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Cell Cycle-dependent Expression of Thyroid Hormone Receptor-β Is a Mechanism for Variable Hormone Sensitivity[D]

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Thyroid hormone receptors (TRs) are ligand-regulatable transcription factors. Currently, little is known about the expression of TRs or other nuclear hormone receptors during the cell cycle. We thus developed a stable expression system to express green fluorescent protein-TRβ in HeLa cells under tetracycline regulation, and studied TR expression during the cell cycle by laser scanning cytometry. Only ~9–15% of the nonsynchronized cell population expressed TR because the majority of cells were in G_1 phase and did not express detectable amounts of TR. However, when cells were synchronized in early S phase with hydroxyurea and then released, TR expression levels increased in a cell cycle-dependent manner and peaked to 30–40% cells expressing TR at late G_2/M phase before declining to nonsynchronized levels. Moreover, we observed a direct correlation between transcriptional activity and TR expression during the cell cycle. Similar cell cycle-dependent findings also were observed for endogenous TR in rat pituitary GH_3 cells. Last, cycloheximide studies demonstrated that the increase in TR expression was primarily due to increased translation. These novel observations of cell cycle-dependent expression of TR suggest that differential hormone sensitivity can occur during the cell cycle and may contribute to cell cycle progression during normal development and oncogenesis.

INTRODUCTION

Thyroid hormone receptors (TRs) belong to a superfamily of transcription factors that include the retinoic acid steroid vitamin D and peroxisomal proliferator activator receptors (McKenna *et al*., 1999; Yen, 2001). There are two major TR isoforms, TRα and TRβ. TRs bind to thyroid hormone response elements (TREs) in the promoters of target genes and regulate their transcription. In the absence of hormone, TRs bind to corepressors such as nuclear receptor corepressor or silencing mediator for retinoic and thyroid hormone receptors, and repress basal transcription by recruiting histone deacetylases, and modifying chromatin structure (Xu *et al*., 1999). In the presence of T_3, corepressor complexes are released from TRs, and coactivator complexes are recruited, leading to increased local histone acetylation and transcriptional activation.

TRs play a vital role during embryonic development and metamorphosis (Sachs *et al*., 2002), and it has been suggested that TR recruitment of corepressors may be a means for suppressing gene expression during metamorphosis. T_3 also has been shown to be mitogenic and is required for cell growth and development in certain cell lines (DeFesi *et al*., 1984; Humes *et al*., 1992; Quintanar-Stephano and Valverde, 1997; Di Fulvio *et al*., 2000). Additionally, T_3 can regulate cell proliferation and differentiation (Robson *et al*., 2000; Miura *et al*., 2002). Recently, it has been shown that human TRβ1 interacts with p53, a tumor suppressor, which plays a critical role in cell cycle regulation and tumorigenesis (Yap *et al*., 1996). TR binding to p53 leads to decreased p53-dependent induction of bax and gadd45 expression but does not affect the expression of p21; suggesting TR can differentially modulate p53-target genes (Barrera-Hernandez *et al*., 1998). This cross talk between two cell-signaling pathways could play an important role in both normal and transformed cells. In this connection, mutant thyroid hormone receptors with defective function have been found in hepatic, renal, and thyroid cancers as well as thyrotropin-secreting pituitary tumors (Lin *et al*., 1996; Ando *et al*., 2001; Yen and Cheng, 2003).

Previous studies suggest that T_3 binding activity changes during the cell cycle; however, these early studies did not directly examine TR mRNA or protein expression during the cell cycle (DeFesi *et al*., 1982; Surks and Kumara-Siri, 1984; Kumara-Siri and Surks, 1985; Kumara-siri *et al*., 1988). Glucocorticoid receptor expression and hormone sensitivity also vary during different stages of the cell cycle (Hsu *et al*., 1992; Hsu and DeFranco, 1995). Moreover, the antiproliferative effects of the estrogen receptor antagonist tamoxifen change in a cell cycle-dependent manner in MCF-7 cells (Dong *et al*., 1991, 1992).

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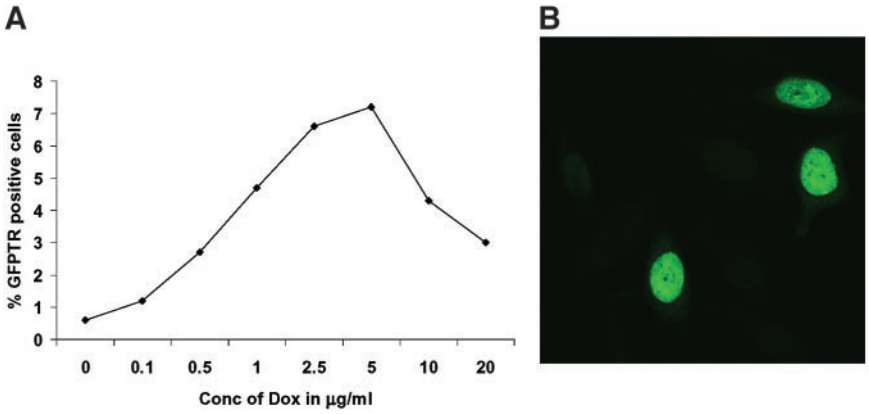

Figure 1. Expression of GFPTR in the presence of doxycycline. (A) Dose-dependent expression of GFPTR in GFP-HeLa cells in the presence of doxycycline. The percentage of cells expressing GFPTR was observed by LSC in the presence of varying concentrations of Dox (0.1–10 μg/ml). (B) Confocal image of GFPTR-expressing cells in the presence of Dox (2.5 μg/ml).

Recently, we used confocal microscopy to study the nucleocytoplasmic shuttling and intranuclear distribution of TRs and other nuclear hormone receptors (NRs) in HeLa cells transfected with vectors expressing green fluorescent protein-NR chimeras. We observed an energy-dependent nuclear uptake of NRs as well as an intranuclear redistribution of NRs upon ligand addition (Baumann *et al*., 2001; Maruvada *et al*., 2003). Currently, there is little information on the intranuclear expression or distribution of NRs during the cell cycle. To address this issue, we established a permanently transfected cell line expressing green fluorescent protein thyroid hormone receptor (GFPTR) and used laser scanning cytometry (LSC) to observe that TR expression and distribution, as well as transcriptional activity, changes during different stages of the cell cycle. Our data thus provide new insights into some of the cellular and molecular events that account for variable hormone sensitivity by NRs during the cell cycle.

