The thyroid hormone (triiodothyronine, T3) is essential for normal brain maturation. To determine the mechanisms by which T3 controls neuronal proliferation and differentiation, we have analyzed the effect of this hormone on the expression and activity of cell cycle-regulating molecules in neuroblastoma N2a-β cells that overexpress the β1 isofrom of the T3 receptor. Our results show that incubation of N2a-β cells with T3 leads to a rapid down-regulation of the c-myc gene and to a decrease of cyclin D1 levels. T3 also causes a strong and sustained increase of the levels of the cyclin kinase inhibitor p27Kip1. This increase is secondary, to the augmented levels of p27Kip1 transcripts as well as to stabilization of the p27Kip1 protein. The increased levels of p27Kip1 lead to a significant increase in the amount of p27Kip1 associated with cyclin-dependent kinase 2 (CDK2), and to a marked inhibition of the kinase activity of the cyclin-CDK2 complexes. As a consequence, the retinoblastoma protein (pRb) and the retinoblastoma protein-related protein p130 are hypophosphorylated in T3-treated N2a-β cells. This study shows for the first time that T3-mediated growth arrest and neuronal differentiation are associated with an increase in the levels of a cyclin kinase inhibitor, which does not allow the inactivation of retinoblastoma proteins required for progression through the restriction point in the cell cycle.

Although the thyroid hormone (triiodothyronine, T3) is essential for the normal development of the central nervous system, the specific mechanisms by which these hormones control neuronal proliferation and differentiation are currently unknown. T3 actions in cells are initiated by binding to nuclear receptors encoded by two genes, α and β, which give rise to different receptor isoforms (1). The neuroblastoma cell line N2a expresses low levels of nuclear receptors (2). However, a stably transfected cell line (N2a-β cells) that overexpresses the nuclear receptor β1 isoform has been established (3). T3 treatment of N2a-β cells blocks proliferation by an arrest of cells in G0/G1, and induces morphological and functional differentiation (3, 4). These findings in neuroblastoma cells correlate with similar effects of T3 in primary neuronal cultures and in the developing brain (5, 6). It has been suggested that T3 may act as a timing clock by pushing the neuroblasts out of the mitotic phase, and a role of nuclear receptor β1 in this effect is supported by the finding that β1 transcripts are predominantly found in zones of neuroblast proliferation (7). However, to our knowledge no studies on the effect of T3 in the expression of proteins involved in the regulation of the cell cycle in neural cells have been reported. To gain some insights into the mechanisms by which T3 regulates neuronal cell growth and differentiation, we have analyzed the effect of this hormone on the expression and activity of cell cycle-regulating molecules. Our results show that incubation of N2a-β cells with T3 leads to a rapid down-regulation of the c-myc gene, to a decrease of cyclin D1 levels, and to a sustained induction of the cyclin kinase inhibitor (CKI)1 p27Kip1. This increase is secondary to the augmented levels of p27Kip1 transcripts as well as to stabilization of the p27 protein. As a consequence of the increased levels of p27Kip1, the kinase activity associated with the cyclin-dependent kinase 2 (CDK2) complexes is inhibited and retinoblastoma protein (pRb) family members are hypophosphorylated in T3-treated N2a-β cells. This study shows for the first time that T3-mediated neuronal growth arrest and differentiation are associated with an increase in the levels of a CKI.

**EXPERIMENTAL PROCEDURES**

**Cell Cultures**—The clonal cell line Neuro-2a stably transfected with the β1 isofrom of the human thyroid hormone receptor (N2a-β cells) was grown as described previously in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% (v/v) T3-depleted serum (3).

**RNA Extraction and Hybridization**—Total RNA was extracted from the cell cultures with guanidine thiocyanate. The RNA was run in 1% formaldehyde-agarose gels and transferred to nylon-nitrocellulose membranes (Nytran) for Northern blot analysis. The RNA was stained with 0.2% methylene blue. The blots were hybridized with labeled mouse cDNA probes for c-myc, cyclin D1, or p27Kip1. Quantification of mRNA levels was carried out by densitometric scan of the autoradiograms. The values obtained were always corrected by the amount of RNA applied in each lane, which was determined by densitometry of the stained membranes.

