

# Basic Transcription Element-binding Protein (BTEB) Is a Thyroid Hormone-regulated Gene in the Developing Central Nervous System

EVIDENCE FOR A ROLE IN NEURITE OUTGROWTH\*

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Robert J. Denver<sup>‡§</sup>, Lydia Ouellet<sup>¶</sup>, Denis Furling<sup>¶</sup>, Akira Kobayashi<sup>||</sup>, Yoshiaki Fujii-Kuriyama<sup>||</sup>, and Jack Puymirat<sup>¶</sup>

From the <sup>‡</sup>Department of Biology, University of Michigan, Ann Arbor, Michigan 48109, the <sup>¶</sup>Department of Human Genetics, Centre Hospitalier de l'Université Laval, Quebec GIV 4G2, Canada, and the <sup>||</sup>Biochemistry Division, Department of Chemistry, Tohoku University, Aoba-Ku, Sendai, 980-8578, Japan

**Thyroid hormone (3,5,3'-triiodothyronine; T<sub>3</sub>) is essential for normal development of the vertebrate brain, influencing diverse processes such as neuronal migration, myelin formation, axonal maturation, and dendritic outgrowth. We have identified basic transcription element-binding protein (BTEB), a small GC box-binding protein, as a T<sub>3</sub>-regulated gene in developing rat brain. BTEB mRNA levels in cerebral cortex exhibit developmental regulation and thyroid hormone dependence. T<sub>3</sub> regulation of BTEB mRNA is neural cell-specific, being up-regulated in primary cultures of embryonic neurons (E16) and in neonatal astrocytes (P2), but not in neonatal oligodendrocytes (P2). T<sub>3</sub> rapidly up-regulated BTEB mRNA in neuro-2a cells engineered to express thyroid hormone receptor (TR)  $\beta$ 1 but not in cells expressing TR $\alpha$ 1, suggesting that the regulation of this gene is specific to the TR $\beta$ 1 isoform. Several lines of evidence support a transcriptional action of T<sub>3</sub> on BTEB gene expression. Overexpression of BTEB in Neuro-2a cells dramatically increased the number and length of neurites in a dose-dependent manner suggesting a role for this transcription factor in neuronal process formation. However, other T<sub>3</sub>-dependent changes were not altered; *i.e.* overexpression of BTEB had no effect on the rate of cell proliferation nor on the expression of acetylcholinesterase activity.**

Thyroid hormone (3,5,3'-triiodothyronine; T<sub>3</sub>)<sup>1</sup> is essential for normal development of the vertebrate central nervous system (CNS). Thyroid hormone deficiency during the period of active neurogenesis (up to 6 months post-partum in humans) results in irreversible mental retardation (*i.e.* cretinism; Refs. 1 and 2). The hormone influences diverse processes in the developing brain including neuronal maturation, neurite outgrowth, synapse formation, and myelination (2). Severe morphological

alterations in the brains of hypothyroid rats are due to impairment of neuronal migration and hypoplasia of neuronal processes which together lead to a reduction in the number of neural circuits (1–3).

Relatively little is known of the molecular mechanisms of T<sub>3</sub> action on the developing brain. The actions of T<sub>3</sub> are mediated by ligand-dependent transcription factors (4, 5). Three functional thyroid hormone receptors (TR $\alpha$ 1, TR $\beta$ 1, and TR $\beta$ 2) encoded by two genes ( $\alpha$  and  $\beta$ ) have been identified (6). In the rat brain TR $\alpha$ 1 expression is detected by embryonic day 14 and remains the major TR isoform expressed before postnatal day 7, while the TR $\beta$ 1 becomes the predominant TR isoform after the second week of life (7). The differential expression of the TR isoforms suggests developmental and brain region-specific functions for these receptors (6–9).

Differential screening experiments have identified several T<sub>3</sub>-regulated genes in the rodent CNS (10–14); but, roles for the protein products of these genes in T<sub>3</sub> action on the brain have not been established. Using a gene expression screen (15) we recently isolated a large set of early T<sub>3</sub>-responsive genes from the *Xenopus* tadpole CNS, several of which encode transcriptional regulatory proteins (16). We identified one of these genes as the *Xenopus* homolog of the mammalian basic transcription element-binding protein (BTEB), a small GC box-binding protein capable of activating or repressing the transcription of genes with GC-box sequences in their promoter (17, 18). BTEB has significant sequence similarity to the Sp family of transcription factors, primarily in the zinc finger region (DNA-binding domain). But beyond the DNA-binding domain the two proteins share little similarity. For example, rat Sp1 is a 788-amino acid protein while rat BTEB is only 244 amino acids in length. Interestingly, while BTEB mRNA is ubiquitously expressed in the rat, the mRNA appears to be translated only in the brain (19).

Using a cross-species hybridization approach we identified BTEB as a T<sub>3</sub>-regulated gene in developing rat brain. The objectives of the present study were to analyze the developmental and T<sub>3</sub>-dependent expression of BTEB in rat brain, and to test for a role for this transcription factor in T<sub>3</sub>-dependent effects on the developing brain.

## EXPERIMENTAL PROCEDURES

**Animal Care and Treatment**—Timed pregnant Harlan Sprague-Dawley rats were obtained from Charles River (Quebec, Canada) and maintained in the animal care facility at Centre Hospitalier de l'Université (CHU) Laval Research Center. Brain tissue from rat fetuses and neonates were used to prepare primary cells (see below). For studies on the effects of *in vivo* manipulation of thyroid status on brain gene expression, rat fetuses and neonates were rendered hypothyroid by continuous administration of the goitrogen propylthiouracil (PTU; Sigma) at 0.005% in the mother's drinking water starting at 12 days of

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§ To whom correspondence should be addressed: Dept. of Biology, 3077 Natural Science Bldg., The University of Michigan, Ann Arbor, MI 48109-1048. Tel.: 313-936-6625; Fax: 313-6747-0884; E-mail: rdenver@umich.edu.

<sup>1</sup> The abbreviations used are: T<sub>3</sub>, 3,5,3'-triiodothyronine; CNS, central nervous system; TR, thyroid hormone receptor; BTEB, basic transcription element-binding protein; PTU, propylthiouracil; AchE, acetylcholinesterase; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide.

gestation and continuing throughout the period of study (to postnatal day 30). The efficacy of PTU treatment in reducing plasma  $T_3$  levels was confirmed by radioimmunoassay (see below). Beginning at birth two groups of *hypothyroid* pups ( $n = 6$  per treatment) received daily subcutaneous injections of either saline (PTU) or 25  $\mu\text{g}$  of  $T_3$ /100 g body weight (PTU +  $T_3$ ) up until the time of sacrifice (24 h after the last injection); one group of *euthyroid* pups received injections of saline (Control). To verify the efficacy of treatments in altering thyroid state, plasma  $T_3$  concentrations were determined in euthyroid, hypothyroid, and  $T_3$ -replaced hypothyroid rat pups using a commercially available  $T_3$  radioimmunoassay (GammaCoat  $^{125}\text{I}$ - $T_3$  RIA kit; Incstar, Stillwater, MN). Animal care was in accordance with the institutional guidelines set by the Animal Care and Use Committee of the CHU Laval Research Center.