MATERIALS AND METHODS

Plasmids and Vectors

Rat TRβ was cloned as a green fluorescent protein fusion into TRE vector (BD Biosciences Clontech, Palo Alto CA). Briefly TRE vector was restriction digested with *Sac*II and then filled in with Klenow fragment followed by treatment with *Eco*RI, whereas the GTR was cut with Eco 47 III and *Eco*RI and ligated with TRE vector.

Stable Cell Lines

HeLa-Teton cells were transfected with GFPTR-TRE vector, and the clones were selected for hygromycin B resistance marker. The clones were finally selected by FACS sorting for positive GFP-expressing cells in the presence of doxycycline (2.5 μg/ml). The stable cells were regularly maintained in DMEM with 10% fetal bovine serum and antibiotics (100 U/ml penicillin and streptomycin, 0.5 mg/ml gentamicin; 0.1 mg/ml) G418 and 0.3 mg/ml hygromycin B as resistance markers (Invitrogen, Carlsbad, CA) and 2 mM L-glutamine in 5% CO_2 incubator at 37°C.

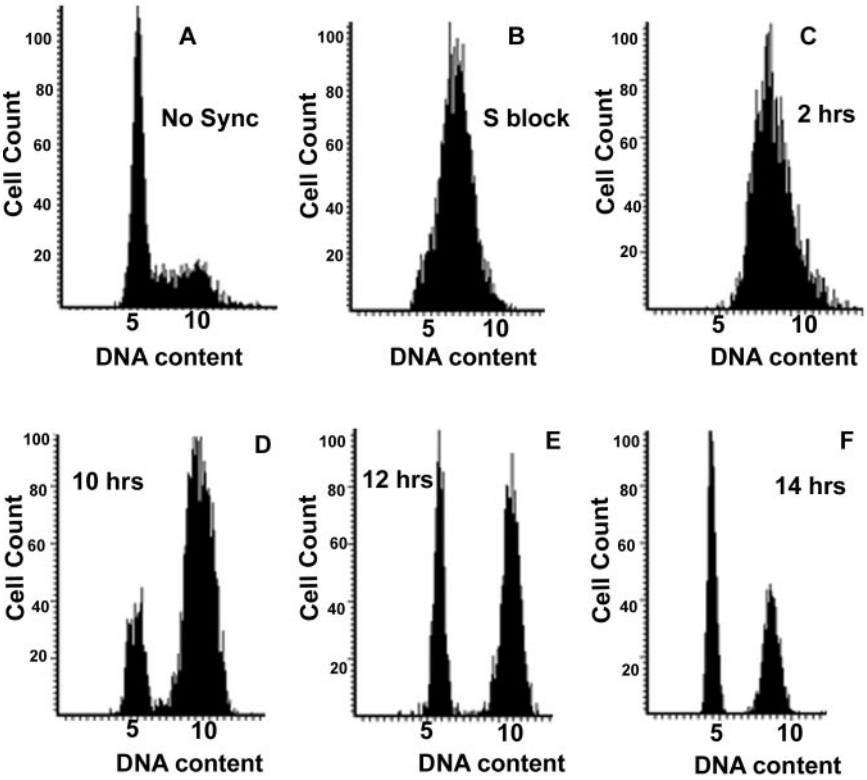

Figure 2. Cell cycle analysis of synchronized GFPTR-HeLa cells (A) unsynchronized GFPTR-expressing cells. (B) GFPTR-expressing cells inhibited in S phase by blocking the cells with hydroxyurea. (C) GFPTR-expressing cells 2 h after release into cell cycle progression by addition of fresh medium. (D) GFPTR-expressing cells 10 h after release into cell cycle. (E) GFPTR-expressing cells 12 h after release into cell cycle. (F) GFPTR-expressing cells 14 h after release into cell cycle.