**Immunoblotting**—The cells were washed in ice-cold phosphate-buffered saline, and lysed in Nonidet P-40 lysis buffer (0.1% SDS, 1% Nonidet P-40, 50 mM Tris-Cl, pH 8, 150 mM NaCl, 5 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, and 10 μg/ml apro- tinin). Protein concentrations were determined with Bio-Rad protein assay reagent. Aliquots containing 50 μg of lysate were boiled in 5× Laemmli sample buffer, and the proteins were separated by SDS-polyacrylamide gel electrophoresis. Proteins were transferred onto nitrocellulose membranes (Schleicher & Schüll), the membranes were then blocked with 5% nonfat milk in Tris-buffered saline and 0.1% Tween 20. This was followed by incubation with the corresponding diluted antibody for 1 h at room temperature. The antisera used were the following: anti-c-myc (N-262, sc-764), anti-cyclin D1 (9, sc-92), anti-p27kip1 (M-197, sc-776), anti-p130 (C-20, sc-137) purchased from Santa Cruz Biotechnology, and anti-Rb (14414A, PharMingen). After washing, the membranes were incubated for 1 h with an appropriate second-

* This research was supported by Grants PB94-0094 and PM97-0135 from the Dirección General de Enseñanza Superior. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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1 The abbreviations used are: CKI, cyclin kinase inhibitor; pRb, retinoblastoma protein; CDK2, cyclin-dependent kinase 2; DMEM, Dulbecco’s modified Eagle’s medium.
ary antibody, and the proteins were visualized by chemiluminescence (ECL, Amersham Pharmacia Biotech). Protein bands were analyzed by densitometry.

**Immunoprecipitation and Kinase Assay**—For immunoprecipitation, 500 μg of cell lysates were incubated overnight at 4 °C with 5 μl of anti-CDK2 antibody-agarose conjugated (M2-G, sc-163-G). The immunoprecipitates were separated by SDS-polyacrylamide gel electrophoresis, and revealed with the anti-p27 antibody. To measure kinase activity, immunoprecipitates were washed twice, resuspended in 40 μl of kinase reaction buffer (50 mM Tris-HCl, pH 7.5, 10 mM MgCl₂, 1 mM dithiothreitol, 0.1 mg/ml bovine serum albumin, and 50 μM ATP) containing 5 μg of histone H1 and 7 μCi of [γ-32P]ATP, and incubated for 30 min at 30 °C. The reactions were stopped by adding Laemmli sample buffer. The samples were boiled, and the proteins were separated by SDS-polyacrylamide gel electrophoresis. 32P-labeled histone H1 was detected by autoradiography and quantified by densitometry.

**33S-Pulse-labeling Chase Experiments**—After a 48-h incubation period in the presence or absence of 5 nM T3, the cells were detached from the culture plates and pulse-labeled for 1 h with a mixture of [35S]methionine and [35S]cysteine (Amersham Pharmacia Biotech) (1.5 μCi/10 ml) in methionine- and cysteine-deficient DMEM. Labeled cells were washed and incubated in DMEM supplemented with 150 μg/ml unlabeled amino acids. After a chase for 0, 3.5, and 7 h, the cells were washed and lysed. The lysates were immunoprecipitated with the anti-p27 antibody, and the immunoprecipitates analyzed by SDS-polyacrylamide gel electrophoresis. After fluorography, the amount of 33S incorporated into p27 was quantitated in an InstantImager (Packard Instruments Co.).

**Proliferation Assays**—N2a-β cells were transfected by calcium phosphate with 0.5 μg of cytomegalovirus β-galactosidase plasmid and 5 μg of pLPC (8) or pLPC-p27 (human p27 cDNA subcloned into the pLPC vector). The DNA precipitates were added to dishes containing coverslips. Cells were exposed to the DNA precipitates for 16 h, washed, and incubated in DMEM containing 1% T3-depleted serum for 48 h. Cells undergoing DNA synthesis were identified by incubation with the thymidine analogue bromodeoxyuridine (BrdUrd) at a final concentration of 10 μM for the last 4 h, and transfected cells were identified by staining for β-galactosidase. Cells were fixed with methanol/acetic acid/water (90:5:5, v/v) for 30 min at room temperature, stained with a 1:500 dilution of β-galactosidase antibody (Promega), and subsequently with a BrdUrd monoclonal antibody (Amersham Pharmacia Biotech) as suggested by the supplier. Fluorescein isothiocyanate-conjugated and rhodamine-conjugated goat anti-mouse antibodies were used as secondary antibodies to visualize β-galactosidase and BrdUrd-stained cells, respectively. Cells were then examined using fluorescence microscopy with the appropriate filters.