**Primary Cell Culture**—Primary neurons were prepared from E16 rat fetal cerebral hemispheres as described by Puymirat and colleagues (20). Cells were mechanically dissociated and plated at a density of  $3 \times 10^6$  cells per 100-mm tissue culture dish. Cells were cultured in chemically defined medium (composition previously described: see Refs. 21 and 22). Tissue culture dishes were coated with gelatin (250  $\mu\text{g}/\text{ml}$ ), incubated overnight with poly-L-lysine (10  $\mu\text{g}/\text{ml}$ ), rinsed with phosphate-buffered saline and preincubated with chemically defined medium containing 10% thyroid hormone-stripped fetal calf serum (stripped fetal calf serum; Ref. 23). Cultures were maintained in a humidified atmosphere of 5%  $\text{CO}_2$ , 95% air at 37 °C. Medium was changed 3 days after plating and every 3 days thereafter. At 6 days into the culture cells were treated with cytosine arabinosine ( $1 \times 10^{-6}$  M) to suppress the overgrowth of astrocytes. After 15 days in culture cells were treated with or without  $T_3$  for 24 h by adding it to the culture medium to a final concentration of 30 nM (this dose was chosen based on previous dose-response experiments; Ref. 22). In these cultures 80–85% of the cells are neurons (based on positive neurofilament immunostaining) and the remainder are astrocytes (based on positive immunostaining with anti-GFAP; Ref. 20).

Primary astrocytes were prepared from 2-day-old rats following the methods of Refs. 24 and 25. Cells were mechanically dissociated and plated at a density of  $4 \times 10^5$  cells/cm<sup>2</sup> in 100-mm diameter culture dishes. Cells were grown at 37 °C in a humidified 10%  $\text{CO}_2$ , 90% air atmosphere in Dulbecco's modified Eagle's medium supplemented with D-glucose (4.5 g/liter), HEPES (3.5 g/liter), and thyroid hormone-stripped horse serum (5%) and newborn calf serum (5%). In all experiments, RNA was extracted from cells for Northern blot analysis after 8 days in culture. Thyroid hormone treatment of cells ( $L$ - $T_3$ , 30 nM) was begun at the initiation of the culture (for cells treated for 8 days) or at various times (5 days, 1 day, 4 h, 2 h, 1 h, and 30 min) prior to the termination of the experiment on day 8. Control cells were maintained in the absence of hormone for 8 days. The medium was changed 3 days after plating and subsequently every 3 days throughout the culture (with fresh  $L$ - $T_3$  added to the medium of the appropriate treatments at each change).

Secondary cultures of oligodendrocytes were prepared from primary mixed glial cell cultures derived from newborn rat cerebral hemispheres as described by Besnard and colleagues (26). Cells were mechanically dissociated and plated in Waymouth's medium (Life Technologies, Inc.) supplemented with sodium bicarbonate (2 g/liter), sodium pyruvate (110 mg/liter), penicillin (50 units/ml), streptomycin-sulfate (50  $\mu\text{g}/\text{ml}$ ), and 10% stripped fetal calf serum. Cells were cultured for 2 weeks (medium changed twice per week) under a humidified atmosphere of 5%  $\text{CO}_2$ , 95% air at 37 °C. At 21 days oligodendrocytes were mechanically dislodged from the underlying astrocytic layer by gently flushing medium over the cultures. Cells were centrifuged at  $100 \times g$  for 10 min, the cell pellet resuspended and replated for 2 h at 37 °C to remove contaminating astrocytes and microglial cells (these cells rapidly attach to the plastic tissue culture dish). Floating cells (O-2A progenitor cells and oligodendrocytes) were aspirated from the cultures and plated on poly-L-lysine-coated tissue culture dishes (35 mm diameter). Two hours after plating the medium was changed to Dulbecco's modified Eagle's medium (Life Technologies, Inc.) supplemented with sodium bicarbonate (2 g/liter), D-glucose (4 g/liter), 15 mM HEPES, bovine insulin (5  $\mu\text{g}/\text{ml}$ ), transferrin (10  $\mu\text{g}/\text{ml}$ ), bovine serum albumin (0.5 mg/ml), and selenium (30 nM). After 6 days in secondary culture 85% of the cells were GC positive attesting to their oligodendrocytic phenotype while 18% were A2B5 positive (O-2A progenitor cells) and 1–3% were GFAP-positive (astrocytes). After 5 days in secondary culture cells were treated with  $T_3$  (30 nM) for various times.

**Production of Stably Transfected Neuro-2a Cells and Cell Culture**—Stably transfected mouse neuroblastoma cells (Neuro-2a) that express either the thyroid hormone receptor  $\alpha 1$  (N-2a[TR $\alpha 1$ ]) or  $\beta 1$  (N-

2a[TR $\beta 1$ ]) were previously prepared by Lebel *et al.* (9). The N-2a[TR $\beta 1$ ] cells respond to  $T_3$  (30 nM) by ceasing proliferation, expressing acetylcholinesterase (indicative of a differentiated neuronal phenotype) and extending perisomal filopodia-like neurites, while the native Neuro-2a and the N-2a[TR $\alpha 1$ ] are unresponsive to the hormone. This is despite the fact that nuclear  $T_3$  binding capacity is elevated to an equivalent extent (~7-fold) in both N-2a[TR $\alpha 1$ ] and N-2a[TR $\beta 1$ ] cells relative to untransfected or empty vector-transfected cells (9). Similar growth rates were observed in parental cells (N-2a), cells stably transfected with empty vector and cells transfected with vectors containing TR $\alpha 1$  or TR $\beta 1$  (9). Stably transfected mouse neuroblastoma cells (Neuro-2a) that overexpress BTEB were prepared as described (9) using the pcDNA3 plasmid (Invitrogen) containing a 720-base pair *Xho* fragment representing the entire coding region of the rat BTEB cDNA (17). Clone selection was performed using Geneticin (1.25 mg/ml; Life Technologies, Inc.). Cell lines were plated in tissue culture dishes at a density of  $10^6$  cells per 60-mm diameter dish and grown at 37 °C in 50:50 Dulbecco's modified Eagle's medium:Ham's F-12 medium supplemented with 10% stripped fetal calf serum under a humidified atmosphere of 5%  $\text{CO}_2$ , 95% air. In experiments with  $T_3$ , 3 days after plating the cells were treated with thyroid hormone for various times by adding  $T_3$  to the medium to a final concentration of 30 nM.

