 1992). pSV2CAT contains the simian virus 40 (SV40) early promoter driving expression of CAT (this native promoter has six GC boxes; Gorman *et al.* 1982). pSV/MC53 (Yanagida *et al.* 1990) and pSV/MC53 + 4GC (Imataka *et al.* 1992) contain a single GC box and five GC boxes, respectively, inserted between the SV40 enhancer and TATA box driving CAT. Expression plasmids used were pCMV-Neo (empty vector), pCMV-xBTEB, pRSV-rBTEB or pRSV-rSp1 (Imataka *et al.* 1992). Following transfection, cells were incubated for 24 h before harvesting for analysis of CAT expression. Each transfection experiment was repeated three times.

RNA isolation and northern blot analysis

RNA was isolated from individual adult brains (diencephalon) and pooled tadpole brains (diencephalon, 10 per pool; intestine, three per pool; and tail, three per pool) using Trizol reagent (Gibco BRL). Northern blots were prepared with 10 μ g total RNA as described by Denver *et al.* (1997). Blots were probed with a 32 P-labeled xBTEB cDNA generated by random priming (Amersham Pharmacia Biotech, Piscataway, NJ, USA). The same blots were stripped and reprobed with a 32 P-labeled cDNA for the *X. laevis* ribosomal protein L8 gene (rpL8; Shi & Liang 1994) to normalize for RNA loading. Each experiment analyzing xBTEB mRNA expression was replicated three times. Blots were digitized and the mean density of the bands was determined using Scion Image software.

Histochemistry

Animal treatment and tissue preparation Premetamorphic (NF stage 52–54) or late prometamorphic (NF stage 60) tadpoles were treated with or without 50 nM T₃ for 48 h before they were killed and tissue was harvested. To analyze cell proliferation in the brain, tadpoles treated with or without T₃ were cultured in 500 μ M bromodeoxyuridine (BrdU; Sigma-Aldrich) 3 h before they were killed. Whole heads (premetamorphic) or brains (prometamorphic) were removed and fixed in 4% paraformaldehyde for 24 h at 4°C and then were saturated in 30% sucrose for 24 h. Tissues were embedded in OCT compound (Sakura [Tissue-

Tek], Torrance, CA, USA), frozen, and cryosectioned sagittally at 20 μ m. Five brains per treatment or developmental stage were used for each histochemical analysis.

In situ hybridization histochemistry for xBTEB mRNA *In situ* hybridization histochemistry (ISSH) for xBTEB mRNA was conducted following methods described by Mansour *et al.* (1994). Linearized pBluescript-xBTEB plasmid templates were prepared and full-length sense or antisense digoxigenin (DIG)-labeled xBTEB cRNA probes were transcribed *in vitro* using a DIG RNA labeling kit (Roche). Cryosections were hybridized overnight at 42°C with 1 μ g probe per 100 μ L hybridization buffer (Hybrisol; Intergen, Norcross, GA, USA). RNA hybrids were revealed using a monoclonal mouse anti-DIG IgG following the manufacturer's protocol (Roche) and fluorescent immunodetection was achieved using a goat anti-mouse secondary antibody conjugated with either fluorescein isothiocyanate (FITC) or Cy3 (1:500; Jackson ImmunoResearch Laboratories, West Grove, PA, USA). Slides were coverslipped with Prolong (Molecular Probes, Eugene, OR, USA) before confocal microscopy.

Combined in situ hybridization histochemistry for xBTEB mRNA and immunohistochemistry for thyroid hormone receptors Double-labeling fluorescent immunohistochemistry (IHC) was used to detect thyroid hormone receptor (TR) protein and xBTEB mRNA on the same brain section. Slides were first processed for ISSH for xBTEB mRNA as described above followed by simultaneous immunodetection of the xBTEB DIG-cRNA probe and TR protein. The rabbit anti-*X. laevis* TR polyclonal serum used was a gift of Dr Yun-Bo Shi (National Institutes of Health, Bethesda, MD, USA). This antiserum was raised against a multiple antigenic peptide corresponding to amino acids 104–123 of *X. laevis* TR β . In western blotting experiments using *in vitro* synthesized *X. laevis* TR α or TR β proteins, we discovered that this antiserum recognizes both receptor subtypes (data not shown). Therefore, our experiments do not distinguish TR α from TR β but rather provide a composite measure of total TR protein.

Cryosections of tadpole brain were incubated with anti-*X. laevis* TR serum (1:1000) for 24 h at 4°C. The secondary antibody used was a goat-anti-rabbit-FITC. Positive staining of xBTEB mRNA and/or TR protein was analyzed with a Zeiss laser scanning confocal microscope (Carl Zeiss, Thornwood, NY, USA). Sections were scanned at 1 μ m using Z-stack to determine if xBTEB mRNA and TR protein were in the

same focal plane, thus ruling out the possibility of false co-localization.

Immunohistochemistry for xBTEB protein and bromodeoxyuridine xBTEB protein was detected by IHC using the affinity-purified anti-xBTEB IgG described above. Cryosections of tadpole brain were prepared and incubated with the purified anti-xBTEB IgG (1:100). Two different secondary antibodies were used: for fluorescence we used an antirabbit-Cy3 (1:500; Jackson ImmunoResearch) and for enzymatic detection (with diaminobenzidine) we used a biotinylated antirabbit IgG (ABC kit; Vector Laboratories, Burlingame, CA, USA) To verify the specificity of the anti-xBTEB IgG we preabsorbed the antiserum with recombinant GST-xBTEB fusion protein (1 µg/mL) at 4°C overnight before application to adjacent tissue sections.

For double-labeling IHC, tadpole brain sections were first processed for detection of BrdU followed by IHC for xBTEB protein. Briefly, tissue sections were denatured with 2 N HCl at 37°C for 30 min, rinsed, and blocked with 5% normal goat serum (NGS) at room temperature for 15 min. Tissue sections were then incubated with anti-BrdU serum (1:500; generated in rat; Serotec, Raleigh, NC, USA) for 24 h at room temperature. Antirat-FITC allowed fluorescent detection of primary anti-BrdU immune complexes. For subsequent detection of xBTEB protein, sections were incubated with anti-xBTEB IgG as described above, and immune complexes were detected using an antirabbit-Cy3 secondary antibody.

Data analysis and statistics

Data were analyzed by Student's unpaired *t*-test or one-way ANOVA using the Systat (version 7.0; SPSS Science) computer program. For ANOVA, data were log-transformed before analysis if the variances were found to be heterogeneous. Scheffe's posthoc test allowed separation of means. $P < 0.05$ was taken as the limit for significance.

Results

Structural comparison of mammalian BTEB1 and X. laevis BTEB proteins

Because *X. laevis* is pseudotetraploid, it possesses two BTEB genes (designated BTEBa and BTEBb; Brown *et al.* 1996). The two frog proteins show greatest similarity in primary structure to the mammalian BTEB1 proteins (Fig. 1). Amino acid sequence alignment of the mammalian BTEB1 and the two

X. laevis BTEB proteins shows that they share several highly conserved regions for which biochemical functions (for the mammalian proteins) have been identified (Fig. 1). Frog BTEBa and rat BTEB1 (292 and 244 amino acids, respectively) share 99% similarity in their DBD, characterized by three Cys₂-His₂ zinc fingers.