**RESULTS**

**T3 Inhibits c-Myc and Cyclin D1 Expression in N2a-β Cells**—T3 treatment of N2a-β cells strongly blocks DNA synthesis and causes an arrest of cells in G0/G1 (Ref. 3, and data not shown). Although the mechanisms connecting c-Myc function to cell cycle control are not well understood, different signals that arrest growth and elicit cell differentiation suppress c-Myc expression. As shown in Fig. 1A, the levels of c-Myc were markedly decreased in N2a-β cells incubated for 48 h in the presence of 5 nM T3. The reduction of c-Myc is a consequence of the decreased c-myc mRNA levels. Incubation with T3 causes a rapid decrease of c-myc transcripts in N2a-β cells. A maximal reduction (approximately 3-fold) was found only after 2 h of incubation with the hormone, and the levels of c-myc transcripts remained low for at least 72 h (data not shown). This is one of the most rapid effects of thyroid hormones on gene expression known. As shown in Fig. 1B, the levels of cyclin D1, another important component of cell cycle progression, were also strongly reduced after 48 h of T3 treatment. As in the case of c-Myc, a reduction of cyclin D1 transcripts was found, showing that T3 also alters cyclin D1 gene expression.

**Induction of p27Kip1 by T3**—Because CKIs are targeted by different growth-inhibitory and differentiation signals, the possibility that T3 could induce the expression of CKIs was also analyzed. In Fig. 2A Northern analysis of RNA from N2a-β cells indicated that p27Kip1 transcripts were induced by T3. Fig. 2B shows that this induction was already observed after 3 h, but the maximal effect was found at 24 h. p27Kip1 mRNA levels decreased thereafter, but were still higher than in the untreated controls after 96 h of incubation with T3. This regulation was specific for p27Kip1 because the level of p21Waf1 mRNA was not affected by T3 (data not shown). To analyze whether de novo protein synthesis was required for T3 induction of p27Kip1 transcripts, treatment with the hormone was performed in the presence and absence of 10 μg/ml cycloheximide. As shown in Fig. 2C treatment with T3 for 4 h caused a detectable increase of p27Kip1 transcripts. Incubation with cycloheximide alone was able to induce p27Kip1 mRNA levels, showing that this induction has characteristics similar to those found with different early response genes, which have transcripts with short half-lives in which labile proteins are implied. T3 was not able to induce further p27Kip1 transcripts in the presence of cycloheximide, indicating that likely activation of p27Kip1 represents an indirect effect of the T3 receptor, which requires previous synthesis of a protein or proteins. The increase of p27Kip1 transcripts could also be secondary to mRNA stabilization. To analyze this point, p27Kip1 mRNA levels were determined in control cells and in cells induced with T3 for 24 h and then incubated for varying times with 5 μg/ml of actinomycin D. Fig. 2D shows that p27Kip1 mRNA disappeared with a similar half-life (2 h) in both untreated and T3-treated N2a-β cells, showing that mRNA stabilization is not involved in the induction of p27Kip1 transcripts. To directly address the question of whether the increase of p27Kip1 transcripts by T3 is caused by an increase of p27 gene transcription, a p27Kip1 reporter construct containing a 1.6-kilobase upstream genomic fragment was used to transiently transfected N2a-β cells (9). Treatment with T3 did not increase p27Kip1 promoter activity. Similar results were obtained with plasmids containing shorter promoter fragments (data not shown). These results suggest that either p27Kip1 is not a transcription-
FIG. 2. T3 induces p27 transcripts. A, total RNA from N2a-β cells treated with and without 5 nM T3 for 24 h was hybridized with a p27Kip1 cDNA probe. The autoradiogram is shown in the top lane and the stained rRNA in the bottom lane. B, quantification of p27Kip1 mRNA levels in cells treated for various intervals with 5 nM T3. The autoradiograms were quantitated by densitometry, and the values obtained were corrected by the amount of RNA applied. Data are mean ± SD values obtained from three independent samples, expressed as fold-induction over the values obtained in control untreated cells. C, Northern blot analysis of RNA from cells treated for 4 h with T3 in the presence or absence of 10 μg/ml cycloheximide (CHX). Quantification of the corrected p27Kip1 mRNA expressed as the percentage obtained in the untreated cells is shown below the autoradiogram. D, determination of the half-life of p27Kip1 transcripts in control and in T3-treated cells. The cells were first incubated in the presence and in the absence of T3 for 24 h. The cultures were then washed (time 0) and incubated with medium containing 5 μg/ml of actinomycin D. At the times indicated, RNA was extracted from duplicate cultures of control and T3-treated cells. The data are expressed as percentages of the corresponding level of p27Kip1 mRNA obtained at time 0.