To determine whether the up-regulation of BTEB mRNA depends on ongoing protein synthesis, N-2a[TR $\beta 1$ ] cells were cultured in the presence or absence of 30 nM  $T_3$  with or without cycloheximide (25  $\mu\text{g}/\text{ml}$ ) for 8 h. This treatment results in >90% inhibition of protein synthesis (27). To distinguish whether the up-regulation of BTEB mRNA by  $T_3$  depends on ongoing RNA synthesis or the stabilization of existing mRNAs actinomycin D was added to cells to block transcription. N-2a[TR $\beta 1$ ] cells were cultured in the presence or absence of  $T_3$  (30 nM) with or without actinomycin D (1  $\mu\text{g}/\text{ml}$ ) for 24 h. This treatment results in >90% inhibition of RNA synthesis.<sup>2</sup>

**RNA Isolation and Northern Blotting Analyses**—RNA was isolated from rat neural tissue using the guanidium isothiocyanate procedure (28). Poly(A)<sup>+</sup> selection was performed using oligo(dT) columns following the procedure of Hartmann *et al.* (29). For most experiments Northern blots were prepared by separating 6  $\mu\text{g}$  of poly(A)<sup>+</sup> RNA per lane by electrophoresis in a 1% formaldehyde-agarose gel and transferring the RNAs to nylon membrane (30). Total RNA (20  $\mu\text{g}/\text{lane}$ ) was used in the experiments in which N-2a[TR $\beta 1$ ] cells were treated with cycloheximide and actinomycin D (see above). Blots were hybridized with rat BTEB or cyclophilin cDNAs labeled with [<sup>32</sup>P]dCTP by random priming (Roche Molecular Biochemicals Corp.) and then washed with  $2 \times \text{SSC}$ , 0.5% SDS at room temperature for 10 min, then  $0.1 \times \text{SSC}$ , 0.5% SDS at 65 °C for 1 h before exposure to x-ray film for 1–14 days.

Initially, in a screen to identify novel  $T_3$ -regulated genes in developing rat CNS using cloned cDNAs for  $T_3$ -regulated genes isolated from *Xenopus* brain, a <sup>32</sup>P-labeled *Xenopus* BTEB (xBTEB) cDNA was used as probe to screen Northern blots under low stringency washing conditions ( $0.25 \times \text{SSC}$ , 0.1% SDS, 55 °C). All subsequent studies of BTEB mRNA expression in developing rat brain were done using the homologous rat BTEB (rBTEB) cDNA as probe (17) with high stringency washing conditions ( $0.1 \times \text{SSC}$ , 0.5% SDS, 65 °C). To control for differences in RNA loading blots were stripped and reprobed with <sup>32</sup>P-cyclophilin cDNA (31).

**Western Blotting Analyses**—Western blots were prepared with extracts obtained from N-2a[TR $\beta 1$ ] cells treated with 30 nM  $T_3$  for 0, 2, and 6 h. Twenty-five  $\mu\text{g}$  of total cellular protein was electrophoresed through 10% SDS-polyacrylamide gels, transferred to nitrocellulose, and probed with a rabbit polyclonal antiserum prepared against rat BTEB (19). Western blots were normalized using a monoclonal antibody (G8) prepared against a 54-kDa nuclear protein. This antibody recognizes 54- and 45-kDa unidentified nuclear proteins that are constitutively expressed in various tissues including neural cells.<sup>2</sup>

**Nuclear Run-on Transcription Analysis**—Nuclei were prepared as described previously (32). Transcription assays were performed as described (33) following the procedure of Stott (34). Briefly, nuclei were incubated at 30 °C for 30 min with 250  $\mu\text{Ci}$  of [ $\alpha$ -<sup>32</sup>P]UTP (Amersham Corp.), 1 mM each of ATP, CTP, and GTP, and 5 mM dithiothreitol. The radiolabeled RNAs were isolated by ethanol precipitation after DNase I and proteinase K treatment. Total incorporation of [ $\alpha$ -<sup>32</sup>P]UTP into RNAs was  $3\text{--}6 \times 10^6$  cpm/ $10^7$  nuclei. The radiolabeled RNAs were hybridized to dot blots containing 5  $\mu\text{g}$  of linearized plasmid DNA. The plasmid containing the 720-base pair rat BTEB insert and a control plasmid (pcDNA3) were linearized with *Xba* and *Hind*III, respectively.

<sup>2</sup> J. Puymirat, unpublished data.

A plasmid containing the cyclophilin insert (31) was linearized with *Pst*I and used for normalization.

**Measurement of Cell Proliferation**—Cells were plated at 25,000 per 60-mm diameter dish (Nunc plastic culture dishes) and grown in the presence or absence of  $T_3$  (30 nM). At different times, cells were incubated with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (250  $\mu$ g/ml) for 3 h at 37 °C, and reduction was measured by colorimetric detection (540 nm) of the blue insoluble formazan product (35). This assay provides an estimate of the number of functioning mitochondria present in the cells; *i.e.* the quantity of formazan product is directly proportional to the number of metabolically active cells in the culture.

**Determination of Acetylcholinesterase (AChE) Activity**—AChE activity was determined by measuring the rate of hydrolysis of acetylcholine using colorimetric detection as described previously (21). Protein determinations were performed by the method of Lowry *et al.* (36).

**Quantification of Neurite Outgrowth**—Neurite outgrowth was estimated as described previously (22). Several randomly chosen fields of the cultures were photographed in a phase-contrast light microscope. The number of neurites on each cell was counted and their lengths were measured.

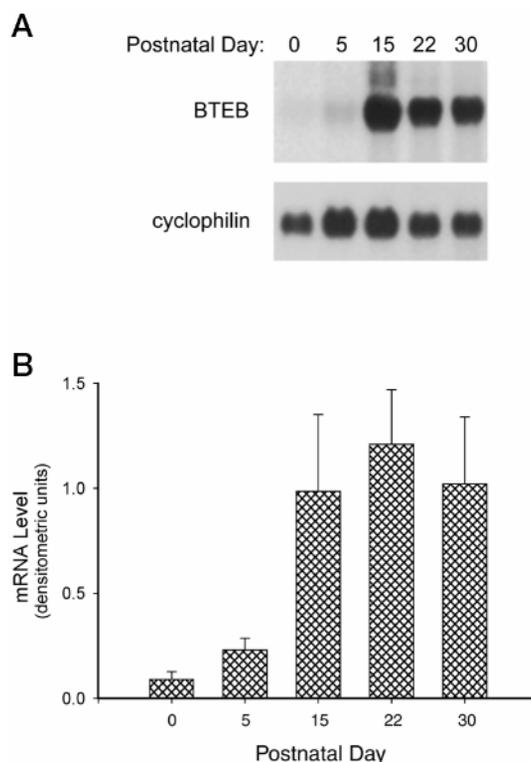
**Data Analysis and Statistics**—All data were  $\log_{10}$ -transformed to achieve homogeneity of variance. Data were analyzed by unpaired *t* test or one-way ANOVA and a post-hoc test (Scheffe's multiple contrast test) was done to determine significant differences among means ( $p < 0.05$ ). The number of replicate experiments is indicated in the text and figure legends. Densitometric analyses of bands on Northern and Western blots were done using a flatbed scanner and Scion Image software.