Less conserved between mammals and frogs are the N-terminal domains, where deletions and/or insertions, in addition to amino acid substitutions, have occurred during evolution. Mutational analysis identified two functional domains in the rat BTEB1 protein that function in transcriptional activation (region A, residues 13–26 and region B, residues 58–68; Fig. 1; Kobayashi *et al.* 1995). Both of these transactivation domains are largely conserved in the frog BTEB proteins. A sequence of four amino acids (Leu-Val-Ser/Thr-Ile) present in both domain A and B of rat BTEB1 (Kobayashi *et al.* 1995) are entirely conserved in the corresponding regions of the frog proteins (Fig. 1).

For biochemical analysis we focused on *X. laevis* BTEBa. There are four amino acid differences between frog BTEBa and BTEBb, and two substitutions occur within the DBD. One substitution falls within the first zinc finger, and while the mammalian BTEB1 and the frog BTEBb proteins have a tyrosine at this position, frog BTEBa has a phenylalanine. However, given that the frog BTEBa and mammalian BTEB1 proteins possess similar DNA-binding properties and transactivational activities, we think that it is unlikely that the two frog proteins differ in their functional properties. Our RNA or protein analyses did not distinguish the two gene products. Here we use the abbreviation xBTEB to refer to frog BTEB proteins, without distinguishing BTEBa or b.

Biochemical analysis of xBTEB

Expression of xBTEB *in vitro* resulted in a band on reducing SDS-PAGE with an apparent molecular mass of approximately 32 kDa: the molecular mass predicted from the nucleotide sequence is 32.8 kDa. The identity of this protein was verified by western blotting using anti-xBTEB serum, and the protein was not present in unprogrammed or empty vector programmed lysate (data not shown). Further resolution of the *in vitro* expressed xBTEB on reducing 12.5% SDS-PAGE revealed that the band consisted of a doublet (Fig. 2a; both proteins reacted with the anti-xBTEB serum, data not shown). This protein heterogeneity could result from run-on transcription in the *in vitro* system, or from post-translational modification.

The *in vitro* expressed xBTEB was tested for its ability to bind the BTE by EMSA. *In vitro* expressed xBTEB formed two supershifted bands (Fig. 2b), and these bands were further supershifted with anti-xBTEB and anti-rBTEB serum, but not with normal rabbit serum (NRS), showing that both protein–DNA complexes contained xBTEB (no supershifted bands were observed with unprogrammed or empty vector programmed lysate; data not shown.)

To determine if the two protein–DNA complexes observed by EMSA were an artifact of expressing xBTEB *in vitro*, we overexpressed xBTEB by transient transfection of XTC-2 cells. For comparison, we also overexpressed rat BTEB1. As with the *in vitro* expressed xBTEB, two protein–DNA complexes were observed by EMSA in extracts from xBTEB-overexpressing cells (Fig. 2c); these complexes were not observed in untransfected or empty vector transfected cell extracts (data not shown). The low mobility complex is tentatively designated frog Sp1 based on its high molecular mass and the ability of antihuman Sp1 serum to supershift it; Sp1 was present in transfected, untransfected or empty vector transfected cell extracts (data not shown). Both protein–DNA complexes obtained by overexpressing xBTEB could be supershifted with anti-xBTEB serum, but not with NRS, supporting the conclusion that both complexes contained xBTEB (Fig. 2c). By contrast, a single protein–DNA complex was observed in cell extracts of

rBTEB-overexpressing cells that could be supershifted by antirat BTEB1 serum (Fig. 2c).

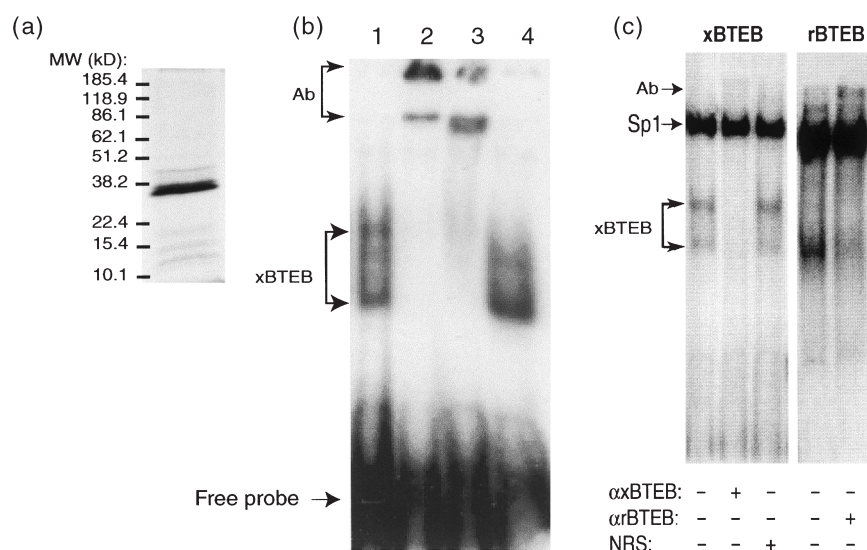
Thus, with two different expression constructs in two different expression systems, we observed heterogeneity with frog BTEB protein–DNA complexes. We have not examined the basis for this protein heterogeneity, but it is possible that it could result from post-translational modification. For example, xBTEB has several unique phosphorylation sites (Fig. 1) that could generate proteins of varying molecular masses. Note that these putative phosphorylation sites are present in non-conserved regions of the frog protein (Fig. 1) and this could explain the lack of heterogeneity in the rat BTEB1.

Competitive binding assays using *E. coli* expressed GST-xBTEB or GST-xBTEB[DBD] showed that both proteins had relatively high and similar affinities for ³²P-BTE, with a mean K_i of 17 nM ± 5.4 nM for GST-xBTEB and 23 nM ± 2.3 nM for GST-xBTEB[DBD] ($n = 3$).

xBTEB activates transcription from GC box-containing promoters

We next tested the ability of xBTEB expressed in mammalian cells to transactivate the SV40 early promoter, a naturally occurring viral promoter containing six contiguous GC box sequences (pSV2CAT). For the transient transfection assays, we followed the

