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Thyroid Hormone-Dependent Regulation of Tα1 α-Tubulin during Brain Development

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Thyroid hormone (T3) is essential for brain development and most of its actions are exerted at the gene expression level after interaction with nuclear receptors. In particular, genes encoding cytoskeletal proteins are influenced by the thyroidal status. Thyroid hormone is involved in the normal downregulation of the Tα1 α-tubulin gene during postnatal growth. The action of T3 on Tα1 tubulin expression is complex and is exerted at least at two levels. In cultured cells, T3 induces a transient and fast decrease of Tα1 mRNA concentration. This effect is enhanced when transcription is blocked by actinomycin D, suggesting that T3 increases mRNA degradation. In transgenic animals T3 affects the expression of β-galactosidase under control of the Tα1 promoter in the same way as the endogenous gene, supporting an effect mediated through the Tα1 promoter. However, the Tα1 promoter is not regulated by T3 in transfected cells and, therefore, the effects of the hormone *in vivo* are likely to be indirect. It is concluded that regulation of Tα1 α-tubulin by thyroid hormone is the result of multiple influences including effects on mRNA half life and indirect effects at the promoter level.

INTRODUCTION

Thyroid hormone (TH) is an important regulator of physiological and developmental processes and plays a crucial role in maturation of the CNS. TH deficiency during critical periods of development produces permanent and severe alterations in the structure and function of the nervous system, including delays of neuronal differentiation with abnormal morphology of dendrites and disturbed outgrowth of neuronal processes (Bernal and Nunez, 1995). Both neurons and oligodendrocytes are cell targets of TH during CNS maturation. These cell types contain developmentally regulated TH receptors and express genes that are regulated *in vivo* and *in vitro* by the hormone (Bernal and Nunez, 1995; Muñoz and Bernal, 1997). Some of these genes are related to key events occurring during neurite outgrowth, such as those encoding cytoskeletal proteins. Alterations of microtubule assembly have been described in a form of congenital hypothyroidism in mice (*hyt/hyt* mice) and also take place after induction of neonatal hypothyroidism in previously normal animals (Biesiada *et al.,* 1996; Nunez *et al.,* 1991). These alterations in microtubule assembly and stabilization are partly mediated through TH-dependent, regulated expression of microtubule-associated proteins (MAPs) (Aniello *et al.,* 1991a; Benjamin *et al.,* 1988) and specific tubulin isoforms (Aniello *et al.,* 1991b; Figueiredo *et al.,* 1993; Lewis *et al.,* 1985). Several studies suggest that TH deficiency maintains an immature composition of microtubules by delaying the developmental program of expression of several tubulin and MAP genes (Aniello *et al.,* 1991a,b; Charriére-Bertrand and Nunez, 1992; Nunez *et al.,* 1991, 1992). In this context, TH is an epigenetic signal required to achieve the construction of a normal neuronal network during development. Indeed TH has been shown to play a role in tubulin gene expression, in particular α-tubulin, during brain development.

Two α-tubulin genes are expressed in the embryonic rat nervous system and are named Tα1 and T26 (Miller *et al.,* 1987), homologous to mouse Mα1 and Mα2, respectively (Lewis *et al.,* 1985). Whereas T26 is constitutively expressed in neurons and in nonneuronal cells, Tα1 is neuron-specific and its expression correlates with neuronal growth (Knoops and Octave, 1997; Miller *et al.,* 1987). Expression of Tα1 α-tubulin is high during

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developmental growth and is subsequently downregulated around the time of target contact and neuronal maturation (Gloster *et al.,* 1999; Mathew and Miller, 1990; Miller *et al.,* 1987; Wu *et al.,* 1997). Tα1 increases in response to different extrinsic cues that induce the growth of mature neurons (Geddes *et al.,* 1990; Ma *et al.,* 1992; Mathew and Miller, 1990, 1993; Miller *et al.,* 1989; Tetzlaff *et al.,* 1991). In this context it has been proposed that Tα1 α-tubulin induction reflects a generalized neuronal growth response that, once initiated, is similar in developing and in mature neurons (Geddes *et al.,* 1990; Mathew and Miller, 1990).

In hypothyroid animals Tα1 α-tubulin expression is increased in cerebellum and decreased in cerebral hemispheres (Aniello *et al.,* 1991b; Figueiredo *et al.,* 1993; Nunez *et al.,* 1991). The information provided by these studies, however, is limited because the analysis of Tα1 α-tubulin expression was done by Northern blotting, thus overlooking any regional-specific variation of Tα1 α-tubulin regulation. In addition the question about the mode of action of thyroid hormone on the regulation of Tα1 α-tubulin expression was not addressed. Given the importance of Tα1 α-tubulin in neuronal growth and regeneration, we have reevaluated its regulation by TH both *in vivo* and *in vitro. In vivo,* studies were aimed at the analysis of the temporal and regional patterns of TH regulation. *In vitro* studies were done with the goal of elucidating the molecular mechanisms of regulation by thyroid hormone.

RESULTS

Tα-1 α-Tubulin Expression in Vivo

We used a specific labeled riboprobe from the 3′ untranslated region of the α-tubulin mRNA to analyze the expression of the Tα1 isoform in normal and hypothyroid rats during postnatal development. Coronal slices from both groups of animals were used for *in situ* hybridization. The patterns of expression in the cerebrum and in the cerebellum are illustrated in Figs. 1 and 2, respectively.

Tα1 tubulin shows a widespread distribution in the brain, with strong regional quantitative differences (Fig. 1). In normal rats, expression in most structures was high during early postnatal ages (P3 and P5) and then decreased progressively in an age-dependent fashion. At P3 and P5, high levels of expression were found in the cortical plate, piriform cortex, and pyramidal field of the hippocampus and thalamus. There was then a decreased expression from P10 onward, and by P20 expression in most parts of the cerebrum was low and near background levels. From this age high expression levels were seen only in the piriform cortex, the pyramidal and granular layers of the hippocampus, and the nucleus of the lateral olfactory tract. This pattern persisted beyond P32.