ally responsive target gene of the T3 receptor, or that the sequences responsible for the transcriptional effect are outside of the promoter fragments examined.

Fig. 3A shows that p27Kip1 protein levels were markedly induced in N2a-β cells incubated with the hormone for different time periods. Quantification of different blots revealed that treatment with 5 nM T3 between 24 and 96 h increased by 8–9-fold the cellular levels of p27Kip1. It has been described that different antiproliferative signals regulate p27Kip1 accumulation by posttranslational mechanisms (10). To determine whether p27Kip1 stabilization could also contribute to the elevated p27Kip1 levels found in T3-treated cells, the incorporation of [35S]methionine and [35S]cysteine into p27Kip1 and the half-life of the labeled protein was determined. As illustrated in Fig. 3B, the half-life of the p27Kip1 protein was much shorter in the untreated N2a-β cells than in the cells treated with T3. Whereas in control cells [35S]-p27Kip1 disappears with an apparent half-life of approximately 5 h, most of the label remained after a 7 h chase in T3-treated cells. Thus, protein stabilization clearly contributes to the accumulation of p27Kip1 protein observed in the cells incubated with T3.

Overexpression of p27Kip1 Blocks Proliferation of N2a-β Cells—To analyze whether an increase in the expression of p27Kip1 protein is able to block cell cycle progression in N2a-β cells, DNA synthesis was determined in cells that overexpress p27Kip1. For this purpose, the cells were transfected with an expression vector encoding p27Kip1. Cotransfection with a plasmid expressing a β-galactosidase gene was used to identify the transfected cells, and cells that entered S-phase were identified by staining for BrdUrd incorporation. Staining with β-galactosidase showed that approximately 20–25% of the total cell population was successfully transfected. As shown in Table I, expression of p27Kip1 caused a dramatic decrease in the percentage of cells in S-phase. Whereas in N2a-β cells transfected with an empty noncoding vector, a large fraction (more than 70%) of the β-galactosidase-positive cells were BrdUrd positive, only 14% of the cells transfected with the p27Kip1-expressing plasmid entered S-phase.

T3 Increases Association of p27Kip1 with CDK2 and Inhibits Kinase Activity—CDK inhibitors are thought to prevent cell proliferation by interaction with cyclin-CDK complexes. To elucidate the significance of the induced p27Kip1 by T3, we studied the complex formation of p27Kip1 with CDK2. As shown in Fig. 4, the levels of CDK2 did not change after incubation of N2a-β cells with T3. However, the amount of p27Kip1 precipitated with anti-CDK2 antibody increased significantly in T3-treated cells concomitantly with the increased content of total p27Kip1 under this condition. It was expected that association of CDK2 with p27Kip1 could result in an impaired kinase activity. Thus, we next examined this activity in the immunoprecipitates using histone H1 as a substrate. Fig. 5A shows that CDK2-associated kinase activity was indeed strongly reduced by T3 in N2a-β cells.

pRB and p130 Are Hypophosphorylated in T3-treated N2a-β Cells—The effects of T3 on pRb family proteins were investigated by immunoblotting. Fig. 5B shows the results obtained with anti-pRB antibody that recognizes specifically the underphosphorylated forms of the protein. These forms were almost undetectable in control N2a-β cells, and T3 produced a significant accumulation of unphosphorylated pRB. In addition, the hormone induced a shift from the slower migrating hyperphosphorylated p130 found in control N2a-β cells to their faster migrating hypophosphorylated forms. These results are consistent with the requirement of CDK2 activity for phosphorylation of pRb and p130 (11, 12), and with the finding that during neuronal differentiation pRB hypophosphorylation is concomitant with the loss of CDK2 activity (13).