## RESULTS

**Expression of BTEB mRNA in Developing Rat Cerebral Hemisphere**—The expression of BTEB mRNA in developing rat CNS was analyzed by Northern blot of poly(A)<sup>+</sup> RNA isolated from cerebral hemispheres of embryonic and neonatal rats. A statistically significant age-dependent increase in BTEB mRNA was observed ( $F_{(4,15)} = 6.34$ ,  $p = 0.008$  [ANOVA];  $n = 4/\text{age}$ ). BTEB mRNA expression was low to nondetectable from day E16 until birth (data not shown) after which time it rose dramatically (>13-fold by postnatal day 22) and remained elevated at 1 month of age (Fig. 1). BTEB mRNA expression was maintained in the adult at the same level obtained by 1 month of age (data not shown).

**Thyroid Hormone Dependence of BTEB mRNA Expression in Developing Rat Cerebral Hemisphere**—Hypothyroidism induced by treatment with the goitrogen PTU and the restoration of euthyroidism in rats treated with PTU +  $T_3$  was confirmed by radioimmunoassay (plasma  $T_3$ : euthyroid controls,  $1.2 \pm 0.7$  nM; hypothyroid group:  $<0.06$  nM; hypothyroid group treated with  $T_3$ :  $11.0 \pm 5.0$  nM; data for postnatal day 22;  $n = 6/\text{treatment}$ ). Northern blots were prepared with poly(A)<sup>+</sup> mRNA isolated from cerebral hemispheres of neonatal rats in the different thyroidal states (euthyroid (Control), hypothyroid (PTU), and hypothyroid with  $T_3$  replacement (PTU +  $T_3$ )) at postnatal days 5, 22, and 30. Blots were then probed for BTEB or cyclophilin (to normalize for RNA loading; Fig. 2). Induction of hypothyroidism by PTU treatment significantly reduced BTEB mRNA levels at postnatal days 5 and 22 relative to the euthyroid controls (by 33 and 26%, respectively ( $n = 6/\text{time}$ );  $p = 0.005$  and  $p = 0.04$ , respectively (*t* test); Fig. 2). Conversely, treatment of hypothyroid animals with  $T_3$  significantly increased BTEB mRNA relative to euthyroid controls at postnatal days 5 and 22 (by 238 and 50%, respectively ( $n = 6/\text{time}$ );  $p = 0.0002$  and  $p = 0.03$ , respectively (*t* test); Fig. 2). Manipulation of thyroid status had no significant influence on brain BTEB mRNA levels by postnatal day 30.

**$T_3$  Up-regulates BTEB mRNA in Primary Neurons and Astrocytes**—Northern blot analysis of primary neurons derived from embryonic day 16 rats demonstrated that  $T_3$  produced a 6.5-fold increase in BTEB mRNA levels relative to untreated controls by 24 h of exposure to the hormone ( $p < 0.05$  (*t* test);  $n = 7$ ; Fig. 3). Astrocytes cultured in the presence of  $T_3$  for 24 h

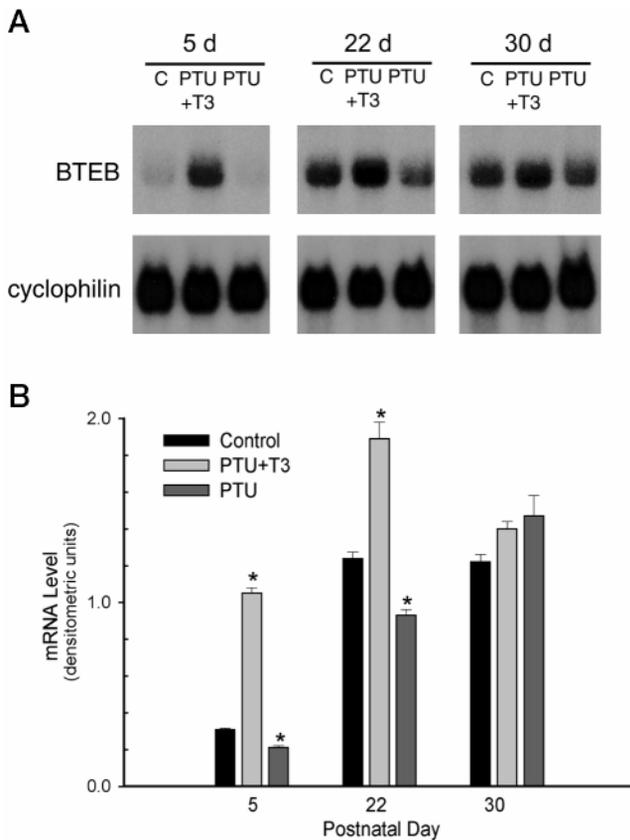


**FIG. 1. Expression of BTEB mRNA in developing rat brain.** Cerebral hemispheres were collected from rats at different embryonic, neonatal, and postnatal stages of development indicated on the *abscissa*. **A**, Northern blot of poly(A)<sup>+</sup> RNA extracted from cerebral hemispheres of developing rat brains. Blots were probed with a random-primed <sup>32</sup>P-labeled probe for rat BTEB (see "Experimental Procedures"). Blots were normalized for RNA loading by stripping and reprobing with a <sup>32</sup>P-labeled probe for cyclophilin. **B**, quantitation of BTEB mRNA expression in developing rat brain. The *graph* shows the mean  $\pm$  S.E.M. ( $n = 4/\text{time}$ ).

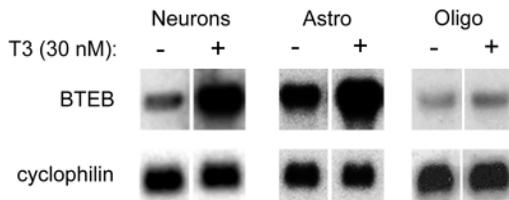
exhibited a small (1.3-fold) but significant increase in BTEB mRNA levels relative to untreated control cells ( $p < 0.05$  (*t* test);  $n = 4$ ; Fig. 3).  $T_3$  did not alter BTEB mRNA expression in primary oligodendrocytes derived from P2 rat cerebral cortex (Fig. 3;  $n = 3$ ).