Tα1 α-tubulin expression in hypothyroid animals was lower than in control animals at early postnatal ages (P3, and also at P1, not shown). Quantification of the data revealed that at P3 levels in hypothyroid animals were about 85% of controls. At P5 there was no difference in expression between both groups of animals. However, the decreased expression that took place in the normal animals after P10 was slowed down in the hypothyroid animals. As a consequence, most structures showed a stronger signal (around 30%) in the samples from hypothyroid animals than in controls at P20. The effect of hypothyroidism was transient, since the differences between both groups of animals nearly disappeared by P32.

In the cerebellum (Fig. 2) there was a diffuse increase in Tα1 α-tubulin expression with postnatal age, which was stronger in the hypothyroid animals at P20. A discrete signal was present in the external germinal layer in control animals at P10 and P20, disappearing by P32. In hypothyroid animals the appearance of label in this layer was delayed. It was strong at P20 (twofold stronger in hypothyroid than in control cerebella) and was still present by P32, reflecting the delayed granule cell migration, a landmark of hypothyroidism.

Regulation of Tα1 α-Tubulin Expression by Thyroid Hormone in Cultured Cells

The influence of thyroid hormone on Tα1 α-tubulin expression *in vivo* could be due to a direct or indirect effect of T3 on the regulatory regions of the Tα1 gene or be a distal consequence of T3 action, far from its primary site of action. As a first approximation to elucidate the mechanism of Tα1 regulation we examined the effect of T3 deprivation and administration in cultured cells. We used two different cell systems: first, the immortalized GT1-7 hypothalamic cell line (Mellon *et al.,* 1990). These cells express physiological amounts of functional T3 receptors, which mediate the regulated expression of the neuronal gene RC3/neurogranin by T3 (Morte *et al.,* 1997). As a more representative system we also used primary cultures of embryonic cortical neurons.

GT1-7 cells were cultured for 4–5 days in TH-depleted medium and then treated with T3 for different periods of time. The effect of T3 on the expression of the Tα1 α-tubulin gene was studied by Northern blot (Fig. 3A). Shortly after T3 addition a sharp decrease on Tα1

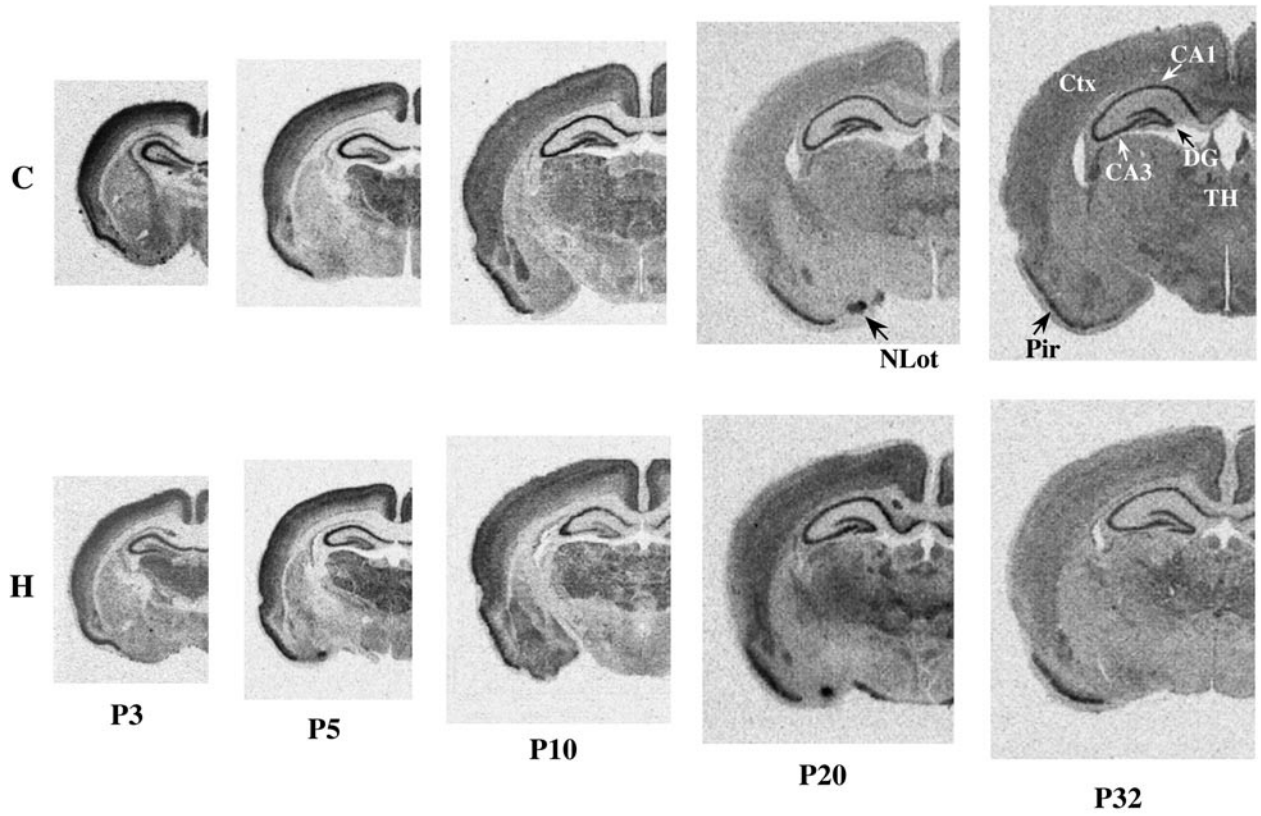

FIG. 1. Pattern of expression of Tα1 α-tubulin mRNA in rat brain. *In situ* hybridization analysis of Tα1 α-tubulin mRNA at different times during postnatal brain development (P3 to P32) in normal control (C, top) and hypothyroid (H, bottom) rats. Coronal slices from the different ages and thyroid conditions were processed simultaneously. Exposure time was 7 days for P3 and P5, 8 days for P10, 10 days for P20, and 15 days for P32. In normal rats there was a decreased signal after P10, which in hypothyroid rats takes place after P20. Abbreviations: Pir, piriform cortex; TH, thalamus; DG, dentate gyrus; CA1, CA3, pyramidal layer of hippocampus; Ctx, cortex; Nlot, nucleus of the lateral olfactory tract.

mRNA levels was observed (49 $\pm$ 11%, mean $\pm$ SD of three experiments). This initial effect of T3 was transient, and from 8 to 24 h Tα1 mRNA concentrations increased, finally reaching control levels. Expression of a neuronal differentiation marker, NSE, was also checked in the same cultures. T3 induced a late increase of NSE mRNA concentration after 16 h of treatment, in parallel to the increase in Tα1 α-tubulin. A similar study carried out with primary cell cultures obtained from E13 mouse cerebral cortex showed the same initial T3-induced inhibition of Tα1 expression (Fig. 3B) and a subsequent increase by 16 h.