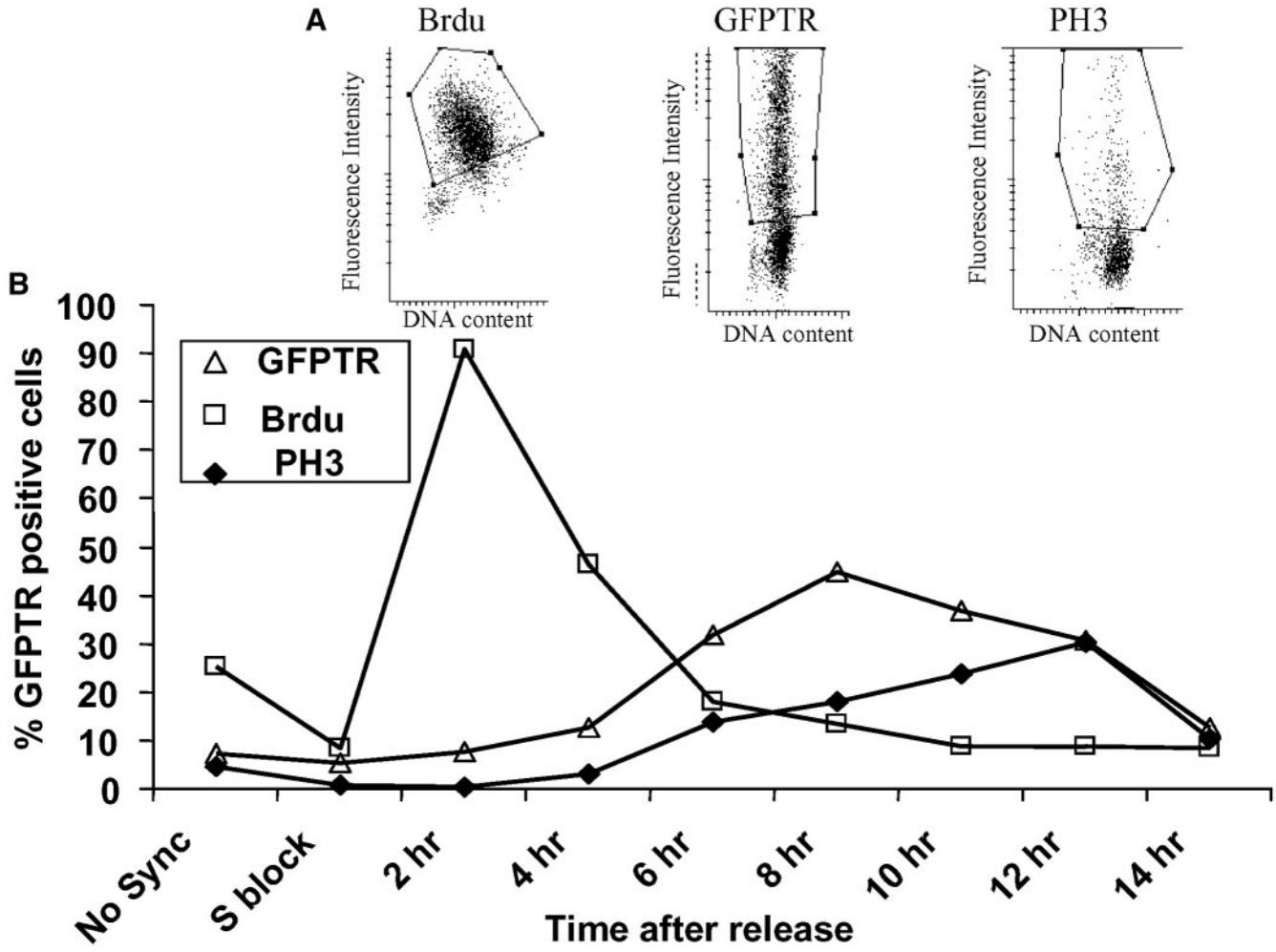

Figure 3. Expression of GFPTR during G_2/M phase of the cell cycle. (A) Representative laser scanning cytograms, as described in MATERIALS AND METHODS; a, GFPTR-expressing cells 2 h after release from S block by hydroxyurea. Cells in S phase are highlighted by anti-BrdU antibody immunofluorescence staining. b, GFPTR-expressing cells 10 h after release from S block by hydroxyurea. Cells expressing GFPTR are highlighted. c, GFPTR-expressing cells 12 h after release from S block by hydroxyurea. Cells undergoing mitosis are highlighted for phosphohistone antibody immunofluorescence staining. (B) Percentage of fluorescent-positive cells by the foregoing methods. Triangles, GFPTR-expressing cells, squares, BrdU-positive cells (S phase), and diamonds, phosphohistone-positive cells (mitosis).

Cell Cycle Synchronization

Exponentially growing cells on coverslips were synchronized by the addition of hydroxyurea (2 mM final concentration) (Sigma-Aldrich, St. Louis, MO) for 16 h at early S phase and released them into S phase by the addition of fresh medium without hydroxy urea and in the presence of doxycycline (Sigma-Aldrich). Cells were harvested at specified time intervals and processed for LSC, confocal microscopy, or analyzed proteins by Western blotting.

LSC

Cells were fixed with 100% methanol at –20°C for 30 min. The fixed cells were washed with phosphate-buffered saline (PBS) three times, permeabilized with 0.1% Triton X-100 for 10 h followed by RNase (1 mg/ml) (Roche Diagnostics, New York NY) treatment for 15 h at room temperature. Finally the cells were treated with propidium iodide (PI) (10 μg/ml) (Sigma-Aldrich) for 5 min and then mounted with 150 μl of antifade (Molecular Probes, Eugene OR.). The cell cycle was analyzed by a laser scanning cytometer (Compucyte, Cambridge MA) by measuring the total PI fluorescence and the peak intensity of fluorescence in the cell nuclei (Dmitrieva *et al.*, 2000, 2001). Total nuclear PI fluorescence is the integral of fluorescence calculated over the entire area of a nucleus and corresponds to DNA content. GFPTRβ expression was measured in the fluorescein isothiocyanate (FITC) channel with 15% laser, and the total FITC fluorescence and the peak intensity were measured. A gate representing the approximate limit of peak fluorescence in cells with negligible GFPTR expression was determined by visual inspection. The data were displayed as bivariate cytograms, plotting peak green fluorescence versus total PI fluorescence in nucleus or particle to determine the number of GFPTR-positive cells (representative cytogram is shown on Figure 3A). For cell cycle distribution analysis, the data were plotted as histograms showing amount of cells with different DNA content (Figure 2).

Analysis of Cells in S Phase of Cell Cycle

To label S-phase cells, they were incubated with 10 μM/ml 5-bromo-2-deoxyuridine (BrdU) for 1 h before fixation. Cells were immunostained with anti-BrdU antibody (FITC-green). DNA was stained with PI (red) and slides were analyzed by LSC. Red Integral fluorescence over the nuclear area was recorded as a measure of PI staining as a direct measure of DNA content. Green integral fluorescence over the nuclear area was recorded as a measure of BrdU content. The data were plotted as bivariate cytograms showing BrdU content versus DNA content (representative cytogram is shown on Figure 3B). The area containing BrdU-positive cells is drawn by eye, and the percentage of cells in this area is determined using WinCyte software. The BrdU cells were viewed by confocal microscopy, and images in both FITC and PI channels were collected simultaneously using TCS NT software and processed by Adobe Photoshop 6.0.

