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Complex Actions of Thyroid Hormone Receptor Antagonist NH-3 on Gene Promoters in Different Cell Lines

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Abstract

It is desirable to obtain new antagonists for thyroid hormone (TRs) and other nuclear receptors (NRs). We previously used X-ray structural models of TR ligand binding domains (LBDs) to design compounds, such as NH-3, that impair coactivator binding to activation function 2 (AF-2) and block thyroid hormone (triiodothyronine, T₃) actions. However, TRs bind DNA and are transcriptionally active without ligand. Thus, NH-3 could modulate TR activity via effects on other coregulator interaction surfaces, such as activation function (AF-1) and corepressor binding sites. Here, we find that NH-3 blocks TR-LBD interactions with coactivators and corepressors and also inhibits activities of AF-1 and AF-2 in transfections. While NH-3 lacks detectable agonist activity at T₃-activated genes in GC pituitary cells it nevertheless activates spot 14 (S14) in HTC liver cells with the latter effect accompanied by enhanced histone H4 acetylation and coactivator recruitment at the S14 promoter. Surprisingly, T₃ promotes corepressor recruitment to target promoters. NH-3 effects vary; we observe transient recruitment of N-CoR to S14 in GC cells and dismissal and rebinding of N-CoR to the same promoter in HTC cells. We propose that NH-3 will generally behave as an antagonist by blocking AF-1 and AF-2 but that complex effects on coregulator recruitment may result in partial/mixed agonist effects that are independent of blockade of T₃ binding in some contexts. These properties could ultimately be utilized in drug design and development of new selective TR modulators.

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There are potentially important applications for thyroid hormone (TH) receptor (TR) antagonists [reviewed in (Baxter, Dillmann et al. 2001; Webb, Nguyen et al. 2002)]. Hyperthyroidism leads to rapid heart rate and arrhythmias, muscle weakness, bone loss in post-menopausal women and anxiety. While this condition can be treated by thyroid gland ablation or drugs that block TH production, thyroxine (T₄), the major circulating form of TH, has a half-life of 8 days so clinical improvement is slow (Braverman and Utiger 2000). Antagonists that act on TRs should provide more rapid relief and could partially supplant existing therapies (Webb, Nguyen et al. 2002). These drugs could also be useful in amiodarone-induced toxicosis, for which current therapies are inadequate (Conen, Melly et al. 2007), and may reverse cardiac arrhythmias in euthyroid conditions (Webb, Nguyen et al. 2002). Finally, TR antagonists could reduce metabolic rate and prevent tissue damage in anoxic states, such as stroke or injury (Webb, Nguyen et al. 2002; Grover, Dunn et al. 2007).

TRs are nuclear hormone receptors (NRs) (Zhang and Lazar 2000; Yen 2001; Laudet and Gronemeyer 2002). There are three major TR isoforms (TR β 1, TR β 2 and TR α 1) that are expressed tissue specifically. Each TR binds to specific DNA sequences (thyroid hormone response elements, TREs) in target gene promoters in the unbound state, often as heterodimers with retinoid X receptors. From this location, TRs recruit coregulators, which, in turn, alter chromatin structure and influence recruitment and processivity of the basal transcription machinery (Lonard and O'Malley 2006). Triiodothyronine (T₃), the active form of TH, modulates TR activity by enhancing packing of C-terminal helix 12 against the core of the ligand binding domain (LBD) (Glass and Rosenfeld 2000; Webb, Nguyen et al. 2002). This simultaneously creates a binding surface (activation function 2, AF-2) for coactivators, including steroid receptor coactivators (SRCs) and p300, and blocks binding of corepressors such as nuclear receptor corepressor (N-CoR) (Feng, Ribeiro et al. 1998; Glass and Rosenfeld 2000; Marimuthu, Feng et al. 2002; Webb, Nguyen et al. 2002; Rosenfeld, Lunyak et al. 2006).