The effect of T3 on the levels of Tα1 α-tubulin protein was also studied in primary cultures of cortical cells obtained from either E13 or E16 mice, using an anti-tubulin antibody. Since Tα1 α-tubulin mRNA comprises greater than 95% of the total population of α-tubulin mRNA in the embryonic nervous system (Gloster *et al.*, 1994; Miller *et al.*, 1987), we may assume that most of the α-tubulin detected by us in the embryonic cortical cultures was Tα1. Tubulin content was measured by Western blotting of cell extracts using Ponceau red staining as a control for protein loading (not shown) and the levels of Erkb by immunoblotting. The result of the Western blots is shown in Fig. 4A. The quantification of the bands is shown on the right-hand side of the figure. In E13 cultures α-tubulin concentration decreased after 4 h of T3 addition and increased thereafter, in agreement with mRNA data. The levels of β3-tubulin and intermediate NF were also measured. Both proteins experienced an initial increase within 8 h of T3 addition, which was followed by a more sustained increased after 16–24 h. The significance of the transient decrease observed for intermediate NF at 16 h is not clear at present. The late increase of both proteins paralleled that of Tα1 α-tubulin, suggesting that T3 stimulated the differentiation of these cells.

To assess in more detail the effect of T3 on neuronal differentiation, the E13 cultures were immunostained for β3-tubulin 24 h after T3 addition. The cultures were

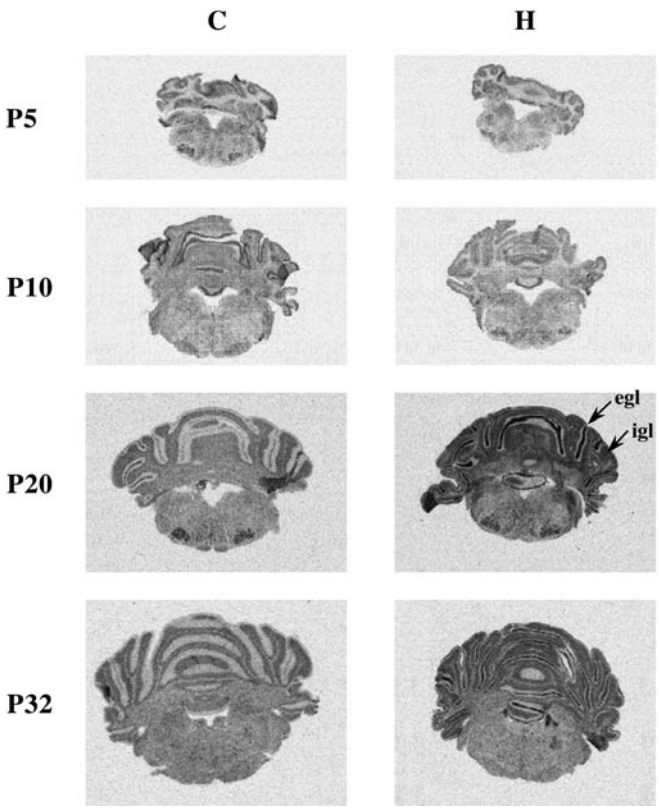

FIG. 2. Pattern of expression of Tα1 α-tubulin in rat cerebellum. Distribution of Tα1 α-tubulin mRNA during postnatal cerebellar development (from P5 to P32) in normal control (C, left) and hypothyroid (H, right) rat cerebellum. Autoradiographs show representative *in situ* hybridization of coronal sections. Note the strong signal present in the external granule layer of hypothyroid animals at P20. igl, internal granular layer; egl, external granular layer.

also counterstained with Hoechst 33258 to visualize the nuclei of all cells. Data from different T3-treated (n = 11) or untreated cultures (n = 9) showed that T3 did not affect the proportion of differentiated cells, i.e., those expressing β3-tubulin in relation to the total number of cells (31.6 ± 11.8% in the untreated cultures versus 27.8 ± 5.9% in the T3-treated cultures). However, addition of T3 increased significantly the proportion of β3-tubulin-positive cells that presented neurites of a length greater than one cell diameter (26.4 ± 6.7% in the T3-treated cultures versus 17.6 ± 6.5% in the untreated cultures, P = 0.016). Therefore, addition of T3 to cultures of cells derived from E13 cultures, which initially contain mainly neuronal progenitors, was associated with an enhanced neuronal differentiation. To assess the effect of T3 in a more differentiated culture we used cells derived from E16 brains (Fig. 4B): in these cells T3 induced an increased tubulin content a few hours after hormone addition without the initial decrease observed in undifferentiated cultures. β3-Tubulin and intermediate NF content were not modified by T3 treatment in these cultures (not shown).