Fig. 2. Biochemical characterization of *Xenopus laevis* basic transcription element binding protein (BTEB). (a) Fluorogram of *in vitro* expressed *Xenopus* BTEB (xBTEB) protein resolved by sodium dodecylsulfate–polyacrylamide gel electrophoresis (SDS–PAGE). The apparent molecular mass of xBTEB is 32 kDa, as predicted from the nucleotide sequence. (b) Electrophoretic mobility shift assay (EMSA) of *in vitro* produced xBTEB protein with labeled basic transcription element (BTE) probe and supershifted with either anti-*X. laevis* or anti-rat BTEB serum (lanes 2 and 3, respectively; Ab, bands supershifted by antisera) or normal rabbit serum (NRS; lane 4). No supershifted bands were observed with unprogrammed or empty vector programmed lysate; data not shown. (c) Overexpression of *X. laevis* and rat BTEB in XTC-2 cells. EMSA analysis of lysate from XTC-2 cells transiently transfected with either *X. laevis* or rat BTEB expression vector. Labeled BTE was used as the probe, and either anti-xBTEB, antiratBTEB or NRS (lanes 2, 3 and 5, respectively) was added to the reaction (indicated below the gel). Arrows show BTEB protein–DNA complexes, BTE supershifted by endogenous Sp1 protein or antibody-supershifted complexes (Ab). These BTEB protein–DNA complexes were not observed in untransfected or empty vector transfected cell extracts (data not shown).



methods, and used the reporter and expression plasmids developed by Imataka *et al.* (1992) to compare results between laboratories. Cotransfection with pCMV-xBTEB produced a statistically significant

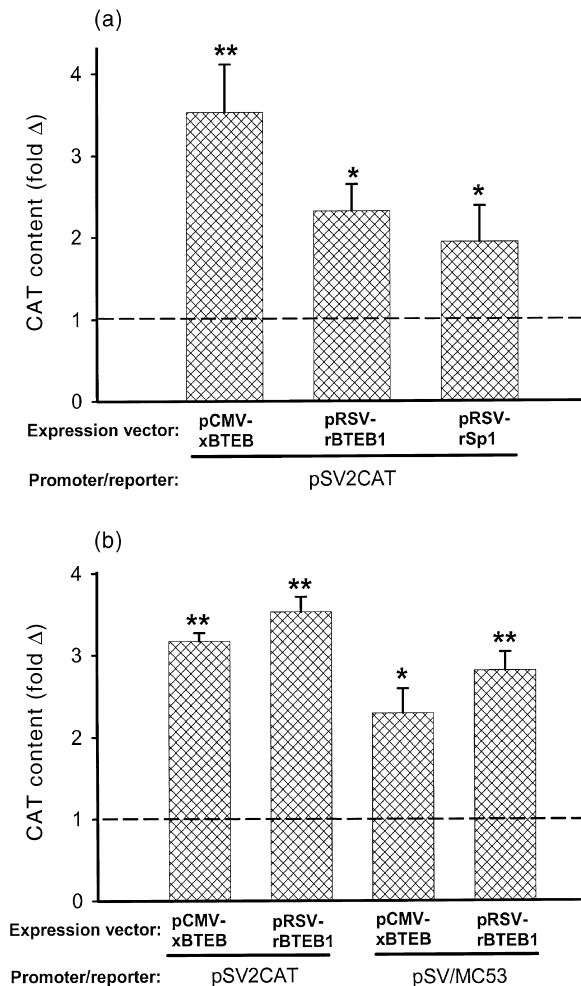


Fig. 3. *Xenopus laevis* basic transcription element binding protein (BTEB) activates transcription from promoters containing single or multiple GC boxes. CV1 cells were transfected as described in the Materials and Methods and cultured for 24 h before harvesting for analysis of chloramphenicol acetyl transferase (CAT) expression. CAT content was normalized to total cellular protein, and the data are expressed as fold change relative to the control (empty expression vector transfected cells = 1). (a) CV-1 cells were co-transfected with the designated expression vectors plus the pSV2CAT promoter/reporter plasmid (this native promoter contains six contiguous GC box sequences.) The data in the graph represent the mean (\pm SEM) of three separate transfection experiments. (b) CV-1 cells were co-transfected with the designated expression vectors and promoter/reporter vectors. The pSV/MC53 vector drives CAT expression and contains the SV40 enhancer and a single GC box sequence. The data in the graph represent the mean (\pm SEM) of three replicates in one transfection experiment. Asterisks in both upper and lower panels designate significant differences from the controls (*, $P < 0.05$; **, $P < 0.01$ by Student's unpaired *t*-test).

($P < 0.01$; *t*-test) elevation in CAT expression driven by the SV40 promoter (pSVCAT), inducing a 3.4-fold increase over empty vector transfected controls (Fig. 3a). The rat BTEB1 or rat Sp1 expression vectors also produced statistically significant increases in CAT expression over controls (2.3-fold and twofold, respectively; $P < 0.05$ for each; *t*-test; Fig. 3a). We also found that xBTEB activated transcription from the synthetic pSV/MC53 + 4GC promoter, which contains five contiguous GC boxes (data not shown).

Imataka *et al.* (1992) reported that CAT expression driven by the synthetic pSV/MC53 promoter (containing a single GC box sequence), was inhibited by rat BTEB but activated by rat Sp1. We therefore compared rat BTEB1 and xBTEB in co-transfection experiments with pSV/MC53. By contrast with the findings of Imataka and colleagues, in our hands both rat BTEB1 and xBTEB significantly activated transcription by pSV/MC53 ($P < 0.01$ and $P < 0.05$, respectively; *t*-test), and we found no evidence for transcriptional repression by either protein (Fig. 3b).

Although equal quantities of DNA were used for each expression vector, we did not quantify the amount of protein expressed and so cannot make quantitative comparisons of the transactivational potencies of each protein. Because of the difficulties of conducting transfection efficiency controls (i.e. most promoters employed to express indicator proteins contain GC box sequences in their promoters and thus would not be appropriate to use as transfection controls; Imataka *et al.* 1992) we repeated our transfection experiments three times with different batches of plasmid DNA to verify the reproducibility of our experiments.

xBTEB[DBD] represses basal and hormone-induced transcription from the X. laevis TR β promoter

We tested whether xBTEB[DBD] could inhibit, in a dominant negative fashion, transcription from the *X. laevis* TR β promoter (Machuca *et al.* 1995) which contains seven GC boxes and is autoinduced during metamorphosis (Tata *et al.* 1993). XTC-2 cells express TR β , and the TR β gene can be autoinduced by the addition of T₃ to the cell culture (Machuca & Tata 1992). Expression of xBTEB[DBD] in XTC-2 cells resulted in a dose-dependent competition for endogenous BTE-binding activity (Fig. 4a). Addition of T₃ (30 nM) induced a greater than 18-fold increase in CAT expression driven by the xTR β -promoter (Fig. 4b). Overexpression of xBTEB[DBD] reduced, in a dose-dependent manner, both basal and T₃-induced CAT expression (Fig. 4b). Basal CAT expression was

reduced to less than 50% of control levels by transfection with 1 μg pCMV-xBTEB[DBD], while transfection with 10 μg of this expression vector rendered basal CAT expression undetectable. Similarly, T_3 -induced CAT expression was reduced to 79 and 39% of controls by transfection with 1 and 10 μg , respectively, pCMV-xBTEB[DBD].