DISCUSSION

Our results show that T3-mediated growth arrest and differentiation of neuroblastoma cells is associated with hypophosphorylation of the retinoblastoma protein pRB and the related protein p130. The pRB family members appear to be essential for neurogenesis and are highly expressed in cell types un-
going neuronal differentiation, whereas loss of pRb affects the ability of neurons to differentiate properly (14–16). One of the molecular events required for cell cycle progression is the inactivation by hyperphosphorylation of pRb family proteins (12,17). Therefore, T3 maintains pRb family proteins in their active form. Under these conditions, they associate with E2F/DP factors and repress transcription of target genes required for progression through the restriction point in the cell cycle (18).

The hyperphosphorylation of pRb family members is catalyzed by the CDKs. It has been shown that during neuronal differentiation the loss of pRb phosphorylation correlates with loss of activity of the pRb kinases CDK2 (associated with cyclins A or E) and CDK4 (associated with D-type cyclins) (13). Our results in neuroblastoma cells demonstrate that T3 causes a strong reduction of CDK2 activity without altering the cellular levels of CDK2. The activities of the CDKs are regulated by various mechanisms including dimerization with cyclins, phosphorylation, and association with a group of inhibitory proteins called CKIs (19, 20). The Cip/Kip family of CKIs, which are targeted by different growth-inhibitory and differentiation signals, includes p21Waf1 (21), p27Kip1 (22, 23), and p57Kip2 (24) that bind to and inhibit all G1-cyclin-CDK complexes. It has been described that during differentiation of neuroblastoma cells with Me2SO, the activities of CDK2 and CDK4 decline by association with p27Kip1 (13) and that differentiation by nerve growth factor and an inhibitor of DNA polymerases is also accompanied by the expression of p21Waf1, another CKI that inhibits cyclin E-associated kinase activity and is required for neuroblastoma cell survival (25). Moreover, neuronal differentiation can be induced by overexpression of p27Kip1 or pRb, suggesting that inhibition of CDK activity and pRb phosphorylation are the major determinants for neuronal differentiation and survival (13, 25). In agreement with the important role of CKIs on neuronal differentiation, we find that T3 induces a strong and sustained increase of the levels of p27Kip1 in N2a-β cells.

Table I

| Vector      | BrdUrd Positive Cells (%) |
|-------------|---------------------------|
| pLPC        | 73 ± 5                    |
| pLPC-p27Kip1| 14 ± 2                    |

FIG. 3. T3 induces the cellular levels of p27Kip1 and increases p27Kip1 half-life. A, lysates from cells treated with 5 nM T3 for various periods were analyzed by immunoblotting with anti-p27Kip1 antibody. The top panel shows a representative blot, and the bottom panel illustrates the quantification obtained from three independent experiments. B, control cells and cells incubated for 48 h with T3 were pulse-labeled for 1 h with [35S]methionine and [35S]cysteine and subsequently chased with an excess of nonradioactive amino acids for the times indicated. Cells were lysed, extracts were normalized for equal counts per minute, and p27Kip1 immunoprecipitated. The autoradiogram of the immunoprecipitates is shown in the top panel, and quantification of the radioactivity of each band expressed as the percentage of the corresponding value found at time 0 is shown in the bottom panel.