Analysis of our X-ray structures of TR-LBDs with agonists suggested a rational strategy for design of TR and NR antagonists (Webb, Nguyen et al. 2002). Since hormone is buried in the core of the domain (Wagner, Apriletti et al. 1995), we proposed that derivatives of agonists with appropriately placed extensions should compete with T₃ for binding and inhibit NR activity by displacing H12 (Scanlan, Baxter et al. 1996; Webb, Nguyen et al. 2002). We used this strategy to identify lead compounds that inhibit TRs (Yoshihara, Apriletti et al. 2001; Baxter, Goede et al. 2002; Nguyen, Apriletti et al. 2002; Nguyen, Apriletti et al. 2005). The best, NH-3, is derived from the TR β selective agonist GC-1 and contains a bulky 5' nitrophenylethynyl extension (Chiellini, Apriletti et al. 1998; Nguyen, Apriletti et al. 2002; Webb, Nguyen et al. 2002). NH-3 binds TRs with nanomolar affinity, blocks TR AF-2 interactions with coactivators *in vitro* and in cell culture and antagonizes T₃ responses at standard reporters in cell culture. NH-3 also inhibits TH mediated metamorphosis of tadpoles, and antagonizes T₃ effects on heart rate, serum cholesterol and plasma thyroid stimulating hormone in rats (Lim, Nguyen et al. 2002; Nguyen, Apriletti et al. 2002; Grover, Dunn et al. 2007).

There are, however, several reasons to suspect that NH-3 will not always act as a pure T₃ antagonist. Unliganded TRs, like many other NRs, bind to DNA in the absence of hormone,

where they have ligand independent actions. Thus, NH-3 dependent alterations in TR conformation could alter coregulator recruitment and gene expression independently of effects on T₃ binding. Indeed, weak partial agonist activity has been observed at high NH-3 doses in rats, but it is not clear whether this involves direct effects on TRs or generation of agonist metabolites (Lim, Nguyen et al. 2002; Grover, Dunn et al. 2007).

How might NH-3 alter coregulator recruitment? We found that NH-3 resembles T₃ in its ability to block TR LBD interactions with the corepressors N-CoR and SMRT in mammalian 2-hybrid assays and cell free systems (Nguyen, Apriletti et al. 2002; Moore, Galicia et al. 2004). If NH-3 prevents TR interactions with corepressors, this effect could result in partial agonist or agonist actions (Glass and Rosenfeld 2000; Webb, Nguyen et al. 2002). Moreover, NRs and TRs contain one at least one alternate activation function (AF-1) in their amino terminal domains (NTD), and possibly other coregulator binding loci (Barettino, Vivanco Ruiz et al. 1994; Wilkinson and Towle 1997; Yang, Hong et al. 1999; Oberste-Berghaus, Zanger et al. 2000; Webb, Nguyen et al. 2002; Tian, Mahajan et al. 2006). While it is not obvious how these surfaces would be affected by an antagonist that acts by displacing H12 on the LBD, it is well known that estrogen receptor (ER) antagonists, including the selective ER modulators (SERMs) tamoxifen and raloxifene, activate AF-1 via release of heat shock proteins that prevent the receptors from binding to DNA and indirect effects on AF-1 itself (reviewed in (Webb, Nguyen et al. 2002)). Understanding these issues would be of clear importance for design of antagonists for TRs and other NRs that bind DNA in the absence of ligand.

In this study, we examined NH-3 effects on TR activation functions in transient transfections and upon gene expression and coregulator recruitment in cultured cells. We find that NH-3 blocks both AF-2 and AF-1 and largely lacks agonist activity, but does induce spot 14 (S14) in HTC liver cells. This effect is accompanied by enhanced coactivator recruitment at the S14 promoter, which is not seen in pituitary GC-1 cells where NH-3 fails to enhance S14 expression. NH-3 and T₃ exert effects on N-CoR interactions that are counter to current dogma (Glass and Rosenfeld 2000), both promote N-CoR recruitment to TR regulated genes, although NH-3 effects vary with cell type. We suggest that antagonists that interact with DNA bound NRs will mostly block agonist actions, but have the potential to exert limited agonist effects and alter some actions of unliganded receptors. These findings have important ramifications for TR and NR antagonist design.