Developmental and hormone-dependent expression of xBTEB mRNA

Northern blotting showed the presence of three xBTEB transcripts (1, 4 and 10 kb) in the tadpole brain, intestine and tail (Fig. 5); however, we did not observe differential regulation of the individual transcripts at

any stage of development in the three tissues analyzed. xBTEB mRNA expression in the brain and intestine was low during premetamorphosis and early prometamorphosis, but rose during prometamorphosis to reach a peak at metamorphic climax (NF stage 60–62). Expression then declined to low levels in the juvenile frog. xBTEB mRNA expression in the tail was low throughout metamorphosis but rose sharply during metamorphic climax, with highest levels at NF stage 64, when the tail undergoes rapid resorption.

Treatment of premetamorphic tadpoles (NF stage 52) with T_3 (5 or 50 nM) produced a dramatic upregulation of xBTEB mRNA in brain and intestine (Fig. 6; only data for the 50 nM dose are shown). T_3 -induced upregulation of xBTEB mRNA was observed by 4–8 h after addition of the hormone and was maximal in both brain and intestine by 12–16 h: xBTEB mRNA expression remained elevated throughout the entire 72 h of exposure to T_3 . xBTEB mRNA expression in the tail was not altered by the addition of 5 or 50 nM T_3 (data not shown). In separate experiments, addition of T_3 to the aquarium water to a concentration of 200 nM resulted in a dramatic increase in xBTEB mRNA in the tail by 12 h (earlier time points were not analyzed) and expression remained high throughout the 48 h of exposure to the hormone (a representative northern blot is shown in Fig. 6c).

To determine if the elevated expression of xBTEB mRNA during prometamorphosis depends on endogenous T_3 , NF stage 58 tadpoles were treated with or without the goitrogen MMI (1 mM) dissolved in the aquarium water for 7 days. Over this time-frame, the average developmental stage of the animals at the end of the experiment was not significantly different between control and MMI-treated tadpoles (data not shown). MMI-induced hypothyroidism reduced the expression of xBTEB mRNA compared to untreated control animals (Fig. 6d).

Several genes that show T_3 -dependent regulation in fetal/neonatal brain of mammalian species, including BTEB1 (Denver *et al.* 1997), become thyroid independent later in life (at 30 days postnatally in rodents; Oppenheimer & Schwartz 1997). We tested whether thyroid hormone could regulate xBTEB mRNA levels in the adult *X. laevis* brain. Adult male frogs were treated with 20 nM T_4 , 1 mM MMI or MMI + T_4 for 2 weeks by adding the hormone or drug to the rearing water. Plasma T_4 was analyzed by RIA to verify the effectiveness of the treatments (note that T_3 is low to non-detectable in adult frog plasma; thus, we treated with and measured T_4 to allow verification of both hypothyroid and hyperthyroid states). A hyperthyroid state was induced by exposure to 20 nM T_4 (plasma T_4 : 5.24 ± 2.05 vs 0.48 ± 0.16 ng/mL in controls)

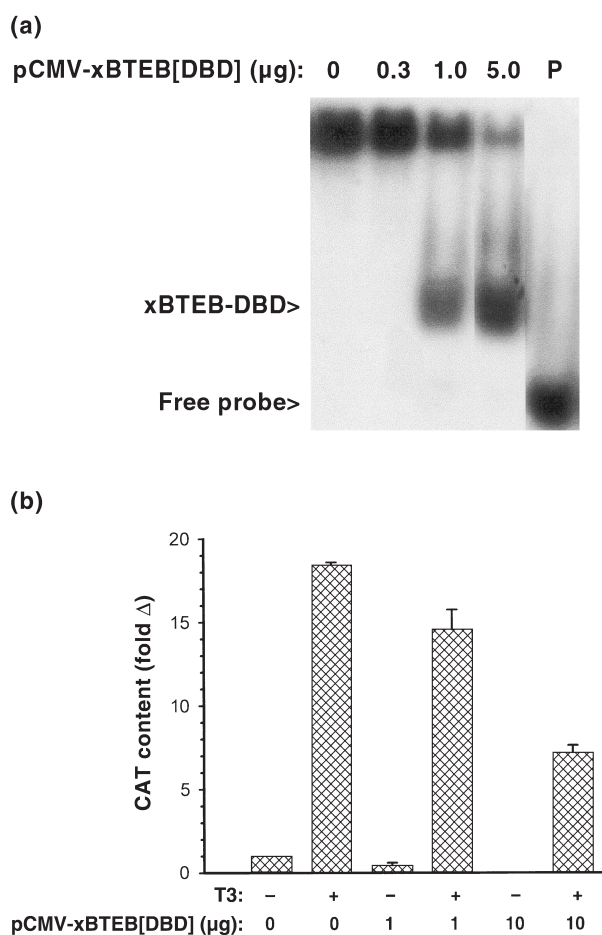


Fig. 4. Dominant negative activity of xBTEB[DBD] on the xTR β promoter in *Xenopus laevis* XTC-2 cells. XTC-2 cells were transfected with 1 μg xTR β -promoter-CAT and varying amounts of pCMV-xBTEB[DBD] as described in the Materials and Methods. Cell extracts were analyzed for (a) basic transcription element (BTE)-binding activity by electrophoretic mobility shift assay (EMSA; P, probe only) or (b) for chloramphenicol acetyl transferase (CAT) expression by CAT ELISA. In (b), cells were treated with or without 30 nM 3,5,3'-triiodothyronine (T_3) for 24 h before harvest. Means of three replicates with SEM are given.

and a hypothyroid state by exposure to MMI (non-detectable plasma T_4). Treatment with MMI + T_4 restored plasma T_4 concentrations to approximately twice the control value (0.98 ± 0.1 ng/mL); however, plasma T_4 concentrations in the T_4 + MMI group were well below those in the T_4 alone group. MMI is an inhibitor of thyroid peroxidase and thus acts to block the organification of iodide within the thyroid gland (Taurog 1996). We currently cannot explain why T_4 replacement was ineffective in the MMI treated frogs, but our findings may point to an extrathyroidal action of this goitrogen in frogs which serves to alter thyroid hormone metabolism/excretion.

xBTEB mRNA levels in the brain were significantly increased by exposure to T_4 (approximately 30% above controls; $F_{(3,17)} = 48.7$, $P < 0.0001$; ANOVA; data not shown). The xBTEB mRNA level was not altered by MMI treatment. However, the level of xBTEB mRNA expression in controls was low, and thus we were unable to quantify decreases from this already low level of expression by northern blotting. xBTEB mRNA levels in the T_4 + MMI group were not different from controls.