Analysis of Cells in Mitosis of Cell Cycle

To find cells in mitosis slides were immunostained with anti-phospho histone H3 antibody (mitotic marker) (Upstate Biochemicals, Waltham, MA) and probed with FITC-labeled secondary antibody. DNA was stained with PI (red) and slides were analyzed by LSC. Red integral fluorescence over the

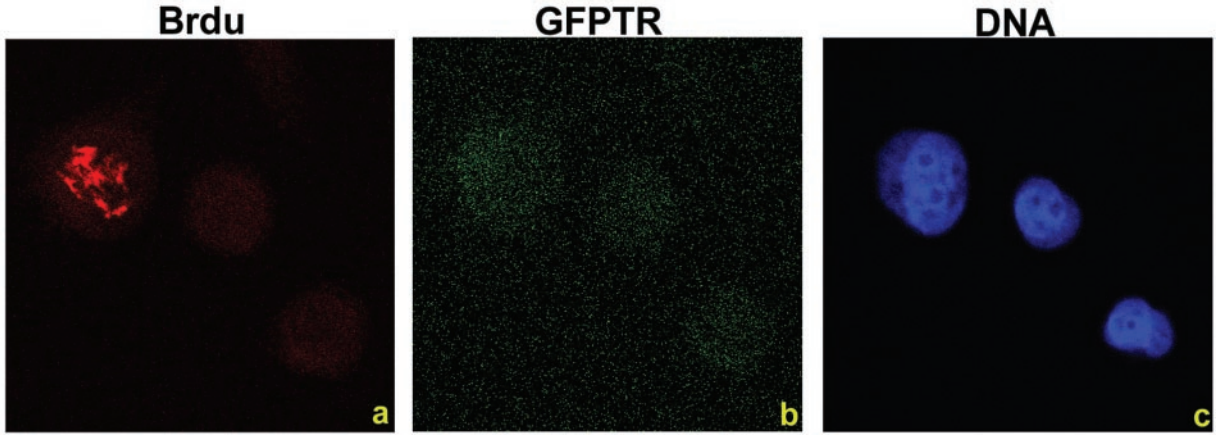

Figure 4. Lack of GFPTR expression during S phase of cell cycle. Cells were synchronized with hydroxyurea for 16 h. One hour before removing the block, the cells were incubated with 1 μg/ml BrdU. At the end of the block, the cells were washed, and fresh medium without hydroxyurea was added. The cells were fixed at 2-h intervals and processed for immunofluorescence by using anti-BrdU antibody followed by Texas Red-labeled anti-mouse IgG secondary antibodies and counterstained with DAPI for DNA staining. The cells were analyzed by confocal microscopy. Images of cells harvested at the end of 2 h after release are presented here. a, BrdU-positive cells labeled with Texas Red in red. b, GFPTR expression in green. c, DAPI staining in blue channels.

nuclear area was recorded as a measure of PI DNA binding (DNA content). Green integral fluorescence over the nuclear area was recorded as a measure of P-H3 amount (P-H3 content). The data were plotted as bivariate cytograms showing P-H3 content versus DNA content (representative cytogram is shown on Figure 3A). The area containing P-H3–positive cells is drawn by eye, and the percentage of cells in this area is determined using WinCyte software. The P-H3 cells were viewed by confocal microscopy and images in both FITC and PI channels were collected simultaneously using TCS NT software and processed by Adobe Photoshop 6.0.

Western Blot Analysis

HeLa-Tet-on cells or GH3 were synchronized with hydroxyurea for 16 h and released into cell cycle progression by adding fresh medium. The cells were harvested at 2-h time intervals and the isolated proteins analyzed by Western blotting by using anti-TRβ antibody (Affinity Bioreagents, Golden, CO) followed by horseradish peroxidase-tagged secondary antibody and detected by enhanced chemiluminescence method. For treatment with cycloheximide, the cells were treated with cycloheximide (50 μg/ml) for specified time intervals, and the cells were harvested and proteins were analyzed by Western blotting.

Reverse Transcription-Polymerase Chain Reaction (RT-PCR) Analyses

Total RNA was isolated from synchronized and released HeLa-Tet-on cells and analyzed by RT-PCR by using primers specific for TRβ1 (Baumann *et al.*, 2001).

RESULTS

Doxycycline (Dox)-dependent Expression of GFPTR

The dose-dependent expression of GFPTR by doxycycline, a stable analog of tetracycline, was observed by LSC (Figure 1). In the absence of doxycycline, there were <1% cells expressing GFPTR, indicating the Tet-On system was tightly regulated in an antibiotic-specific manner. As the concentration of doxycycline increased, there was a dose-dependent increase in GFPTR-expressing cells, which reached maximal levels at 2.5–5.0 μg/ml, beyond which the percentage of expressing cells declined due to antibiotic toxicity. On the basis of these data, all subsequent studies were conducted at 2.5 μg/ml doxycycline. Of note, only 5–10% of the cell population expressed GFPTR even in the presence of optimal concentrations of doxycycline. The mechanism for this limited expression is not known; however, it is possible that cell cycle-dependent differences in GFPTR expression could potentially account for the limited expression within the cell population of the GFPTR clonal cell line.

Cell Cycle Analysis of HeLa-GFPTR Cell Line

To examine this possibility, GFPTR cells were synchronized with hydroxyurea for 16 h and released into the cell cycle by the addition of fresh medium without hydroxyurea. Cells then were harvested and sorted according to their cell cycle phase by LSC (Figure 2). The majority of nonsynchronized cells was in G_1 phase and occurred as a major peak on the cytogram (Figure 2A). When the cells were synchronized with hydroxyurea, they were arrested at the beginning of S phase and showed as one peak (Figure 2B). They then progressed into S phase after 2 h as the peak shifted toward the right (Figure 2C). After 10 h, a major G_2 peak occurred in the cytogram (Figure 2D), indicating that most of the cells were in the G_2/M phase of the cell cycle. After 12 h, there were two nearly equal populations of G_1 and G_2/M observed on the cytogram (Figure 2E). Cells finally completed one round of the cell cycle after ~14 h (Figure 2F). Addition of T_3 did not change the progression of these cells through the cell cycle (our unpublished data).