Results

NH-3 Blocks TR-LBD Interactions with Coactivators and Corepressors

We confirmed that NH-3 blocks TR-LBD interactions with coactivators and corepressors in cells that express endogenous TRs, rat GC pituitary and HTC liver cells (Nguyen, Apriletti et al. 2002). T₃ activated a GAL4 reporter in the presence of a GAL-TR β LBD fusion protein in both cell types, an effect requiring coactivator association with TR AF-2 (Fig. 1). By contrast, NH-3 failed to activate the reporter and blocked T₃ induction, confirming that it functions as a TR antagonist. Both ligands also inhibited N-CoR interactions with the TR LBD (Fig. 2); NH-3 and T₃ reduced transcriptional readout from the GAL4 responsive reporter in the presence of a GAL4 DBD bait linked to N-CoR NR interacting domains and

a VP16-tagged TR β LBD trap. Similar results were also obtained with a GAL-SMRT fusion (not shown), as previously observed in HeLa cells (Nguyen, Apriletti et al. 2002). Thus, NH-3 blocks TR LBD interactions with coactivators and corepressors.

TR AF-1 and AF-2 Cooperate in Gene Induction in GC and HTC Cells

Data in Fig. 3 show effects of NH-3 on activities of full length TR and individual TR transactivation functions (AF-1 and AF-2) at T₃-activated TRE-dependent reporter in GC and HTC cells. In GC cells, T₃ activated a TRE-dependent reporter (containing an inverted palindrome, IP-6; (Velasco, Togashi et al. 2007)) in the presence of endogenous TRs whereas NH-3 inhibited basal activity (Fig. 3, upper panel). Transfected TR β 1 amplified T₃ response, but did not alter the ability of NH-3 to suppress basal reporter activity. In HTC cells; T₃ response was only obtained with transfected TR β 1. Here, NH-3 failed to repress basal activity (Fig. 3, lower panel).

Both TR activation functions are active in GC and HTC cells; a TR β truncation that lacks AF-1 (TR β 1 DBD-LBD) and a TR β point mutant that lacks AF-2 activity (TR β E457K (Feng, Ribeiro et al. 1998)) exhibit reduced T₃-induction relative to TR β . As expected, NH-3 blocked the activity of the TR truncation that only contained AF-2. However, NH-3 also blocked activity of the TR β E457K mutant that retains AF-1 but lacks AF-2, suggesting that NH-3 must indirectly inhibit TR AF-1 activity in this context.

NH-3 also failed to act as an agonist at standard reporters in a variety of related experimental conditions (not shown). NH-3 did not induce the IP-6 reporter in GC and HTC cells transfected with TR β 2 or TR α 1, with any TRs or TR truncations at T₃-activated reporters driven by other TREs (Velasco, Togashi et al. 2007), a direct repeat (DR-4) and a palindrome (Pal), and with transiently transfected coactivators that potentiate T₃ response, including SRCs and p300. Thus, NH-3 exhibits one cell-specific effect; it suppresses basal TR activity in GC cells but not in HTC cells, but generally lacks agonist activity at standard reporters and can inhibit both AF-1 and AF-2.

NH-3 Is a Cell and Promoter-Specific TR Agonist

We compared effects of T₃ and NH-3 on endogenous T₃-responsive genes. Similar to results of others (Sharma and Fondell 2002; Liu, Xia et al. 2006), we observed transient 2–4 fold increases in steady state mRNA levels of growth hormone (GH), spot 14 (S14) and sarcoplasmic endoplasmic reticulum calcium dependent ATPase 1 (SERCa1) in GC cells after T₃ treatment (Fig. 4A). There were variations in kinetics of hormone induction; GH and S14 mRNA levels peaked 1 hr after T₃ administration, declined at 3 hrs and rose again at 6 hrs, whereas SERCa1 mRNA levels peaked at 3hrs and declined thereafter. T₃ also induced S14 mRNA 2–3 fold in HTC cells, with peak induction 1 hr after hormone administration (Fig. 4B; GH and SERCa1 transcripts were not detected in this cell type; not shown). Glyceraldehyde phosphate dehydrogenase (GAPDH) transcripts were unaltered in both cell types, confirming that T₃-effects were specific.