**$T_3$  Up-regulates BTEB mRNA in N-2a[TR $\beta$ 1] Cells**—Neuro-2a cells engineered to express TR $\beta$ 1 responded to  $T_3$  (30 nM) by up-regulating BTEB mRNA (Fig. 4). BTEB mRNA was increased 1.3-fold by 30 min after exposure to the hormone and continued to rise thereafter; however, a statistically significant increase in the mRNA level was not achieved until 6 h ( $F_{(4,18)} = 3.21$ ,  $p = 0.045$  (ANOVA);  $n = 4$ ). The highest level of gene induction (4.3-fold) was obtained at 24 h. We did not find a significant effect of  $T_3$  on BTEB mRNA levels in these cells within the first 48 h of culture (data not shown); *i.e.* the cells reached confluence before they became responsive to  $T_3$ . By contrast with the N-2a[TR $\beta$ 1] cells, the low expression level of BTEB mRNA was not significantly altered by  $T_3$  in N-2a[TR $\alpha$ 1] cells ( $n = 3$ ; data not shown). In these experiments we also examined whether the increase in BTEB mRNA by  $T_3$  in N-2a[TR $\beta$ 1] cells is associated with an increase in BTEB protein. Total protein extracts of N-2a[TR $\beta$ 1] cells treated with  $T_3$  (30 nM) for 0, 2, and 6 h were analyzed by Western immunoblot for BTEB protein expression using a polyclonal antiserum to rat BTEB (19). BTEB protein increased by approximately 2-fold by 2 h after exposure to the hormone and continued to increase up to 6 h (approximately 4-fold induction; Fig. 5). This level of BTEB protein (at 6 h) corresponds to an approximately 3.4-fold induction of the mRNA.

**$T_3$  Regulates the Rate of Transcription of the BTEB Gene in**

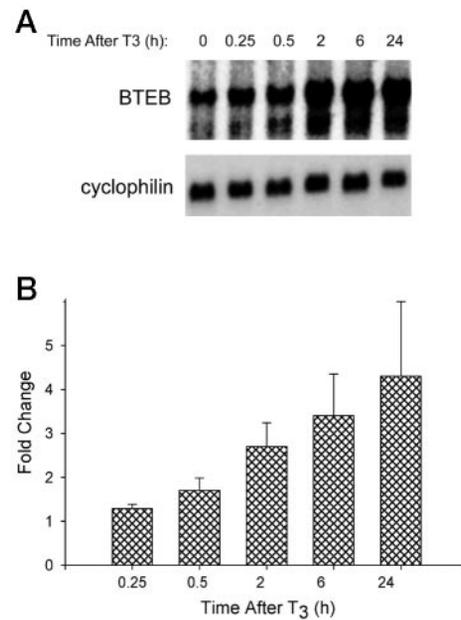


**FIG. 2. Thyroid status alters BTEB mRNA levels in neonatal rat brain.** Rat fetuses and neonates were rendered hypothyroid by continuous administration of 0.005% PTU in the mother's drinking water. PTU treatment was initiated at 12 days of gestation and continued throughout the period of study (day of sacrifice indicated on the *abscissa*). The PTU-treated (hypothyroid) pups received daily subcutaneous injections of either saline or  $T_3$  (25  $\mu\text{g}/100$  g body weight); one group of euthyroid pups (non-PTU treated) received injections of saline. Injections were given from birth to 24 h before sacrifice. *A*, Northern blot analysis of total RNA extracted from developing rat brain under different thyroidal states. Northern blots were prepared following the methods described under "Experimental Procedures" and in the legend of Fig. 1. *B*, quantitation of BTEB mRNA expression under different thyroid states. The *graph* shows the mean  $\pm$  S.E.M. ( $n = 5/\text{time}$ ).

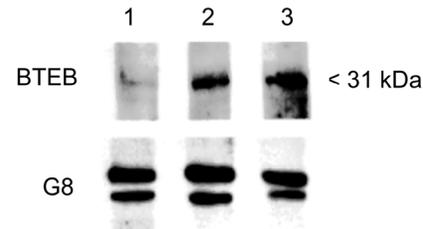


**FIG. 3. Effects of exposure to  $T_3$  on BTEB mRNA levels in primary neurons, astrocytes (*Astro*), and oligodendrocytes (*Oligo*).** Primary neurons were isolated from E16 rat brain and primary astrocytes and oligodendrocytes from P2 rat brain and cultured as described under "Experimental Procedures." For each experiment cells were cultured in the absence or presence  $T_3$  (30 nM) for 24 h before harvesting for RNA analysis. Poly(A)<sup>+</sup> RNA was isolated and analyzed by Northern blot as described under "Experimental Procedures" and the legend of Fig. 1.

***N-2a[TR $\beta$ 1]* Cells**—To determine if the up-regulation of BTEB mRNA depends on ongoing protein synthesis, *N-2a[TR $\beta$ 1]* cells were cultured in the presence or absence of  $T_3$  (30 nM) with or without cycloheximide (25  $\mu\text{g}/\text{ml}$ ) for 8 h and BTEB mRNA expression was analyzed by Northern blot. As in previous experiments,  $T_3$  up-regulated BTEB mRNA in the *N-2a[TR $\beta$ 1]* cells by 8 h following exposure to the hormone. Inhibition of protein synthesis by cycloheximide did not significantly alter



**FIG. 4.  $T_3$  up-regulates BTEB mRNA in *N-2a[TR $\beta$ 1]* cells.** Confluent monolayers of *N-2a[TR $\beta$ 1]* cells were cultured in the presence of  $T_3$  (30 nM) for various times before harvesting for poly(A)<sup>+</sup> RNA isolation. *A*, Northern blot (see "Experimental Procedures" and the legend of Fig. 1 for details.) *B*, quantification of normalized BTEB mRNA expression in *N-2a[TR $\beta$ 1]* cells. *Bars* in the *graph* represent the mean  $\pm$  S.E.M. ( $n = 4$ ).



**FIG. 5.  $T_3$  up-regulates BTEB protein in *N-2a[TR $\beta$ 1]* cells.** Confluent monolayers of *N-2a[TR $\beta$ 1]* cells were cultured in the presence of  $T_3$  (30 nM) for 0, 2, or 6 h before harvesting for protein analysis. BTEB protein was analyzed by Western blot using a polyclonal antiserum to rat BTEB (see "Experimental Procedures"). Blots were normalized with the G8 monoclonal antibody which recognizes two unidentified nuclear proteins (54 and 45 kDa) that are constitutively expressed.

either the basal or the  $T_3$ -induced levels of BTEB mRNA in these cells (cycloheximide: 90% of control; cycloheximide +  $T_3$ : 119% of  $T_3$  treated).

To determine if blockade of transcription reduces the accumulation of BTEB mRNA induced by  $T_3$ , *N-2a[TR $\beta$ 1]* cells were treated with or without actinomycin D for 24 h. Actinomycin D reduced the basal level of BTEB mRNA (when normalized for the cyclophilin signal) to 14% of control.  $T_3$  up-regulated BTEB mRNA in the absence but not in the presence of actinomycin D (actinomycin D +  $T_3$ : 20% of  $T_3$ ).