Distribution of xBTEB mRNA and regulation by T_3 in the tadpole brain

In situ hybridization histochemical analysis of xBTEB mRNA expression was conducted on brains obtained from premetamorphic tadpoles (NF stage 52) treated with or without T_3 (50 nM) for 48 h. Hybridization with the sense cRNA xBTEB probe gave no hybridization signal (data not shown). The specificity of the anti-sense xBTEB cRNA probe is further demonstrated by the dramatically increased signal following exposure to T_3 (Fig. 7, compare panel A vs B [green] or panel C vs D [red]). Treatment with exogenous T_3 resulted in a dramatic increase in xBTEB mRNA expression in several brain areas (an example of this induced expression is given for the optic tectum and the lateral motor column in Fig. 7B and D, respectively). Areas of highest xBTEB mRNA expression were observed in the telencephalon, diencephalon, optic tectum, cerebellum and spinal cord (the neural retina and the olfactory bulbs were not included in the analysis). In all brain regions, xBTEB mRNA expression was observed in the subventricular zones, just outside of

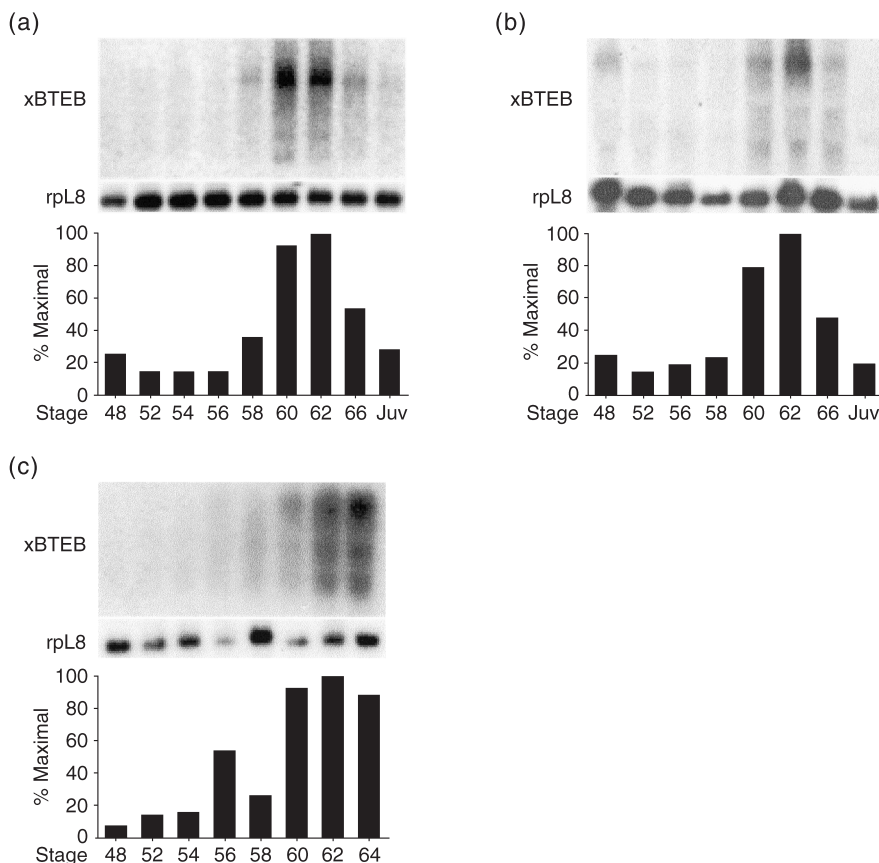


Fig. 5. Expression of the *Xenopus* basic transcription element binding protein (xBTEB) mRNA in the brain (diencephalon), intestine and tail during tadpole metamorphosis. Tissues were collected from *Xenopus laevis* tadpoles at different developmental stages and analyzed by northern blot. Representative northern blots of the developmental expression of xBTEB mRNA in the tadpole (a) diencephalon, (b) intestine and (c) tail. Graphs show the densitometric quantitation of northern blot data ($n = 3$ per tissue). Bars in the graph represent the mean mRNA level, expressed as a percentage of maximal expression.

the proliferative zones (zones of proliferation were identified by BrdU labeling or by IHC for phosphohistone 3 expression; L. Huang and R. J. Denver, unpubl. data, 2001). An example of xBTEB mRNA expression in the subventricular zone and in deep cellular layers of the tectum is shown in Fig. 7B.

xBTEB mRNA co-localizes with thyroid hormone receptor protein

Combined histochemical analysis showed that xBTEB mRNA and TR protein are co-localized in cells that upregulate xBTEB mRNA (an example from the motor column is given in Fig. 7C,D). Both xBTEB mRNA and TR protein are strongly upregulated by 48 h T₃ treatment. The xBTEB mRNA signal is exclusively cytoplasmic while the TR protein is nuclear, as expected. Note the dramatic increase in xBTEB mRNA expression (red) and the detection of TR protein (green towards the center of the nuclei and yellow near the nuclear envelope owing to the merge) following T₃ treatment. The TR immunoreactivity, while restricted to cell nuclei, occurred in a punctate pattern, with the majority of the immunoreactive material located at the periphery of the nucleus.

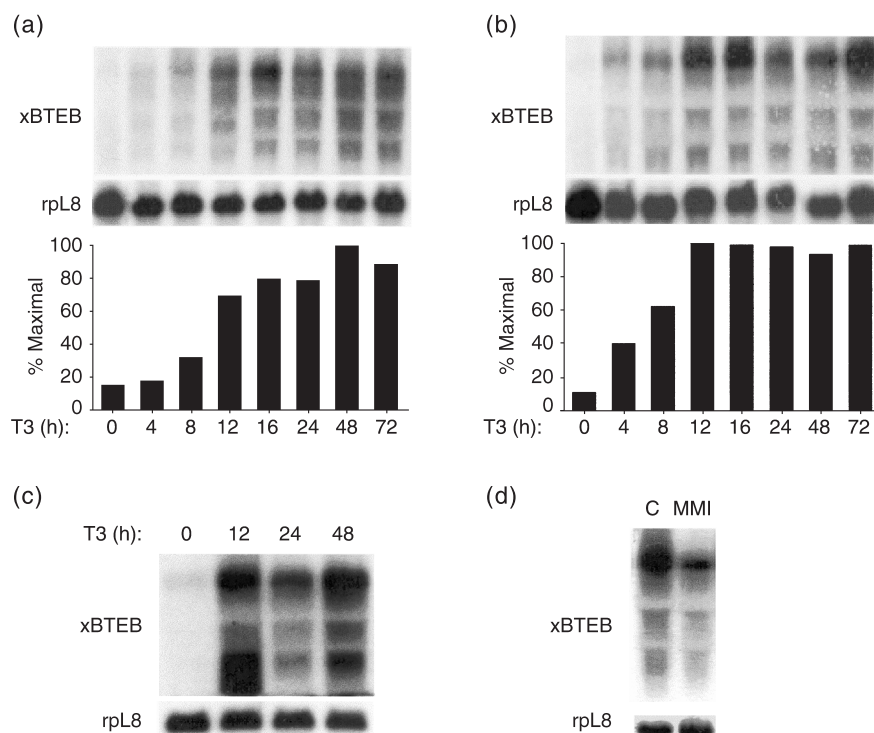
Distribution of xBTEB immunoreactivity in the late prometamorphic tadpole brain

We conducted IHC using the affinity subtracted anti-xBTEB IgG to analyze xBTEB protein expression in the late prometamorphic tadpole brain (when xBTEB mRNA expression is high; based on northern blotting analysis). We confirmed the specificity of the histochemical reaction by conducting preabsorbtion studies with recombinant xBTEB protein (data not shown). In the late prometamorphic/early climax stage tadpole brain (NF stage 60) xBTEB immunoreactivity was strong and restricted to cell nuclei. xBTEB immunoreactivity was observed in the telencephalon, optic tectum, diencephalon, cerebellum and spinal cord, corresponding to brain regions that showed xBTEB mRNA expression. The highest xBTEB immunoreactivity was observed in the hypothalamus and the lateral motor column (data not shown).