To analyze whether the short-term inhibition of Tα1 tubulin expression by T3 was due to inhibition of transcription or increased mRNA degradation, we treated the GT1-7 cells with T3 in the presence or absence of actinomycin D (AcD), an inhibitor of transcription, or cycloheximide (Chx), an inhibitor of translation, as previously described (Morte *et al.*, 1997). In the presence of AcD (Fig. 5A) Tα1 mRNA was decreased by 40% at 6 h compared with the untreated control cells, suggesting a

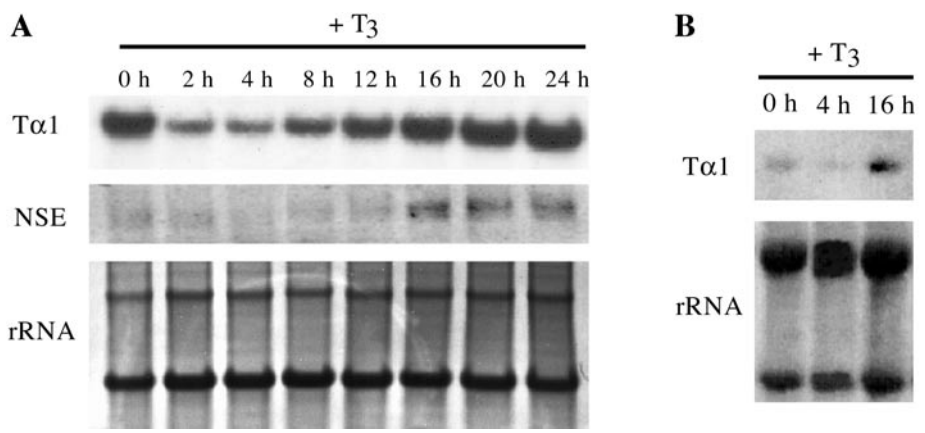

FIG. 3. Time course of the effect of T3 on Tα1 α-tubulin and NSE mRNA content of GT1-7 cells. (A) Cells were incubated in T3-free serum with or without addition of T3 at a final concentration of 150 nM for the times indicated. After the cells were harvested, poly(A)$^+$ RNA was isolated and Northern blots were performed. The membranes were hybridized with a probe from the 3′ untranslated region of the Tα1 α-tubulin cDNA or from the full-length NSE cDNA. Each lane was loaded with 5 μg of poly(A)$^+$ RNA. The bottom shows the ribosomal RNA stained with methylene blue. (B) Cortical cells obtained from E13 embryos in primary culture were incubated in the absence or in the presence of 150 nM T3 for 4 and 16 h. Northern blotting was performed using total RNA and the filters were hybridized with the Tα1 α-tubulin probe.

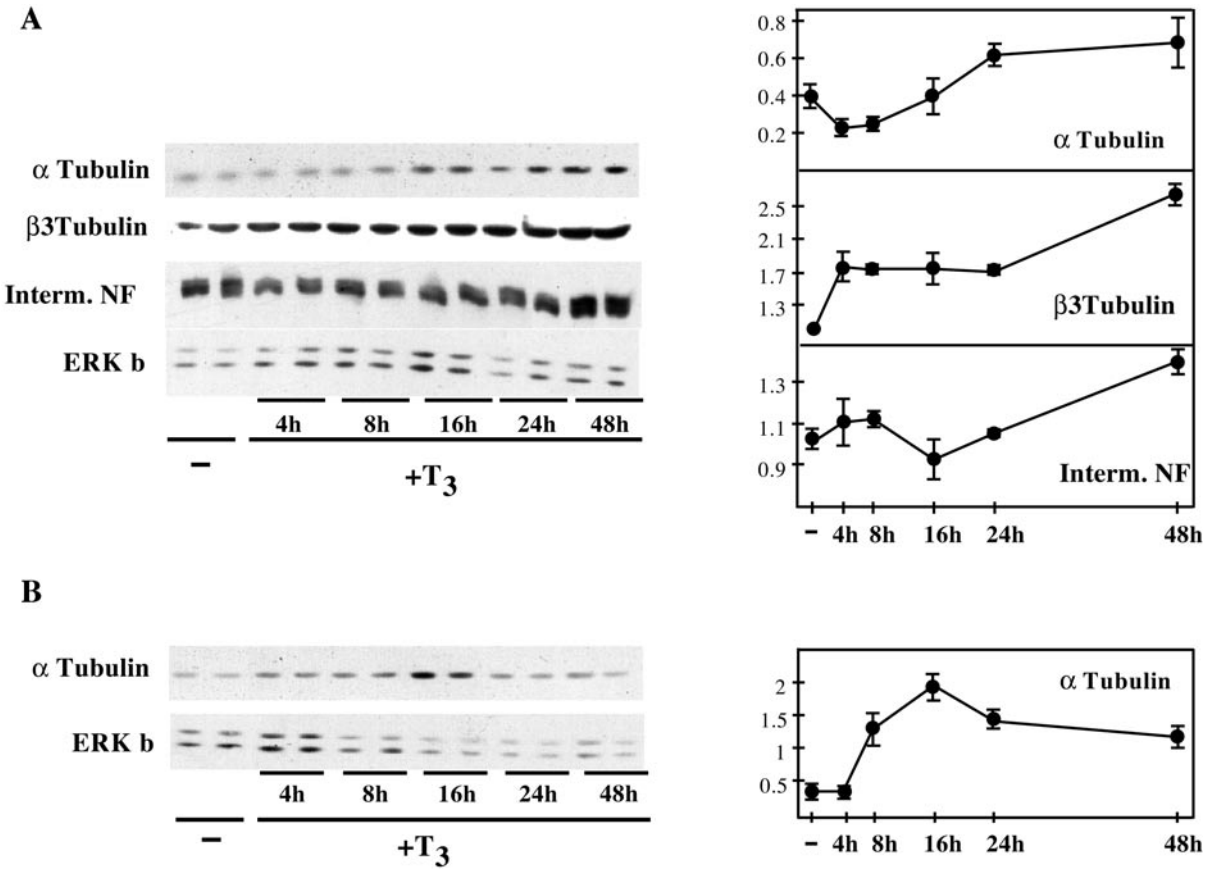

FIG. 4. Effects of T3 on α-tubulin levels in mouse cortical primary cultures. Western blot analysis of the effect of T3 addition on the protein levels of α-tubulin, β3-tubulin, and intermediate neurofilament in E13 (A) and E16 (B) primary cultures (left). Each time point was analyzed in duplicate. T3 was added sequentially to the cultures, so that all cells were harvested at the same time. Changes in Erkb concentration represent culture variations in the concentration of total protein and were used to normalize the tubulin and neurofilament concentrations.

half-life for the mRNA of around 8–9 h, compatible with other estimates (Bachurski *et al.*, 1994). T3 alone induced a similar Tα1 mRNA decrease. In the presence of both AcD and T3, the decrease of Tα1 mRNA was more pronounced than with either agent alone, suggesting a destabilization of Tα1 mRNA in addition to a blocked transcription. That the concentration of Tα1 mRNA is also regulated at the level of mRNA stabilization is supported by the effect of Chx (Fig. 5B). In its presence Tα1 mRNA experienced a sharp decrease (more than 60% at 6 h), suggesting that Chx prevented the synthesis of short-lived proteins involved in Tα1 mRNA stability. The effect of Chx was more pronounced than that of T3 alone, and addition of both Chx and T3 did not decrease Tα1 mRNA any further.