To directly test whether the  $T_3$ -induced accumulation in BTEB mRNA resulted from an increased rate of transcription, we performed nuclear run-on measurements with nuclei isolated from cells maintained for 24 h in the presence or absence of  $T_3$  (30 nM). In two experiments the transcriptional rate of the BTEB gene showed a 1.6- and 2.8-fold increase with  $T_3$  treatment; whereas, there was no change in the transcriptional rate for the cyclophilin gene in either experiment (Fig. 6).

***Overexpression of BTEB Induces Neurite Outgrowth in N-2a Cells***—We showed previously that treatment of *N-2a[TR $\beta$ 1]* cells with 30 nM  $T_3$  for 48 h blocks cellular proliferation and induces differentiation (*i.e.* increases acetylcholinesterase ac-

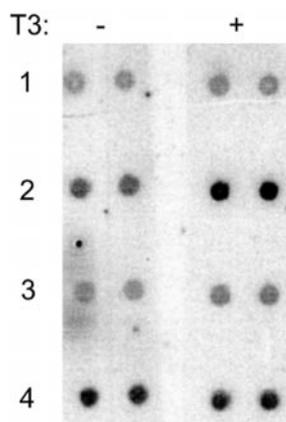


FIG. 6.  $T_3$  enhances transcription of the rat BTEB gene in N-2a[TR $\beta$ 1] cells. Confluent monolayers of N-2a[TR $\beta$ 1] cells were cultured with 250  $\mu$ Ci of [ $\alpha$ - $^{32}$ P]UTP per well in the presence or absence of  $T_3$  (30 nM) for 24 h before harvesting nuclei for nuclear run-on analysis (see "Experimental Procedures"). Radiolabeled RNAs were hybridized to dot blots containing 5  $\mu$ g of linearized plasmid DNAs (Rows 1 and 3, pcDNA3 (control); Row 2, pcDNA3-rBTEB; Row 4, pGEM-cyclophilin (for normalization)). In this experiment  $T_3$  increased the rate of BTEB transcription by 1.6-fold; whereas, there was no effect of  $T_3$  on transcription of the cyclophilin gene. Similar results were obtained in a replicate experiment (2.8-fold induction of BTEB; data not shown).

tivity and the number of perisomatal filopodia-like neurites; see Ref. 9). We examined whether overexpression of BTEB in N-2a cells in the absence of  $T_3$  can reproduce some of these  $T_3$ -dependent effects. We established stable transfectants that overexpress BTEB at two different levels (3- or 10-fold; clone number 10 or clone number 4, respectively; analyzed by Northern blot; data not shown) relative to cells transfected with empty vector (which exhibited comparable basal BTEB mRNA levels to those of the parent cell line and the N-2a[TR $\beta$ 1] cell line). The N-2a[BTEB] cell lines allow analysis of dose-dependent effects of BTEB overexpression. The rate of proliferation of N-2a[BTEB] (clone number 4) and N-2a[TR $\beta$ 1] cells was assessed at different times using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide test (see "Experimental Procedures"). There was no significant difference observed between the proliferative rate of N-2a[BTEB] and untreated N-2a[TR $\beta$ 1] cells (Table I). We have shown previously that N-2a[TR $\beta$ 1] cells proliferate at a similar rate as parental cells or cells transfected with empty vector (9). As in earlier experiments (9), treatment of N-2a[TR $\beta$ 1] cells with  $T_3$  (30 nM) decreased their rate of proliferation by 50% after 24 h of exposure to the hormone.

We also determined whether BTEB overexpression alters the level of AchE activity in N-2a cells. While treatment of N-2a[TR $\beta$ 1] cells with  $T_3$  (30 nM) for 48 h elevated AchE activity (control:  $1.1 \pm 0.05$ ;  $T_3$ -treated:  $3.1 \pm 0.12$  nmol/min/mg protein; as shown previously; Ref. 9) there was no effect of overexpression of BTEB on AchE ( $0.75 \pm 0.2$  nmol/min/mg of protein).

In contrast to the lack of effect on cellular proliferation and AchE activity, overexpression of BTEB in N-2a cells significantly increased both the number and length of perisomatal filopodial-like neurites. N-2a[BTEB] cells were compared with N-2a[TR $\beta$ 1] cells (cultured in the presence or absence of  $T_3$ ) after 5 days in culture (Table II; Fig. 7). The two clones that overexpressed BTEB at different levels (clone number 10, 3-fold; clone number 4, 10-fold) were compared. No distinct neurite outgrowth was observed during the first 3 days in cultures in N-2a[BTEB] cells. After 5 days in culture, both the number and length of neurites were increased in a dose-dependent manner by overexpression of BTEB (Table II; Fig. 7).

Only 3.4% of control cells (N-2a[TR $\beta$ 1]) exhibited neurites, whereas this level rose to 30 and 40% in cells overexpressing BTEB by 3- and 10-fold, respectively. The index of neurite length was also increased by BTEB overexpression in a dose-dependent manner (Table II). The percentage of neurites with lengths greater than 2 cell diameters was increased from 0 in control cells to 6.5 and 37% in cells overexpressing BTEB by 3- and 10-fold, respectively. By comparison,  $T_3$  treatment of N-2a[TR $\beta$ 1] for 5 days caused 31% of the cells to extend neurites with 11% of the cells possessing neurites greater than 2 cell diameters (Table II). This morphological change is comparable to that observed in N-2a[BTEB] cells (clone number 10, 3-fold overexpression).

#### DISCUSSION

We have identified the small GC box-binding transcription factor BTEB as a  $T_3$ -regulated gene in the developing rodent CNS. The expression of BTEB mRNA in the developing rat brain depends on thyroid status; the induction of hypothyroidism in neonatal pups resulted in reduced expression that was restored by  $T_3$  replacement therapy. Furthermore, the gene is developmentally regulated, exhibiting a distinct elevation in expression that parallels developmentally-dependent increases in plasma  $T_3$  levels (2). Similar to other  $T_3$  responsive genes in the developing CNS,  $T_3$  regulation of BTEB gene expression disappears in the adult (Ref. 37; see Fig. 2).