FIG. 4. T3 increases the association of p27Kip1 with CDK2. N2a-β cells were cotransfected with the CMV β-galactosidase plasmid and pLPC-p27Kip1 vector, which directs the expression of p27Kip1, or with the pLPC vector as a negative control. Transfected cells were analyzed by fluorescence microscopy and the percentage of BrdUrd-positive cells was determined in β-galactosidase-positive cells. Data are the average ± S.D. of counting at least twenty different microscopy fields from two separate experiments.
p27Kip1 mRNA levels in N2a-β cells is found as soon as 3 h after treatment with the hormone, which suggests a direct effect on p27Kip1 gene transcription. Transcriptional effects of thyroid hormones are mediated by binding of nuclear receptors to hormone-response elements located normally in the promoter region of regulated genes and several potential nuclear receptors binding sequences are present in the 5'-flanking region of the p27Kip1 gene. However, we did not identify a functional response element, because T3 did not increase the activity of the p27 promoter in N2a-β cells. Moreover, T3 was not able to induce p27Kip1 transcripts above the levels found in the presence of cycloheximide, suggesting the requirement of de novo protein synthesis for this induction. This suggested that T3 could increase p27Kip1 expression primarily by stabilization of the gene transcripts, but the hormone did not increase the half-life of p27Kip1 mRNA, which was approximately 2 h, regardless of the treatment. The short half-life as well as the implication of labile proteins in the mRNA turnover demonstrated by the induction in the absence of protein synthesis could explain the rapid induction by T3. The findings that the induction in p27Kip1 levels by T3 was more pronounced than that of p27 mRNA levels, and that p27 transcripts decline between 24 and 72 h of incubation with T3, when the protein levels are maximally induced, suggested that p27Kip1 stabilization could also contribute to the elevated p27Kip1 levels. This fact was demonstrated by pulse-chase experiments, which showed that T3 significantly increased p27Kip1 half-life in N2a-β cells.

It has been reported that accumulation of p27Kip1 protein is not sufficient on its own to arrest the cell cycle or to induce differentiation in oligodendrocytes (33). In this cell model, the levels of p27Kip1 progressively increase in the precursor oligodendrocytes as they proliferate, but despite high levels of the inhibitor the cells tend to keep dividing and not differentiate in the absence of hydrophobic signals such as T3. In contrast, our results clearly show that overexpression of p27Kip1 is sufficient in itself to trigger an exit from the cell cycle in N2a-β cells. Our results are in agreement with the finding that expression of p27Kip1 blocks cell cycle progression in G1 in different cell lines tested (17).

Although the hypophosphorylation of pRb and p130 caused by T3 is most likely secondary to the increase in p27Kip1 and the consequent inhibition of CDK2 activity, cyclin D-dependent kinases also induce pRb hyperphosphorylation (34). Cyclin D is rate limiting for the G1/S transition, and it has been reported that expression of cyclin D1 advances the G1/S transition and leads to the immediate appearance of hyperphosphorylated pRb. Induction of cyclin D1 promotes cell cycle progression not only by activating CDKs but also by sequestering p27Kip1 (35–37). Because T3 reduces cyclin D1 expression, this decrease as well as the induction of p27Kip1 might contribute to maintain pRb family proteins in their hypophosphorylated state.

p27Kip1 and the c-myc proto-oncogene generally have opposite roles in cell growth control, and c-Myc antagonizes the growth arrest induced by p27Kip1 (38). Expression of c-myc is suppressed by different growth-inhibitory or differentiation signals (39). Therefore, it was not surprising that T3 reduced c-myc gene expression in N2a-β cells. It has been shown that c-Myc induces an as yet unknown cellular activity leading to sequestration of p27Kip1, derepression of cyclin/CDK activity and pRb hyperphosphorylation and thus allowing the escape from the restriction point control in the cell cycle (38). Suppression of c-Myc levels by T3 assuredly contributes to growth arrest and differentiation of neuroblastoma cells.

In summary, our results indicate that T3 coordinately regulates the expression of several genes that play a key role in cell cycle control and differentiation of neuronal cells. The induction of a CKI, which inhibits the activity of cyclin-dependent kinases and does not allow progression through the restriction point in the cell cycle, provides a mechanism to explain the crucial effects of thyroid hormones on neuroblast growth and differentiation in the developing brain.

Acknowledgments—We thank J. L. Jorcano, J. Paramio, and A. A. Nordin for materials used in this study.

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J. Biol. Chem. 1999, 274:5026-5031.
doi: 10.1074/jbc.274.8.5026

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