Role of the Tα1 Tubulin Promoter in T3 Action

The Tα1 tubulin promoter has been isolated and characterized (Gloster *et al.*, 1994). In transgenic mice, a DNA fragment containing the proximal Tα1 promoter and the upstream region up to −1.1 kb from the start of transcription was able to direct β-gal expression in a manner closely resembling that of endogenous Tα1 tubulin (Bamji and Miller, 1996; Gloster *et al.*, 1994, 1999; Miller *et al.*, 1996; Wu *et al.*, 1997). Since this DNA fragment apparently contains most of the regulatory sites for physiological Tα1 expression we analyzed the role of the promoter in the effect of thyroid hormone on Tα1 tubulin expression both *in vitro* and *in vivo*. First we checked the possibility that the promoter is directly responsive to T3. A construct containing the CAT gene under control of the proximal Tα1 promoter described above was transfected in GT1-7 cells that express physiological amounts of functional T3 receptors. We did not observe any effect of T3 treatment (not shown). The experiments were then performed using PC12 cells, a cell line in which addition of NGF induces upregulation of Tα1 tubulin mRNA together with neurite outgrowth.

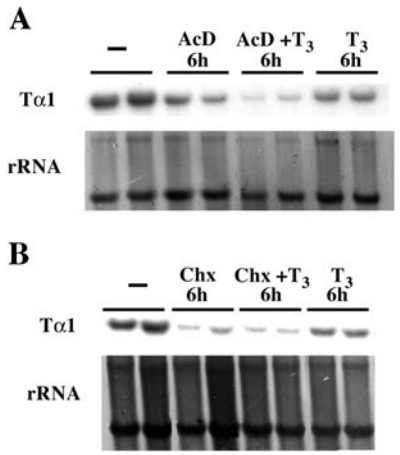

FIG. 5. Effect of T3, actinomycin D (AcD), and cycloheximide (Chx) on Tα1 α-tubulin expression. GT1-7 cells were cultured for at least 4 days in T3-free serum and were then incubated for 6 h with T3 (150 nM), with or without AcD (5 μg/ml, A), or Chx (8 μg/ml, B). AcD and Chx were added to the cultures 30 min before T3 addition. Northern blots containing 3 (A) or 5 μg (B) mRNA per lane were hybridized with the Tα1 α-tubulin probe. RNA loading was controlled by staining with methylene blue (rRNA).

The cells therefore allowed for the control of the responsiveness of the transfected Tα1 promoter to NGF. PC12 cells were transfected with the Tα1 promoter together with expression vectors encoding T3 receptors (Fig. 6A). Addition of T3 did not result in a stimulation of CAT activity. As controls, addition of NGF was able to induce the reporter gene (Fig. 6A) and T3 was able to activate the thymidine kinase (*tk*) promoter through a TRE (Fig. 6B). It can be concluded therefore that T3 does not influence directly the activity of the Tα1 α-tubulin promoter.

T3, however, could exert indirect effects *in vivo* on the Tα-1 α-tubulin promoter as part of a distal response to a primary action of T3 elsewhere. This possibility was checked using the transgenic mouse line K6, in which the β-gal gene is under control of the Tα1 promoter. In these mice, expression of Tα1:nlacZ is closely similar to that of the endogenous gene, suggesting that the 1.1-kb promoter fragment contains all sequences necessary for the regulated expression of Tα1 tubulin (Bamji and Miller, 1996). The mice were made hypothyroid using a combination of two antithyroid drugs, as described under Experimental Methods. The reduced weight of the animals and the strong alterations in Purkinje cell organization (not shown) confirmed the effectiveness of the treatment.

Expression of the transgene was analyzed by histochemistry for β-galactosidase and that of the endogenous gene by *in situ* hybridization, and the results are illustrated in Fig. 7. In Fig. 7A, it is shown that β-gal staining was stronger in the slices from hypothyroid mice at P15. These differences were not observed at P20 and P35 (not shown). In Figs. 7B and 7C, expression of the endogenous tubulin (Mα1 tubulin) mRNA is shown. There was a stronger signal in the hypothyroid brains at P15 than in the control animals and the effect of hypothyroidism was not observed at P35. Therefore the effects of hypothyroidism on the endogenous tubulin gene in mice were similar to those observed in rats and also similar to the effects on the promoter-driven reporter gene, suggesting that the effect of thyroid hormone is exerted, at least partially, through the α-tubulin promoter.

DISCUSSION

In this work we have studied the role of thyroid hormone on the expression of the α1 tubulin isoform

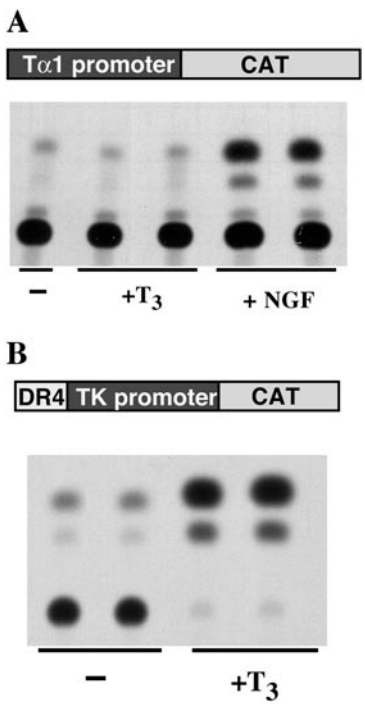

FIG. 6. The Tα1 α-tubulin promoter is not responsive to T3. In A, PC12 cells were cotransfected with the Tα1 promoter driving CAT expression and plasmids encoding the T3 receptor β1 isoform and RXRα. After transfection, the cells were treated with T3, at a final concentration of 150 nM, for 24 h and CAT activity was assayed. No increase in CAT activity was observed after T3 addition to cells transfected with the Tα1:CAT construct. However, treatment with NGF increased CAT activity. To control for the action of T3, a reporter vector containing a DR4 TRE upstream of the thymidine kinase promoter was assayed (B).