Cell Cycle-dependent Expression of GFPTR

The expression of GFPTR under synchronized conditions was studied using markers for different stages of the cell cycle (Figure 4). More than 90% of the cell population was in S phase 2 h after release as determined by BrdU staining, which then decreased to <10% 6 h afterward (squares). Phosphohistone staining (marker for mitosis) increased after 6 h and peaked at 12 h, indicating that most cells were in mitosis during this time (triangles). Interestingly, GFPTR expression increased at 4 h and peaked at 8 h before decreasing to <10% at 14 h (diamonds). In conjunction with Figures 2 and 3 as well as previous studies examining DNA cell content during the cell cycle (Dmitrieva *et al.*, 2002), these results indicate that increased cell expression of TR started in late S phase and peaked in G_2 phase of the cell cycle before finally decreasing to baseline levels in G_1 phase after 14 h. Addition of T_3 did not change significantly the expression pattern or amount in these cells (data not shown).

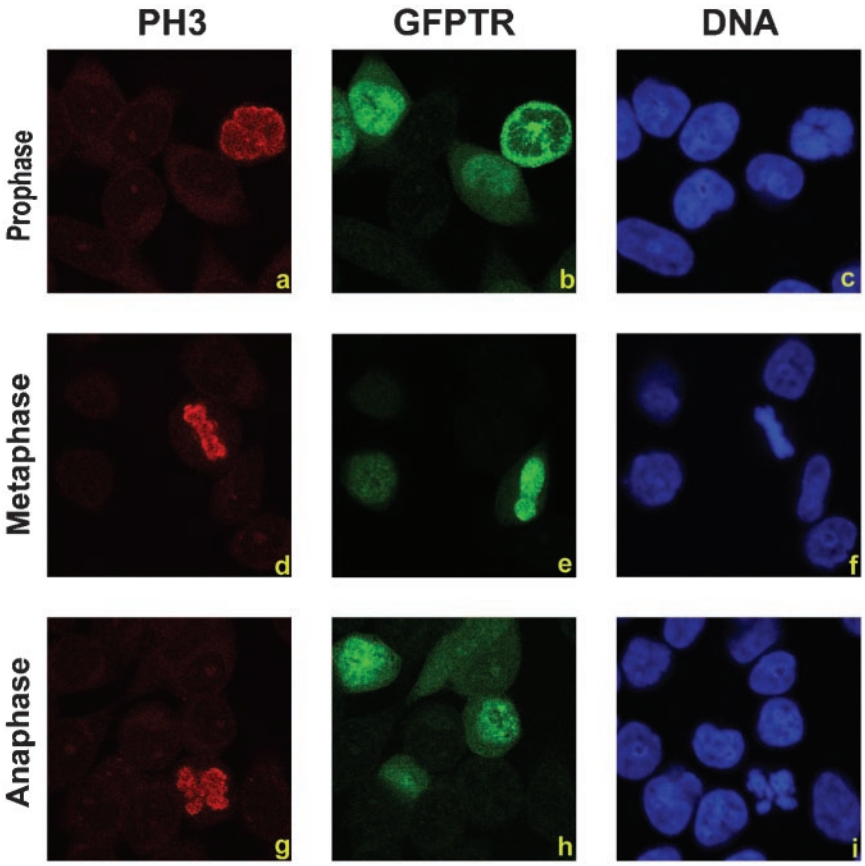

Figure 5. GFPTR expression during early mitosis. Cells were synchronized with hydroxyurea for 16 h. At the end of the block, cells were washed with PBS, and fresh medium was added. The cells were fixed at 2-h intervals and processed for immunofluorescence by using anti-phosphohistone antibodies, which specifically recognize mitotic cells, followed by Texas Red-labeled anti-mouse IgG antibodies and counterstained with DAPI for DNA content. The cells were analyzed by LSC simultaneously. Images of cells harvested between 10 and 12 h after release are presented here. PH3-positive cells are stained in red, GFPTR expression in green, and DAPI staining in blue channels, respectively. a, d, and g show red channel imaging of PH3-positive cells. Cells are in prophase metaphase and anaphase, respectively (circled). b, e, and h show green channel imaging of GFPTR-expressing cells in prophase, metaphase, and anaphase, respectively. c, f, and i show blue channel imaging of DAPI-positive staining cells in prophase, metaphase, and anaphase, respectively.

GFPTR Is Not Expressed during S Phase

To determine whether GFPTR is expressed in S phase, immunofluorescence studies were performed on GFPTR cells with anti-BrdU antibodies, which specifically stain cells in S phase of the cell cycle. Under these conditions, there was no concomitant green fluorescence with BrdU staining, indicating that GFPTR was not expressed in S phase (Figure 4, a and b). DNA counterstaining also was performed with 4,6-diamidino-2-phenylindole (DAPI) (Figure 4c).

GFPTR Is Expressed in Early Mitosis

The expression of GFPTR was studied along with immunofluorescent staining with anti-phosphohistone antibody, which stains for mitotic cells. Prophase cells had both positive phosphohistone staining and GFPTR expression (Figure 5, a and b). Interestingly, both metaphase and anaphase cells did not express GFPTR to any significant extent (Figure 5, d, e, g, and h). These findings suggest that GFPTR expression disappeared between prophase and metaphase. Additionally, intranuclear GFPTR was expressed along the nuclear membrane in prophase, in contrast to the more diffuse, homogenous pattern observed in G_2. These findings demonstrate cell cycle-dependent redistribution of TRs. DNA counterstaining with DAPI (Figure 5, c, f, and i) confirmed the immunofluorescent staging of the cells.

Transactivation by GFPTR during G_2 Block

We examined whether T_3-mediated transcriptional activity could be modified during the cell cycle by changes in TR expression levels. Accordingly, the transactivation by GFPTR was studied by synchronizing the cells in G_2 and transfecting a TRE luciferase reporter gene into the GFPTR cell line. We previously showed that GFPTR had similar T_3-mediated transcriptional activity as wild-type TRβ in cotransfection studies (Baumann *et al.*, 2001). The cells were synchronized with hydroxyurea after 24 h and released into S phase by adding fresh medium. When the cells were about to reach the G_2/M phase, they were blocked with nocodazole, which inhibits cell mitosis. Under these conditions, the transcriptional activity of GFPTR was studied in the presence and absence of T_3 by analyzing the luciferase activity in the lysates after 24 h. As shown in Figure 6, the T_3-mediated transcriptional activity was further increased by almost twofold, suggesting that increased transcriptional activity occurred when GFPTR expression was maximal.