Developmental and hormonal regulation of xBTEB immunoreactivity in the tadpole brain

Treatment of premetamorphic tadpoles with T₃ resulted in a dramatic increase in xBTEB immuno-

Fig. 6. 3,5,3'-triiodothyronine (T₃) upregulates *Xenopus* basic transcription element binding protein (xBTEB) mRNA in the brain (diencephalon), intestine and tail of premetamorphic *Xenopus laevis* tadpoles. Stage 52 *X. laevis* tadpoles were treated with either 50 nM (for brain and intestine blots) or 200 nM T₃ (for tail blot) in the aquarium water and tissues were collected at various times thereafter and analyzed by northern blot. Representative northern blots of xBTEB mRNA expression in the (a) diencephalon, (b) intestine and (c) tail, respectively. Graphs show the densitometric quantitation of northern blot data (*n* = 3 per tissue). Bars in the graphs represent the mean mRNA level, expressed as a percentage of maximal expression (*n* = 3 blots per tissue). (d) xBTEB gene expression during metamorphosis depends on thyroid hormone. Northern blot of xBTEB mRNA expression in the diencephalon of prometamorphic (Nieuwkoop and Faber stage 58) *X. laevis* tadpoles treated with the goitrogen methimazole (1 mM) for 7 days.



reactivity in the same brain regions that had shown enhanced xBTEB mRNA with T_3 (Fig. 8; shown for the optic tectum and the cerebellum). xBTEB immunoreactivity increased during metamorphosis and was high in the late prometamorphic tadpole brain (Fig. 8). Exposure to exogenous T_3 failed to alter xBTEB immunoreactivity in late premetamorphic tadpoles, probably owing to the maximal level of xBTEB expression at this later developmental stage.

xBTEB immunoreactivity is excluded from proliferative cells in the tadpole brain

Thyroid hormone induces proliferation in the tadpole brain (Weiss & Rossetti 1951; Chibon & Dournon 1974). To test whether xBTEB protein is expressed in brain cells undergoing mitosis, we conducted dual labeling IHC for xBTEB and BrdU. T_3 treatment caused a robust increase in the number of BrdU-positive cells in the periventricular zone of the optic tectum (Fig. 8). Combined histochemistry for BrdU and xBTEB showed that xBTEB immunoreactivity is

excluded from proliferative cells in the tadpole brain (Fig. 8, compare panels D [xBTEB immunoreactivity], E [BrdU immunoreactivity] and F [merge]).

Discussion

Thyroid hormone induces dramatic morphological and biochemical changes during amphibian metamorphosis, and these tissue transformations depend on changes in gene expression. A central challenge to understanding the mechanistic basis for this process is to establish the identities and functions of genes that are induced or repressed in the transforming tissues. Towards this goal, we analyzed the biochemical characteristics, functional properties, and expression patterns of the T_3 -inducible gene BTEB in *X. laevis*. We found that xBTEB binds DNA with high affinity and can function as a transcriptional activator on GC box-containing promoters. xBTEB gene expression is developmentally and hormonally regulated, and xBTEB protein parallels changes in its mRNA. We hypothesize that BTEB participates in the

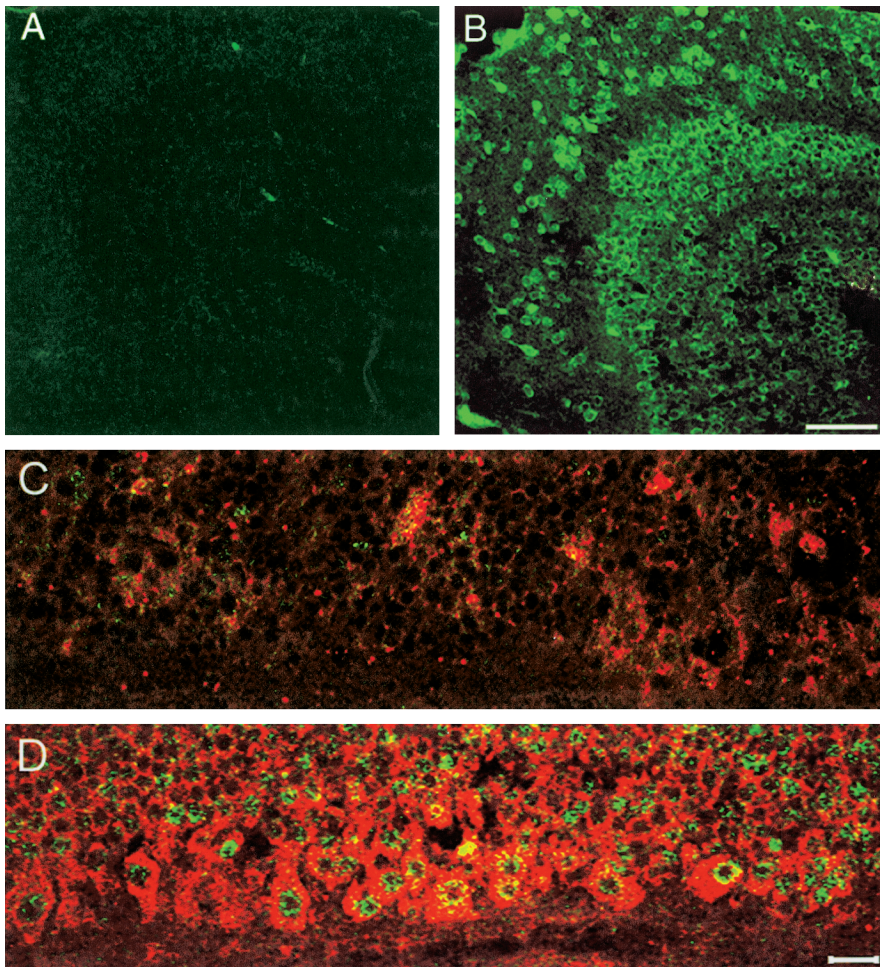


Fig. 7. *Xenopus* basic transcription element binding protein (xBTEB) mRNA expression is induced by 3,5,3'-triiodothyronine (T_3) in premetamorphic tadpole brain. Nieuwkoop and Faber stage 52 tadpoles were treated with or without 50 nM T_3 for 48 h before they were killed. Sagittal sections (10 μ m) were hybridized with anti-sense digoxigenin-labeled cDNA probes for xBTEB (optic tectum, xBTEB mRNA is green in panels A and B [bar, 100 μ m]); lateral motor column, xBTEB mRNA is red in panels C and D [bar, 50 μ m]); hybridization with sense probe showed no staining (data not shown). xBTEB mRNA and thyroid hormone receptor (TR) protein are expressed in the same cells. Dual histochemistry for xBTEB mRNA (red) and TR protein (green; analyzed by immunohistochemistry) was conducted (the lateral motor column is shown in panels C and D). Note the low level of xBTEB mRNA expression (cytoplasmic red staining) and the absence of detectable TR protein (lack of nuclear staining) at this developmental stage. Hormone treatment was as described above (C, untreated; D, 50 nM T_3).