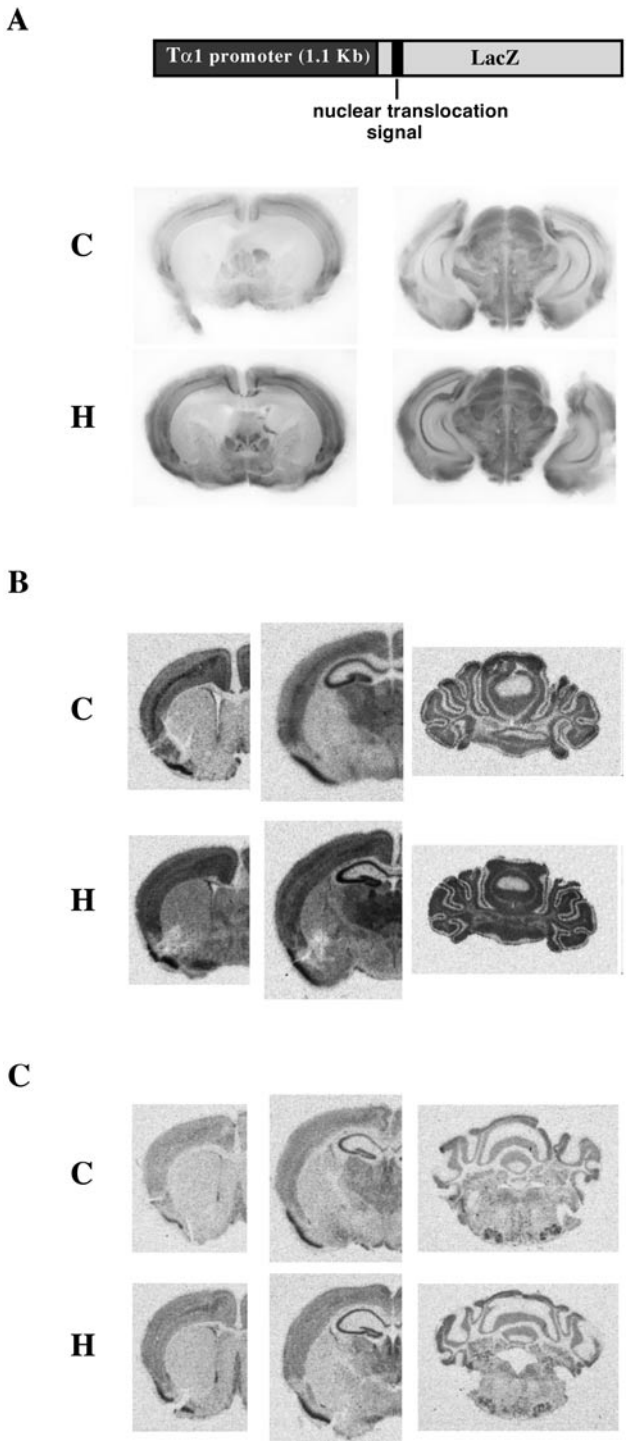

FIG. 7. Pattern of expression of the Tα1:nLacZ transgene. Shown is the scheme of the construct used for generation of transgenic mice expressing the LacZ gene under control of the Tα1 promoter. (A) Histochemical assay for β-galactosidase activity in P15 intact (C) or hypothyroid (H) mice. (B and C) Expression of Mα1 α-tubulin mRNA in transgenic K6 mouse brain at P15 (B) and P35 (C), either control (C) or hypothyroid (H). Autoradiographs show representative *in situ* hybridizations in coronal sections of cerebrum and cerebellum.

(Tα1 or Mα1 α-tubulin) in the rodent brain during postnatal development. Although Tα1 α-tubulin is expressed in other tissues such as lung or testis (Lewis *et al.*, 1985), in the brain it is neuron-specific and strongly dependent on the stage of neuronal growth and differentiation (Mathew and Miller, 1990, 1993; Miller *et al.*, 1987, 1989, 1991; Wu *et al.*, 1997). Although widely expressed, Tα1 mRNA levels vary considerably among different regions during brain maturation (Miller *et al.*, 1987). We found that during early postnatal development expression is high in neocortex, piriform cortex, hippocampus, thalamus, and granular layers of the cerebellum, in agreement with data from Miller *et al.* (1987) for P3 animals. As development proceeds, Tα1 mRNA levels decrease as maturational processes progress in each region (Paxinos, 1985). The main consequence of hypothyroidism was to delay this progressive decrease of Tα1 mRNA with age, leading to differences between normal and hypothyroid rats that were more pronounced around P20 in most brain regions. These results generally agree with previous data from other authors using Northern blotting analysis of α-tubulin expression (Aniello *et al.*, 1991b; Figueiredo *et al.*, 1993).

The mechanism of thyroid hormone action on Tα1 α-tubulin expression is complex. The previously described, putative thyroid hormone receptor-responsive element in the α-tubulin gene (Iglesias *et al.*, 1996) has been found by us to be nonfunctional (unpublished results from our laboratory). Therefore, we have looked for alternative mechanisms of T3 action by analyzing tubulin expression in cultured cells and the role of the tubulin promoter in transgenic mice. For the analysis *in vitro* we used the neuronal cell line GT1-7, expressing physiological amounts of functional T3 receptors (Morte *et al.*, 1997). In these cells T3 exerts a fast and transient decrease of tubulin mRNA, followed by a recovery to control levels. This effect was also observed in primary cultures isolated from E13 embryonic brains. In both cases the recovery of tubulin levels was parallel to an increased differentiation of the cells induced by T3 as shown by induction of differentiation markers, such as NSE in the GT1-7 cells, or β3-tubulin and intermediate neurofilament in cortical neurons.