Posttranscriptional Regulation of GFPTR Expression

The increased expression of GFPTR during G_2 phase of the cell cycle could be due to increased mRNA expression and/or increased protein synthesis. To determine the cause for increased GFPTR expression, we studied the protein expression of GFPTR by Western blot analysis and its mRNA expression by RT-PCR (Figure 7). Protein expression patterns were similar to those obtained by LSC, and thus provided further evidence for cell cycle changes in TR expression (Figure 7A). However, there was no significant change in the mRNA expression (Figure 7B). Together, these findings suggest that posttranscriptional changes primarily regulate the expression of GFPTR during the cell cycle.

Western blotting analysis also was performed on extracts of GH_3 cells, which express endogenous TRβ. Endogenous TRβ expression was low during S phase and increased dur-

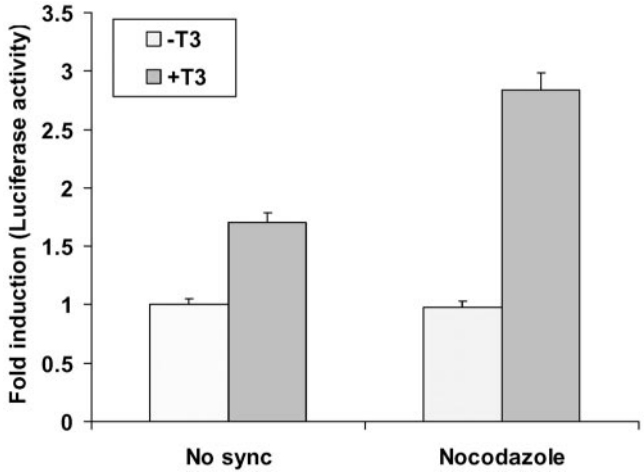

Figure 6. Transcriptional activity of GFPTR-expressing cells in G_1 and G_2/M phases. GFPTR-expressing cells were transfected with TRE luciferase reporter gene by LipofectAMINE method. After 24 h, cells were synchronized with hydroxyurea for 16 h. At the end of the block the cells were washed with PBS and fresh medium added. At the end of 4–6 h, when the cells moved into G_2 phase of cell cycle, nocodazole (0.5 ng/ml) was added to block cells at G_2/M. The cells were incubated for 24 h with or without T3, harvested, and assayed for luciferase activity as described in MATERIALS AND METHODS. Luciferase activity in the absence and presence of T3 is shown. Dotted light bars, luciferase activity in the absence of T3; striped dark bars, luciferase activity in the absence of T3.

ing G_2 (Figure 8). Thus, the endogenous TRβ expression pattern during the cell cycle in GH_3 cells was similar to that observed in GFPTR cells. In both Figures 7 and 8, the TRβ protein levels were low at time 0 as the majority of cells were in G1 phase and TRβ is not significantly expressed during this phase (Figures 2 and 3). Additionally, as observed in Figure 7, we did not observe significant changes of endogenous TRβ mRNA expression during the GH_3 cell cycle (our unpublished data).

New Protein Synthesis Required for GFPTR Expression

The increased GFPTR protein expression may be due to increased protein synthesis or decreased protein degradation, or to a combination of both these processes. To determine whether new protein synthesis is required for the increased levels of GFPTR during the cell cycle, cells were treated with cycloheximide 4 h after release, and their expression determined by both Western blot analysis (Figure 9A) and LSC (Figure 9B). These results suggest that increased GFPTR expression is primarily due to increased translation.

DISCUSSION

Our studies demonstrate that TR expression varies according to the cell cycle. From cell synchronization studies, we observed virtually no expression of TR in early S phase, but a progressive increase in its expression during late S and G_2 until maximal levels were reached at G_2/M. TR was expressed during prophase of mitosis but then disappeared during anaphase and telophase. It reappeared at low levels during G_1. Similar patterns were observed in Western blots of TRβ in GFPTR and GH_3 cells (the latter of which contains endogenous TRs). The transcriptional activity in response to T_3 also varied during the cell cycle and correlated with TR expression.