BTEB is the first  $T_3$ -responsive gene to be identified that is differentially regulated by  $T_3$  in specific neural cell types. We showed that BTEB mRNA expression is up-regulated by  $T_3$  in primary neurons and astrocytes derived from embryonic and neonatal rat brain, respectively, but not in oligodendrocytes. The effect of  $T_3$  on BTEB mRNA levels in primary neurons is unlikely to be due to contaminating astrocytes (which represent ~15% of the cell population) since the degree of BTEB mRNA induction in neuronal cultures is much greater than that observed in the astrocyte cultures (6.5 versus 1.3-fold). Because most of the neurons cease division after 8 days *in vitro*, our results indicate that the effect of  $T_3$  on BTEB gene expression occurs in post-mitotic neurons. This result is consistent with the up-regulation of BTEB mRNA by  $T_3$  observed in the brain of the P22 rat (see Fig. 1). Similarly, we have found that the effect of  $T_3$  on BTEB mRNA levels in N-2a[TR $\beta$ 1] cells is absent during the first 48 h of culture and is not expressed until the cells are confluent. This suggests that the cells must cease division before BTEB can be up-regulated by  $T_3$ . We have not yet determined whether neurons and astrocytes express BTEB and are responsive to  $T_3$  *in vivo*; however, it is noteworthy that our previous work has shown that these primary neurons express their program of differentiation similar to that observed *in vivo* (20, 32).

Our findings demonstrate the utility of using a cross-species hybridization approach to identify novel hormone-regulated genes in the developing rodent brain. Recent attempts to isolate  $T_3$ -regulated genes in neonatal rodent brain by differential screening procedures have met with limited success. These studies have led to the identification of several mitochondrial genes (12 S and 16 S rRNAs, cytochrome *c* oxidase subunit III and NADH dehydrogenase subunit 3; Refs. 11 and 12), a tubulin and NCAM (13), a novel synaptogamin-related protein (involved with regulating neurotransmitter release) and a zinc finger protein related to the product of a recently identified mouse gene, hairless (14, 38). Given the dramatic and pleiotropic effects of  $T_3$  on CNS development, this set of genes likely represents only a small fraction of the total number of genes controlled by the hormone. Also, given that thyroid and steroid hormones bring about developmental changes by activating gene regulation programs, it is surprising that so few transcrip-

TABLE I  
Comparison of the proliferative rate of N-2a cells expressing the thyroid hormone receptor  $\beta 1$  (N-2a[TR $\beta 1$ ]) with that of N-2a cells overexpressing BTEB (N-2a[BTEB]; clone number 4)

Cells were seeded at  $50 \times 10^5$  cells per well (22-mm diameter wells). Two days after initiating the cultures T<sub>3</sub> (30 nM) was added to half of the N-2a[TR $\beta 1$ ] wells and the MTT assay was performed (see "Experimental Procedures") at different times (0, 2, 6, 12, and 24 h). Values are the mean  $\pm$  S.E.M. of triplicate samples from two different experiments.

	0	2 h	6 h	12 h	24 h
N-2a[TR $\beta 1$ ] - T <sub>3</sub>	0.55 $\pm$ 0.04	0.54 $\pm$ 0.04	0.70 $\pm$ 0.06	1.68 $\pm$ 0.02	1.99 $\pm$ 0.012
N-2a[TR $\beta 1$ ] + T <sub>3</sub>	0.59 $\pm$ 0.24	0.56 $\pm$ 0.01	0.46 $\pm$ 0.15	1.33 $\pm$ 0.02 <sup>a</sup>	0.94 $\pm$ 0.007 <sup>b</sup>
N-2a[BTEB]	0.54 $\pm$ 0.05				1.97 $\pm$ 0.047

<sup>a</sup> Significantly different from untreated cells by Student's *t* test at  $p < 0.004$ .

<sup>b</sup> Significantly different from untreated cells by Student's *t* test at  $p < 0.007$ .

TABLE II  
Effect of overexpression of basic transcription element binding protein (BTEB) on the number and the length of neurites

N-2a cells that overexpressed BTEB by 3- (clone number 10) or 10-fold (clone number 4), or the thyroid hormone receptor  $\beta 1$  (TR $\beta 1$ ); cultured with or without 30 nM T<sub>3</sub> were grown for 5 days. At that time cells were photographed and neurites were counted and measured. Five hundred (500) cells were counted for each experimental condition. Results for neurites per cell represent the percentage of cells with the indicated number of neurites. The index of neurite length was determined by counting the number of cells with either neurite length under 2-fold cell diameter (<2) or with at least one neurite with a length above 2-fold cell diameter (>2).

Clones	Neurites per cell, %					Neurite length, %	
	0	1	2	3	>3	<2	>2
N-2a(BTEB), clone number 10	70	13	5.8	1.6	0.6	93.5	6.5
N-2a(BTEB), clone number 4	60	13.4	16.6	5.0	5.0	62.6	37.4
N-2a(TR $\beta 1$ ) - T <sub>3</sub>	96.6	2.6	0.8	0	0	100	0
N-2a(TR $\beta 1$ ) + T <sub>3</sub>	69	13	14.5	3	3	89	11

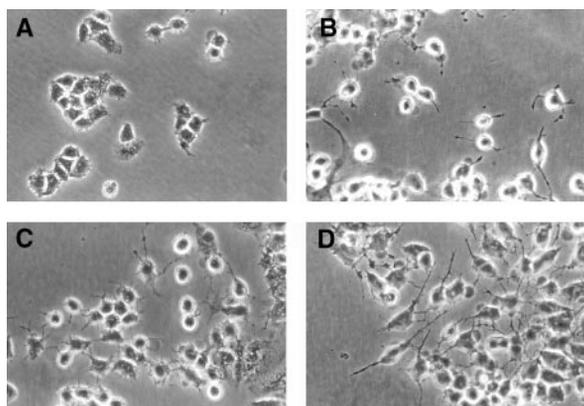


FIG. 7. **BTEB overexpression in N-2a cells induces neurite outgrowth.** Stably transfected N-2a cells that overexpress BTEB were prepared as described under "Experimental Procedures." Cells were cultured for 5 days before microscopic analysis. A and B, N-2a[TR $\beta 1$ ] cells cultured in the absence (A) or presence (B) of T<sub>3</sub> (30 nM) for 5 days; C and D, N-2a[BTEB] cells overexpressing BTEB mRNA at 3-fold (C, clone number 10) or 10-fold (D, clone number 4) above basal level. Cells close to the periphery of the confluent monolayers were photographed to allow visualization of the neurites.

tional regulatory proteins have been isolated from rodent brain as T<sub>3</sub>-responsive genes. We previously isolated by subtractive hybridization 34 cDNAs that correspond to T<sub>3</sub>-regulated genes in the *Xenopus* tadpole CNS (16). Several of these genes are transcriptional regulatory proteins, and we hypothesized that similar sets of genes would be regulated by the hormone in mammal brain. BTEB is the first such gene that we have analyzed and found to be similarly regulated in frog and rodent brain. Our success raises the possibility that other rat homologs of frog genes isolated by our gene expression screen will also be found to be regulated by T<sub>3</sub> in the developing mammal brain.

**BTEB Gene Regulation May Be Specific to the TR $\beta 1$  Isoform**—Because TR $\alpha 1$  and TR $\beta 1$  are differentially expressed in the developing brain we hypothesized that each TR isoform might regulate distinct sets of genes in different brain regions.