The initial effect of T3 on Tα1 α-tubulin mRNA levels appears to be due to increased mRNA degradation, perhaps through inhibition of mRNA-stabilizing proteins. The experiments involving Chx suggest that the Tα1 α-tubulin mRNA levels are regulated, at least partially, by stabilizing proteins. As a matter of fact, the regulation of tubulin mRNA levels occurs primarily at the level of mRNA stability, which is coupled to tubulin translation (Gonzalez-Garay and Cabral, 1995, 1996; Theodorakis and Cleveland, 1992). The secondary effect

of T3, increasing Tα1 expression, was likely due to a general stimulation of cellular differentiation, as suggested by the increased expression of cell differentiation markers. This contention is supported by the observation that the effect of T3 on cortical neurons depends on the differentiating stage of the cultures. In E13 cultures in which most of the cells are neuronal progenitors the regulation of Tα1 α-tubulin is similar to that observed in GT1-7 cells, with an initial decrease of mRNA followed by an increase. This effect, observed at the mRNA level in GT1-7 cells also occurred at the protein level in cortical cultures. On the other hand, in E16 cultures, which contain primarily postmitotic neurons, T3 induced an increase in tubulin expression as soon as 4 h after T3 addition, reaching a plateau after 16 h of treatment. A possible explanation is that the increase in α-tubulin expression is secondary to a more general effect of T3 on neuronal maturation, which should take place more rapidly in neurons from E16 brains than in those from E13. Deprivation of T3 from differentiating cultures retards axodendritic outgrowth and also alters neuronal morphology (Biswas *et al.*, 1997). The T3-dependent increase of α-tubulin levels may reflect the need to rapidly increase the pool of α-tubulin demanded by the stimulation of differentiation.

Thus we may postulate the existence of a double regulation of Tα1 by T3 in cultured cells: an initial decrease in Tα1 mRNA levels, probably mediated through a reduction in the half-life of the mRNA, followed by T3 stimulation of neuronal maturation, which induces an upregulation of Tα1. This is in line with the role of T3 as a neural induction agent, as has been shown in cell lines such as PC-12 cells expressing TRα1 (Muñoz *et al.*, 1993) or N2a cells expressing TRβ1 (Lebel *et al.*, 1994).

T3 also exerts influences through the tubulin promoter *in vivo.* A 1.1-kb fragment of the tubulin promoter was able to direct expression of a reporter gene in transgenic mice with a pattern similar to that of the endogenous gene (Bamji and Miller, 1996; Gloster *et al.*, 1994, 1999). Therefore it presumably contains sequences necessary for full physiological expression *in vivo.* As in rats, hypothyroidism in mice delayed the developmental downregulation of both the endogenous α-tubulin gene and the reporter gene under control of the tubulin promoter. However, the promoter was not responsive to T3 in cells cotransfected with thyroid hormone receptors. The results therefore indicate that thyroid hormone exerts indirect influences on the activity of the tubulin promoter, which are involved in the physiological downregulation of the tubulin gene.

EXPERIMENTAL METHODS

Animals

CD1 mice and Wistar rats were used to map the expression of the respective endogenous Mα1 and Tα1 α-tubulin mRNAs by *in situ* hybridization. Maintenance and handling of animals were as recommended by the European Communities Council Directive of November 24, 1986 (86/609/EEC). The Tα1:nlacZ transgenic mouse line K6, in which 1.1 kb of the 5′ upstream region of the Tα1 α-tubulin gene regulates the expression of a β-galactosidase marker gene, was used to map the expression of the Tα1:nlacZ transgene by using X-gal staining (Bamji and Miller, 1996; Gloster *et al.*, 1994). Hypothyroidism was induced by the continuous administration of 0.02% methyl mercaptoimidazol (Sigma Chemical Co., St. Louis, MO) and 1% $KClO_4$ in the drinking water, starting on day 9 of pregnancy, until the end of the experiments.

LacZ Staining

Tα1:nlacZ transgenic mice were deeply anesthetized with sodium pentobarbital (35 mg/kg) and perfused transcardially with 4% paraformaldehyde in phosphate buffer. The brains were removed, postfixed, and sliced into 1- to 2-mm-thick sections. They were then rinsed three times for 30 min each with a wash buffer containing 2 mM $MgCl_2$, 0.01% sodium deoxycholate, 0.02% NP-40, 0.1 M NaH_2PO_4, pH 7.3. The staining reaction was performed by incubating the tissue at 37°C in a reaction mixture containing all the components of the wash buffer with the addition of 1 mg/ml X-gal, 5 mM $K_3Fe(CN)_6$, 5 mM $K_4Fe(CN)_6$, pH 7.3–7.6, for 30–90 min as previously described (Bamji and Miller, 1996; Gloster *et al.*, 1994). All sections from the same experiment were allowed exactly the same time for color development.

In Situ Hybridization

Two animals from each experimental group (control and hypothyroid) were analyzed at each of the following postnatal ages: P3, P5, P10, P20, and P32. All procedures, including killing the animals, treatment of tissue, sectioning, and *in situ* hybridization using floating sections, were exactly as described previously (Iñiguez *et al.*, 1996). ^{35}S-labeled sense and antisense riboprobes were prepared from the 3′ untranslated region of Tα1 α-tubulin complementary DNA. After exposure to X-ray films, the radioactive signal was quantified by densitometry as described (Guadaño-Ferraz *et al.*, 1999). To have comparable signals in the autoradiographs, the

exposure time was 7 days for P3 and P5, 8 days for P10, 10 days for P20, and 15 days for P32. For identification of brain structures, the rat atlas of Paxinos and Watson was followed (Paxinos, 1985).