autoinduction of TR β gene expression during metamorphosis, and we found that TR protein and xBTEB mRNA are upregulated in the same cells in the central nervous system. xBTEB immunoreactivity was not found in proliferating cells in the tadpole brain, supporting the hypothesis that this protein does not play a role in thyroid hormone-induced proliferation, but may function in neural cell differentiation.

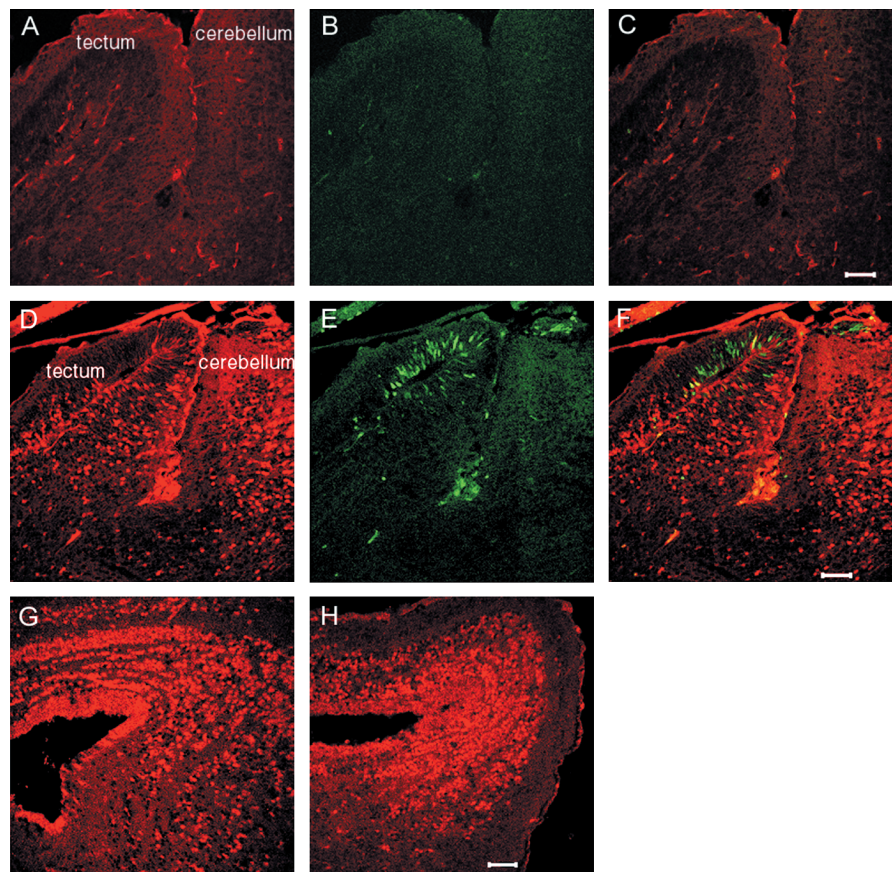
Xenopus laevis BTEB possesses similar DNA-binding characteristics to rat BTEB1 (Sogawa *et al.* 1993b). In transient transfection assays, we found that xBTEB activated transcription from the SV40 promoter, which contains six GC boxes in tandem (pSVCAT; Imataka *et al.* 1992). We also found that xBTEB activated transcription from a promoter containing a single GC box (pSV/MC53). Thus, while we cannot rule out the possibility that xBTEB can function as a transcriptional repressor on native promoters, our data support the hypothesis that xBTEB functions as a transcriptional activator.

Our findings with the pSVCAT plasmid, which contains six GC boxes, is consistent with the findings of Imataka *et al.* (1992). However, we did not find that

BTEB (frog or rat) repressed transcription on a promoter possessing a single GC box (pSV/MC53), as reported by Imataka *et al.* (1992). Instead, we found that both proteins activated transcription from this promoter. Although we attempted to carefully replicate the experimental design of Imataka and colleagues (i.e. using CV-1 cells, identical plasmids and DNA concentrations, same transfection method) we cannot rule out the possibility that there were slight differences in our protocol, or the way that our CV-1 cells behaved, that might be responsible for the different results. However, it is noteworthy that we found that both rat and frog BTEB activated transcription from this single GC box-containing promoter.

Previous studies showed that the *X. laevis* TR β gene is autoinduced by T₃ during metamorphosis (Tata *et al.* 1993) and in XTC-2 cells (Machuca & Tata 1992), and a thyroid hormone response element was identified in its promoter (Machuca *et al.* 1995). Furthermore, the *X. laevis* TR β promoter described by Machuca *et al.* (1995) contains seven GC boxes that could serve as binding sites for xBTEB or other Krüppel or Sp family members. Notably, the

Fig. 8. *Xenopus* basic transcription element binding protein (xBTEB) in the tadpole brain is developmentally and hormonally regulated and is not expressed in proliferating cells. Double labeling immunohistochemistry was used to analyze xBTEB protein and bromodeoxyuridine (BrdU)-labeled cells. Premetamorphic tadpoles (Nieuwkoop and Faber [NF] stage 52; panels A–F) were reared without (A–C) or with (D–F) 50 nM T₃ for 48 h. BrdU was added to the aquarium water to a final concentration of 500 μ M 3 h before tadpoles were killed, to label mitotic cells. Sagittal sections (10 μ m) were analyzed by double labeling immunohistochemistry for xBTEB (red) and BrdU (green; the optic tectum and cerebellum are shown). Note that xBTEB protein is not expressed in proliferating cells identified by BrdU labeling. Late prometamorphic/early climax stage tadpoles (NF stage 60) were reared in the (G) absence or (H) presence of 50 nM T₃ for 48 h before they were killed. The panels show xBTEB immunoreactivity in the optic tectum. Bars, 100 μ m.



expression of TR β and xBTEB mRNAs have similar expression patterns in tadpole tissues (compare Yaoita & Brown 1990 and this study). We also demonstrated by histochemistry that xBTEB and TR are coordinately upregulated in the same brain cells. These observations led us to hypothesize that the coordinate upregulation of TR β and xBTEB is functionally related; that is, TR β , in the presence of its ligand, regulates xBTEB expression and, subsequently, xBTEB influences TR β expression. We further hypothesize that the upregulation of TR β gene expression by T₃ (autoinduction) is dependent upon, or at least enhanced by, the coordinate upregulation of xBTEB.