Lebel and colleagues (9) developed neuronal cell lines (Neuro-2a cells; derived from a mouse neuroblastoma) that overexpress either the TR $\alpha 1$  or TR $\beta 1$  isoform (N-2a[TR $\alpha 1$ ] or N-2a[TR $\beta 1$ ]). Our results using these two cell lines suggest that BTEB gene regulation is specific to the TR $\beta 1$  isoform. These two cell lines express similar levels of T<sub>3</sub> binding activity (~7-fold over the parental cell line). Furthermore, the TR $\alpha 1$  expression plasmid produces a transcriptionally active receptor in a transient transfection assay using a luciferase reporter gene construct under control of a thyroid hormone response element (9). It is noteworthy that when constructing the overexpressing cell lines, Lebel and colleagues screened 20 TR $\alpha 1$ -expressing clones and none were responsive to T<sub>3</sub> in terms of its antiproliferative and differentiative effects. Conversely, the majority of TR $\beta 1$ -expressing clones were responsive to T<sub>3</sub>. Taken together, our results support the view that BTEB is regulated by T<sub>3</sub> through a specific interaction with the TR $\beta 1$  in neural cells. Whether such differential gene regulation by TR isoforms occurs in the developing brain has not yet been determined. However, correlative evidence suggests that BTEB may be regulated by T<sub>3</sub> through an interaction with TR $\beta 1$  in the developing brain. For example, the expression of BTEB mRNA and its regulation by T<sub>3</sub> correlates more closely with the appearance of TR $\beta 1$  than it does with TR $\alpha 1$  in primary neuronal cultures (20) and in the developing rat brain (7).

**Transcriptional Regulation of BTEB Gene Expression by Thyroid Hormone**—The rapid kinetics of T<sub>3</sub> up-regulation of BTEB mRNA in N-2a[TR $\beta 1$ ] cells (see Fig. 4) suggested that T<sub>3</sub> might directly regulate BTEB gene transcription. Our finding that the up-regulation of BTEB mRNA by T<sub>3</sub> occurs in the presence of the protein synthesis inhibitor cycloheximide provides further support for this hypothesis. The effect of T<sub>3</sub> is probably not due to the stabilization of the mRNA since treatment with actinomycin D abolished the T<sub>3</sub> effect on the level of BTEB mRNA. In addition, nuclear run-on analysis experiments confirm that T<sub>3</sub> alters the rate of transcription of the BTEB gene (Fig. 6). Taken together, these results provide strong support for a direct transcriptional action of T<sub>3</sub> on BTEB gene expression.

**Functional Consequences of BTEB Gene Expression**—The expression of the BTEB gene in the developing rat brain coincides with the neonatal rise in TR $\beta$ 1 and with the neonatal surge in the level of serum T $_3$ , suggesting that BTEB could mediate some of the postnatal effects of T $_3$  on neural cell differentiation. Although there is evidence for a role for thyroid hormones in the control of the duration of the proliferative phase of neuroblasts and oligodendrocyte progenitors, our results argue against a role for BTEB in these actions. T $_3$  regulates BTEB gene expression in post-mitotic cultured neurons and, BTEB is expressed essentially postnatally in the developing cerebral hemispheres during a period where most neuronal cells have ceased mitosis. It is also unlikely that BTEB is involved in the effect of T $_3$  on the control of the proliferative phase of oligodendrocyte progenitors (O-2A) since these cells do not express TR $\beta$ 1 (39).

It is well established that the absence of thyroid hormone during the neonatal/postnatal period causes profound alterations in CNS development, including deficiency in myelination, neurite outgrowth, and the development of specific neuronal functions. It is unlikely that BTEB is involved with T $_3$  action on myelination since the gene does not appear to be regulated by T $_3$  in oligodendrocytes, at least not *in vitro*. A major effect of T $_3$  on primary cultured neurons is on neurite outgrowth (21); similar effects of the hormone are seen in N-2a[TR $\beta$ 1] cells (9). In the present study, in addition to increasing the number of neurites (as reported by Lebel *et al.* (9)) we found that T $_3$  treatment of N-2a[TR $\beta$ 1] cells also increased neurite length. This difference is most likely due to the different incubation times in the two studies (48 h exposure to T $_3$  in the study by Lebel *et al.* (9) versus 5 day exposure to T $_3$  in the present study).

Our findings support a role for BTEB in mediating some of the postnatal effects of thyroid hormone. Here we show that expression of BTEB induces neurite outgrowth in N-2a cells. This effect was observed after 5 days in culture when most cells are confluent, suggesting that N-2a cells must cease division, probably by contact inhibition, before the up-regulation of BTEB can exert its effects on neurite outgrowth. It is noteworthy that the level of BTEB mRNA expression in clone number 10 (3-fold) is comparable to the level of T $_3$ -induced expression of this gene in both primary neurons (Fig. 3) and N-2a[TR $\beta$ ] cells (Fig. 4). Furthermore, the number and length of neurites observed with N-2a[BTEB] clone number 10 was comparable to that observed in N-2a[TR $\beta$ 1] cells treated with T $_3$  for 5 days (see Table II; Fig. 7). These observations argue against the generation of a neomorphic phenotype as a result of overexpression of this transcription factor. Because our results with BTEB overexpression were obtained in a stably transfected neuroblastoma cell line, at present we can only speculate on the role that BTEB plays *in vivo* in the developing brain. However, taken together, our findings suggest that BTEB could be a critical component of the signaling pathway induced by the hormone in the developing brain; studies are underway to test this hypothesis in primary neurons. Furthermore, the parallel between the effect of T $_3$  on neurite outgrowth and the associated elevation of BTEB mRNA in primary neuronal cultures is consistent with this hypothesis (21).

The transcriptional regulation of BTEB gene expression by T $_3$  suggests that up-regulation of this transcription factor is one of the earliest events (following TR activation) in T $_3$ -induced neurite outgrowth. The absence of an effect of BTEB overexpression on AchE activity in N-2a cells indicates that different transcriptional regulatory pathways mediate the action of T $_3$  on neurite outgrowth and the induction AchE activity. These findings illustrate the complexity of the molecular

mechanisms that underly the action of the hormone in specific neural cell types. The earliest observed effect of T $_3$  in N-2a[TR $\beta$ ] cells is to block, by an unknown mechanism, cellular proliferation (9). AchE activity is then stimulated and this involves the activation of a serine/threonine protein kinase pathway (33). The cessation of cellular proliferation is followed by an increase in neurite outgrowth, most probably through activation of BTEB gene expression. Our results indicate that T $_3$  can produce a variety of cell-type and temporally-specific effects through an interaction with the same thyroid hormone receptor. Our findings provide a first step toward understanding the complex gene expression changes induced by the hormone in the developing brain.

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