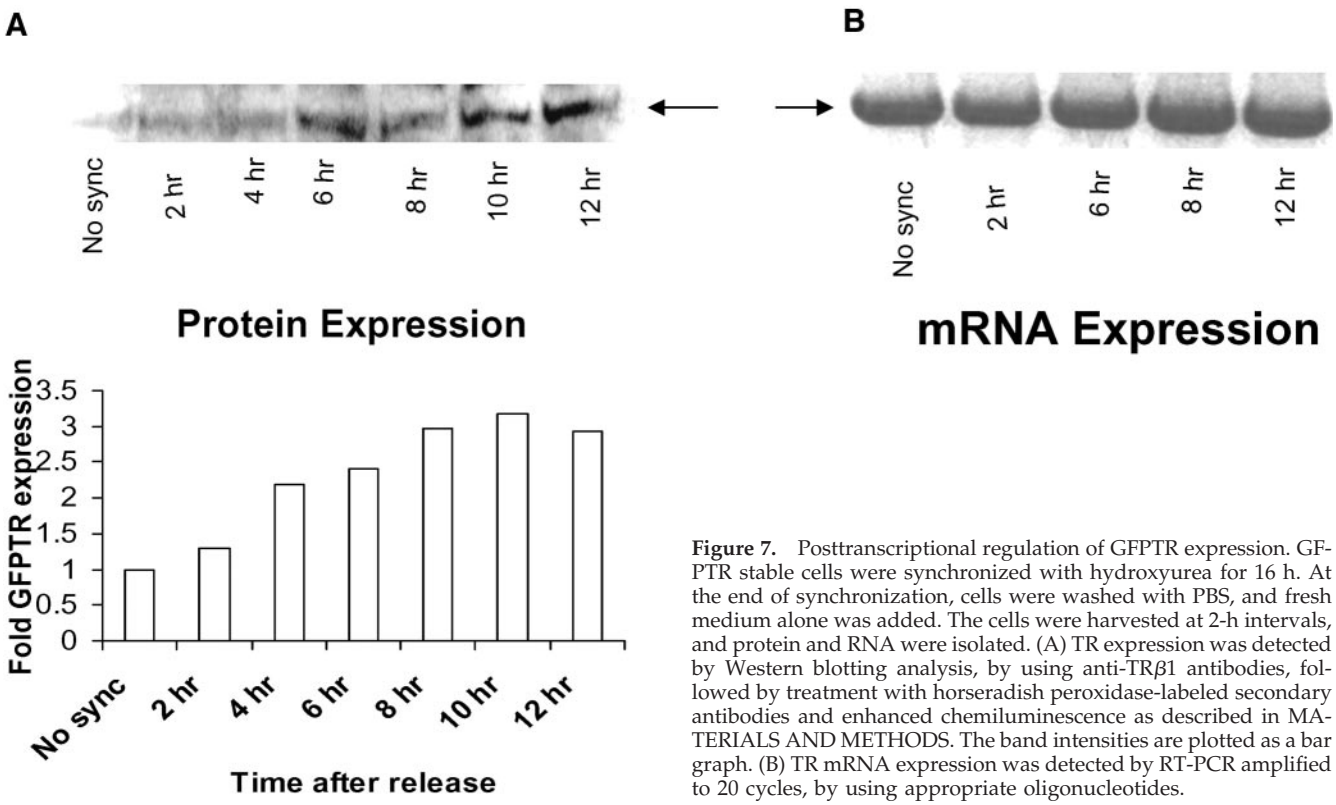

Figure 7. Posttranscriptional regulation of GFPTR expression. GFPTR stable cells were synchronized with hydroxyurea for 16 h. At the end of synchronization, cells were washed with PBS, and fresh medium alone was added. The cells were harvested at 2-h intervals, and protein and RNA were isolated. (A) TR expression was detected by Western blotting analysis, by using anti-TRβ1 antibodies, followed by treatment with horseradish peroxidase-labeled secondary antibodies and enhanced chemiluminescence as described in MATERIALS AND METHODS. The band intensities are plotted as a bar graph. (B) TR mRNA expression was detected by RT-PCR amplified to 20 cycles, by using appropriate oligonucleotides.

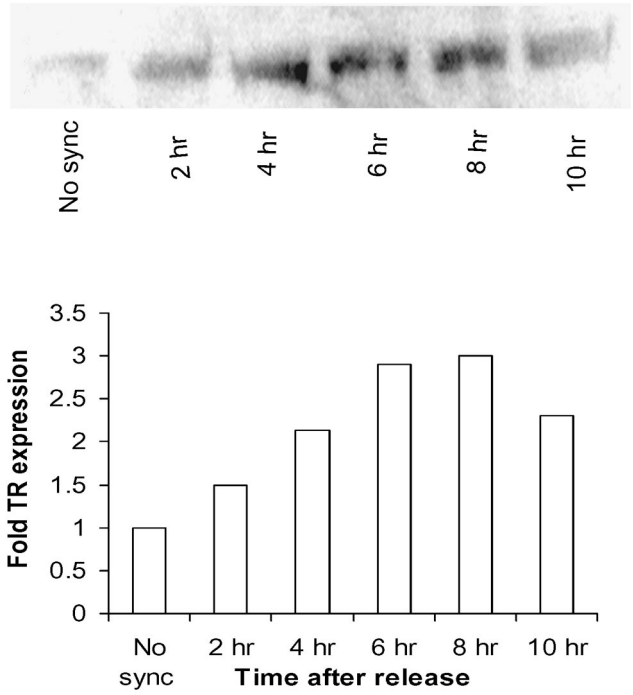

Figure 8. Cell cycle-dependent expression of TRβ in GH3 cells. GH3 cells were synchronized with hydroxyurea for 16 h. At end of the block, cells were washed, and fresh medium was added. The cells were harvested at 2-h intervals, and the protein was isolated. TR expression studied by Western blotting analysis, by using anti-TRβ1 antibody followed by treatment with horseradish peroxidase-labeled secondary antibodies and detection by enhanced chemiluminescence. The band intensities were plotted in a bar graph.

Previous studies with GR and TR showed increased hormone binding in S phase and G_2 (Cidlowski and Cidlowski, 1982; Surks and Kumara-Siri, 1984; Filipcik *et al*., 1992). These observations were based upon radioactive hormone binding after a population of cells was released from blockade in G_1 phase due to thymidine treatment. Direct measurement or visualization of receptor proteins in the released cells was not performed. In contrast, we used BrdU staining to identify individual cells in S phase and found virtually no GFPTR coexpression. These findings strongly support the notion that TRs are not significantly expressed during S phase. A decline in dexamethasone binding by GRs also has been observed during mitosis (Cidlowski and Cidlowski, 1982; Necela and Cidlowski, 2002). Up until now, there have not been studies of NR expression and their intranuclear patterns in individual cells during distinct phases of mitosis.

The mechanism for the increased TR expression during G_2 is likely due to posttranscriptional mechanisms because mRNA levels were stable, whereas TR proteins levels increased. The blockade of the increase in TR protein expression by cycloheximide suggests that translational regulation is critically important for TR expression during this stage of the cell cycle. Because only the TRβ1cDNA was used in the GFP construct, translational regulation cannot occur via the 5′ or 3′-untranslated region of TRβ1 mRNA. Recently, several examples of translational regulation by proteins that bind to the coding sequence of mRNAs have been described (Spencer and Eberwine, 1999; Xu and Grabowski, 1999). Thus, it is possible that cycloheximide could have blocked the expression of translational regulatory proteins that promote translation of TRβ mRNA by this or a similar mechanism. However, cycloheximide also decreases general translation, so its effect on TRβ translation may not have been specific. Additionally, it is theoretically possible that cycloheximide may have decreased the expression of a protein that prevents TRβ protein degradation. Alternatively, it is possible that proteosome-mediated or other degradation pathways may also contribute to the cell cycle-dependent disappearance of TRs during late mitosis and S phase (Dace *et al*., 2000; Chen *et al*., 2003).