To begin to test this hypothesis, we overexpressed the DBD of xBTEB in XTC-2 cells and found that it competed for endogenous GC box-binding activity and reduced both basal and T₃-induced TR β promoter activity. These findings suggest that GC box-binding proteins play a role in regulating transcription of the frog TR β gene, and could be important for the autoinduction of TR β during metamorphosis. GC box sequences in the human TR β 1 promoter were shown to be necessary for transcriptional activity (Suzuki *et al.* 1995). Furthermore, proteins present in fetal rodent brain that bind to these sequences were not supershifted by antibodies to Sp1-4 or Egr 1 or 2, suggesting that other GC box-binding factors present in the developing brain regulate this promoter (Nagasawa *et al.* 1997). In this regard, we have shown that BTEB1 is strongly upregulated in a thyroid-dependent manner in the neonatal rat brain (Denver *et al.* 1999).

Comparison of the postembryonic developmental expression of xBTEB mRNA in three tissues (brain, intestine and tail) showed that this gene is developmentally regulated, similar to other metamorphosis-associated genes (Shi 2000). The stage of maximal expression in the three tissues differs, and parallels the period of most active biochemical and morphological transformation in that tissue (Shi 2000). The correlation of xBTEB expression with active tissue remodeling/regression suggests that xBTEB could play an active role in mediating T₃ action during metamorphosis.

Addition of T₃ to the rearing water of premetamorphic tadpoles resulted in the precocious upregulation of xBTEB mRNA in each of the three tissues examined. Elevated xBTEB mRNA expression was observed by 4–8 h following addition of the hormone to the water, which is consistent with a previous report from our laboratory in which xBTEB mRNA levels in the brain were analyzed (Denver *et al.* 1997). Previous work has supported the view that xBTEB is a direct T₃

response gene (Wang & Brown 1993; Brown *et al.* 1996; Denver *et al.* 1997; see also Denver *et al.* 1999 for evidence for direct T₃ regulation of the rat BTEB1 gene.) Furlow & Brown (1999) reported that one of the frog BTEB genes (not specified) possesses a functional TRE in its promoter. Expression of xBTEB mRNA remained elevated throughout the 72 h that T₃ was present in the rearing water. A dose of 5 nM T₃ was sufficient to upregulate xBTEB mRNA expression in the brain and intestine. However, we were unable to detect a change in xBTEB mRNA expression in the tail following addition of either 5 or 50 nM T₃ (data not shown). By contrast, the addition of 200 nM T₃ caused a strong increase in xBTEB mRNA accumulation in the tail. This finding is consistent with previous findings that the tail is less sensitive to T₃ than other tadpole tissues (i.e. brain, liver, hindlimb, intestine), and thus this tissue does not show metamorphic changes until late in metamorphic climax when T₃ production is maximal (Shi 2000).

In addition to the induction of xBTEB mRNA by the addition of exogenous T₃, we showed that the upregulation of xBTEB during prometamorphosis depends on endogenous T₃. We also found that thyroid hormone could upregulate xBTEB mRNA in the adult frog brain. However, while plasma T₄ concentrations were increased 10-fold more than controls in the T₄-treated group, xBTEB mRNA expression increased only 30%. Nevertheless, our findings demonstrate thyroid hormone regulation of a metamorphic gene in adult amphibians, and suggest that the decline in the expression of metamorphic genes following metamorphic climax is related to the decline in thyroid hormone production, and not due to an irreversible change in the ability of thyroid hormone to regulate these genes. This contrasts with thyroid hormone-regulated brain genes, including BTEB1, in rats, whose continued expression becomes independent of the thyroid after postnatal day 30 (Oppenheimer & Schwartz 1997; Denver *et al.* 1999).

Previous work of ours has provided evidence for a role of BTEB in neuronal process development (Denver *et al.* 1999; Cayrou *et al.* 2002). For example, overexpression of BTEB in N-2a cells causes a dramatic increase in the number and length of neurites, and this effect is dose-dependent (Denver *et al.* 1999). Also, knockdown of BTEB in embryonic rat neurons with antisense oligonucleotides blocks T₃ effects on neurite branching *in vitro* (Cayrou *et al.* 2002). For these reasons we focused on the tadpole brain for a detailed analysis of xBTEB mRNA and protein. We showed that xBTEB mRNA is virtually undetectable in premetamorphic tadpole brain (analyzed by ISHH), but T₃ produced a strong

increase in expression throughout the brain, with highest expression in the telencephalon, optic tectum, hypothalamus, cerebellum and spinal cord. In the optic tectum, the expression of xBTEB mRNA was strongest in the subependymal and deep cellular layers, with less expression in the periventricular zones where proliferative responses to T_3 are observed (L. Huang and R. J. Denver, unpubl. data, 2001).

Immunohistochemistry using specific, affinity-subtracted anti-xBTEB IgG demonstrated nuclear xBTEB immunoreactivity in the same brain regions as those identified by ISHH. Highest levels of xBTEB immunoreactivity were observed in the hypothalamus and the lateral motor column, two brain regions previously reported to be most sensitive to T_3 action, and regions that undergo demonstrable morphological and biochemical changes during metamorphosis (Denver 1996). Similar to the effects of T_3 on xBTEB mRNA expression, T_3 dramatically increased xBTEB immunoreactivity in the premetamorphic tadpole brain. Comparison of xBTEB immunoreactivity in the optic tectum of premetamorphic and late prometamorphic tadpoles showed a significantly higher level of expression during spontaneous metamorphosis. We conclude that changes in xBTEB mRNA largely parallel changes in xBTEB protein.

In double labeling experiments for BrdU and xBTEB protein, and using confocal microscopy, we found that xBTEB immunoreactivity is excluded from proliferating cells in the tadpole brain. xBTEB immunoreactivity was found predominantly in cell layers just distal to the proliferative zone which undergo differentiation and migration. Berry *et al.* (1998) reported that xBTEB mRNA was expressed in proliferating cells in the tadpole brain. However, they did not show their data for xBTEB in the brain, nor did they use confocal microscopy to verify co-localization of BTEB and BrdU. We hypothesize that BTEB is upregulated in cells undergoing differentiation and plays a role in the upregulation of proteins necessary for the expression of the adult neuronal phenotype. In addition to the present expression results, support for this hypothesis comes from our previous findings showing that overexpression of BTEB causes neurite outgrowth, and antisense treatment reduces neurite branching induced by T_3 in neural cells (Denver *et al.* 1999; Cayrou *et al.* 2002). A primary effect of T_3 on the developing brain is on neuronal process development. Thyroid hormone has been shown to be essential to the proper maturation of dendritic spines and axons, and synaptic junctions in the developing brain. Our findings support the view that BTEB, through its positive action on the transcription of

essential genes, is a critical intermediate in the action of T_3 on the developing brain.

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