Cell Cultures

GT1-7 cells were supplied by Dr. P. Mellon (University of California at San Diego). Cells were routinely cultured in Dulbecco's modified Eagle's medium with 4.5 mg/ml glucose and 10% fetal calf serum. To study the influence of thyroid hormone, the cells were incubated in serum depleted of thyroid hormone by treatment with charcoal and Dowex resin as previously described (Morte *et al.*, 1997). The chemicals used for cell treatments were from Sigma Chemical Co. and were added to the cultures at the following final concentrations: T3, 150 nM; Chx, 8 μg/ml; and AcD, 5 μg/ml. When different time points were recorded, the cells were plated and T3 was added at different times before harvesting. In this way the cells corresponding to each time-point were all incubated for the same time period and the cells were harvested at the same time.

Cortical progenitors from CD1 mice were cultured as described (Slack *et al.*, 1998). Briefly, cortical tissue obtained from E13 or E16 embryos was dissected in ice-cold Hanks' balanced salt solution (Gibco). The tissue was disaggregated by being passed through a fire-polished Pasteur pipette into cell clusters that were plated in multiwell tissue culture dishes precoated with laminin and poly-d-lysine (Collaborative Biomedical Products). The cells were cultured in Neurobasal medium (Gibco) containing 500 μM glutamine, N2-supplement, and penicillin–streptomycin (Gibco); the medium was supplemented with bFGF for the first 2 days. Thereafter B27 was substituted for bFGF. Cell density ranged between 30,000 and 100,000 cells per well. To quantitate the effect of T3 on neuronal differentiation, T3-treated and untreated cultures were stained for β3-tubulin and counterstained with Hoechst 33258 to visualize the nuclei of cells, as previously described (Gloster *et al.*, 1999). Total number of cells and β3-tubulin-positive cells were counted in 11 T3-treated cultures and 9 untreated cultures, and each field examined contained 50–100 cells. The results were analyzed statistically using the Student *t* test.

Western Blot Analysis

Neurons were lysed in TBS lysis buffer containing 13 mM NaCl, 20 mM Tris–HCl, pH 8, 1% (vol/vol) NP-40, 10% (vol/vol) glycerol, 1 mM PMSF, 10 μg/ml aprotinin, 0.2 μg/ml leupeptin, 5 mM phenanthroline, and 1.5 mM sodium vanadate. Samples were rocked for 20 min at 4°C and centrifuged for 15 min at 15,000 rpm. The supernatant was normalized for protein concentration using a BCA Protein Assay Reagent (Pierce Chemical Co., Rockford, IL). Supernatants were boiled in sample buffer (2% sodium dodecyl sulfate (SDS), 100 mM dithiothreitol, 10% glycerol, and 0.05 bromophenol blue) for 5 min and electrophoresed on 8% SDS–polyacrylamide minigels. After electrophoresis, proteins were transferred to nitrocellulose filters for 1 h at 0.5 A, and the membrane was washed twice for 10 min in TBS. The filters were stained with Ponceau red. Membranes were then blocked in 5% nonfat dry milk in TBS for 2.5 h and washed twice for 10 min in TBS. The membranes were incubated with the primary antibodies overnight at 4°C, at a dilution of 1:10,000 for anti-α-tubulin, 1:1000 for anti-β3-tubulin, 1:500 for anti-intermediate NF, and 1:10,000 anti-Erkb. Incubation with secondary antibodies (goat anti-mouse HRP for anti-αtubulin and anti-β3-tubulin and goat anti-rabbit HRP for anti-intermediate NF and anti-Erkb antibodies) was for 1.5 h at room temperature at a 1:10,000 dilution. Detection was carried out using enhanced chemiluminescence (Amersham Corp., Arlington Heights, IL) and XAR X-ray film (Eastman Kodak, Rochester, NY). Results were quantitated by image analysis.

Preparation of RNA and Northern Blot Analysis

Isolation of poly(A)$^+$ RNA was done as described (Vennström and Bishop, 1982). RNA samples were carefully checked by densitometry and by electrophoresis to ensure that the RNA loaded for each sample was identical. Northern blots were performed on nylon membranes (Nytran; Schleicher and Schuell) according to standard protocols (Sambrook *et al.*, 1989) using 5 μg RNA per lane. As a further control for RNA loading the filters were stained with 0.02% methylene blue in 0.3 M sodium acetate. Probes were labeled by the random priming method using available kits (Rediprime; Amersham, Buckinghamshire, UK). Hybridizations were performed overnight at 65°C in 7% SDS, 500 mM sodium phosphate buffer, pH 7.2, and 1 mM EDTA (Church and Gilbert, 1984). Filters were washed twice for 30 min each in 1% SDS and 40 mM sodium phosphate buffer, pH 7.2, at 65°C. Before rehybridization of the nylon membranes with other probes, the radioactive probe was stripped off the membrane by placing in a 75°C water bath for 5 min. Sizes of respective mRNAs were calculated by using an RNA ladder as marker. Membranes were exposed to Hyperfilm MP (Amersham). Results were quantitated by densitometry.

Transfections and CAT Assays

PC12 cells were grown in DMEM supplemented with thyroid hormone-depleted 10% fetal calf serum. Cells were transfected, using the calcium phosphate procedure, with 4 μg of plasmids containing the CAT gene under control of either the Tα1 or the *tk* promoter. The latter construct also contained the strong thyroid hormone-responsive element present in the LTR of the Moloney murine leukemia virus (Sap *et al.*, 1989). Cells were cotransfected with or without 0.2 μg of TRβ and 0.2 μg of RXRα expression plasmids and 1 μg of plasmid pCH110 (Pharmacia LKB Biotechnology). This plasmid contains the β-galactosidase gene under the control of the SV-40 early promoter and was used as an internal control to correct for transfection variability. At 16–18 h after DNA addition, the cells were washed with PBS and replenished with medium containing serum depleted of thyroid hormones. Where appropriate, 150 μM T3 or NGF was added and the cells were incubated for 24 h before harvesting. Preparation of cell extracts and measurements of CAT and β-gal reporter gene activities were done as described by Sambrook *et al.* (1989). To correct for transfection variability, the cell extracts used for determination of CAT activity were diluted according to the β-gal measurements.

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