The changes in TR expression during the cell cycle and consequent effects on hormone sensitivity may have significant biological implications. First, it is possible that proliferating cells have increased receptor expression and therefore greater hormone sensitivity. Higher nuclear receptor expression in proliferating cells may influence oncogenesis because they may be more sensitive to the mitogenic effects of hormones. It also could explain differences in developmentally regulated responses or tissue-specific sensitivities to hormone. For example, neonatal and adult brains have markedly different sensitivities to thyroid hormone (Koibuchi and Chin, 2000). Also, TH plays critical roles during important stages of embryogenesis and metamorphosis (Su *et al*., 1999). Additionally, it is possible that the particular cell cycle composition in a given tissue may contribute toward the tissue response in pathological states of hormone excess such as the syndrome of resistance to thyroid hormone (Yen, 2003).

Although receptor expression often correlates with transcriptional responsiveness to hormone (Nyborg *et al*., 1984; Yaffe and Samuels, 1984), other processes must be involved, because increased expression of estrogen or glucocorticol receptors or GR per se, does not invariably lead to increased transcription or cell progression (Darbre and King, 1987; Planas-Silva *et al*., 1999). The competency of NRs to respond to hormones in a given cell may require other effects, some of which may be cell cycle specific, such as phosphorylation of receptors, expression of coactivators, or DNA methylation or histone modifications of target genes (Hsu and DeFranco, 1995; Garcia-Villalba *et al*., 1997; Planas-Silva *et al*., 2001; Berger and Daxenbichler, 2002)

We and others previously have shown that NRs are dynamic because they shuttle between the cytoplasm and nucleus, rapidly diffuse within the nucleus, continuously exchange between the DNA enhancer elements, and change their intranuclear distribution in response to hormone (McNally *et al*., 2000; Baumann *et al*., 2001; Bunn *et al*., 2001; Maruvada *et al*., 2003). Our present studies show that not only does the expression of TRs vary during the cell cycle but also TRs can redistribute during the cell cycle as intranuclear TR changes from a diffuse homogeneous pattern in G_2 to a peripheral pattern within the nucleus during anaphase. The mechanism and purpose of this change in intranuclear distribution of TR currently is not known but may be due to association with insoluble nuclear components (Baumann *et al*., 2001; Stenoien *et al*., 2001; Reid *et al*., 2003). Recently, several laboratories have noted cyclical recruitment of nuclear hormone receptors and cofactors to hormone response elements in chromatin immunoprecipitation assays, although the timing and periodicity can vary (Brown *et al*., 1995; Sharma and Fondell, 2000; Reid *et al*., 2003). Although some of these observed effects may be due to differences in the cell types used in these studies, it also is possible that cell cycle differences may contribute to this variability, particularly given our present findings.

In summary, we have observed cell cycle-dependent changes in TR expression and distribution, which in turn can

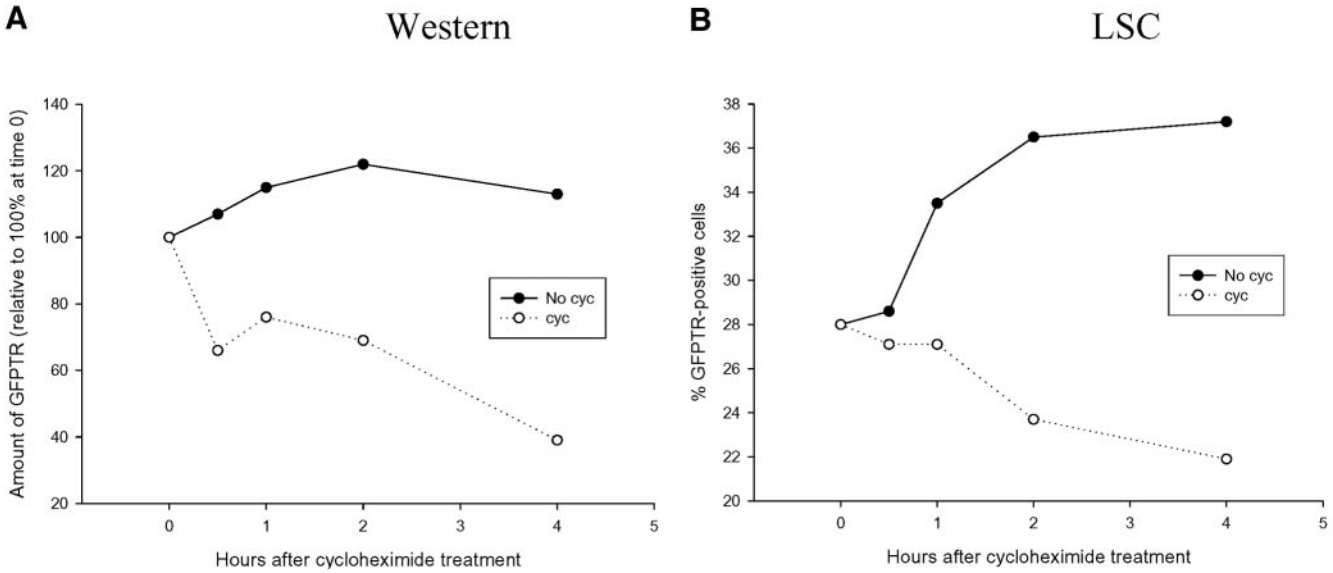

Figure 9. New protein synthesis required for GFPTR expression. GFPTR-expressing cells were synchronized with hydroxyurea for 16 h. At the end of the block, the cells were washed with PBS, and fresh medium was added. Four hours after release, cells were treated with 50 μg/ml cycloheximide and either fixed or harvested for protein at 0.5, 1, 2, and 4 h after release. (A) TRβ protein expression detected by Western blotting. (B) The percentage of cells expressing GFPTR was analyzed by LSC.

affect transcriptional response to T_3. These novel observations of cell cycle-dependent effects on TR expression may occur for other nuclear hormone receptors. Furthermore, they suggest that differential hormone sensitivity may occur during the cell cycle and thus contribute to hormonal effects in cell cycle progression during normal development and oncogenesis.

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