NH-3 exhibited different effects. It failed to induce GH, S14 and SERCa1 transcripts in GC cells at short times and weakly suppressed mRNA levels of these genes at 18hrs (Fig. 4A). By contrast, NH-3 induced S14 mRNA about 3-fold in HTC cells, slightly more efficiently

than T₃ (Fig. 4B). There were no effects on GAPDH mRNA in either cell type confirming that NH-3 effects were specific. Thus, NH-3 shows cell type specific agonist activity at S14.

NH-3 Induces Histone H4 Acetylation at the S14 Promoter in HTC Cells

To explore effects of NH-3 on the S14 promoter, we performed ChIP experiments with GC and HTC cell extracts. First, we used an antibody against acetylated histone 4 (H4), a marker for transcriptional activation (Jenuwein and Allis 2001). T₃ increased H4 acetylation at the S14 TRE in GC cells whereas NH-3 had a lesser effect (Fig. 5B). Similar results were obtained with the GH and SERCa1 promoters (not shown). By contrast, T₃ and NH-3 enhanced H4 acetylation at S14 in HTC cells to similar extents (Fig. 5C). This suggests that NH-3 induces a transcriptionally active state at the S14 promoter in HTC cells.

NH-3 Enhances TR DNA binding in Cultured Cells

While T₃ did not affect TR/DNA interactions, NH-3 enhanced TR binding to target promoters. As reported by others (Yen 2001), ChIP analysis showed that both TRβ2 and TRβ1 occupy the S14 TRE in GC and HTC cells, respectively, and that TR binding is only minimally affected by T₃ (Figs. 6A and 6B). By contrast, NH-3 promoted transient increases in TRβ2 at all three target promoters in GC cells (Fig. 6A and not shown) and weaker but qualitatively similar increases in TRβ1 levels at the S14 promoter in HTC cells (Fig. 6B). These effects could not be recapitulated in gel shifts *in vitro* (Fig. 6C). Like T₃, NH-3 promoted TR dissociation from IP-6 (although slightly less than T₃, shown) and DR-4 elements (not shown) and failed to affect RXR-TR interactions with either TRE. Thus, NH-3 alters TR DNA binding in cultured cells but not *in vitro*.

NH-3 and T₃ Promote Different Patterns of Coactivator Recruitment to Target promoters

To explore the idea that NH-3 could induce S14 by altering TR transcription complex formation, we analyzed recruitment of representative TR coactivators to each promoter. We chose three coactivators that are recruited early in transcriptional activation, the p160s SRC2 and SRC1, and the histone acetyl-transferase p300 (Sharma and Fondell 2002; Liu, Xia et al. 2006).

T₃ promoted recruitment of all three coactivators to target promoters but the precise pattern varied with cofactor and cell type (Fig. 7). In GC cells (Fig. 7A), SRC2 was rapidly recruited to the S14 promoter, dismissed at 40 minutes and recruited again at 60 minutes. SRC1 was present before T₃ induction, dismissed at 20 minutes, re-recruited at 40–60 minutes and dismissed again at 180 minutes. Finally, p300 was also present prior to T₃ induction, dismissed at 40 minutes and recruited again at 1–3 hrs. The same profile was obtained with GH and SERCa1 (not shown). Broadly similar results were obtained with the S14 promoter in HTC cells (Fig. 7B); the sole distinction from GC cells is that the second wave of SRC2 and p300 recruitment occurred later in HTC cells.

NH-3 effects on coactivator recruitment differed from T₃. In GC cells, NH-3 promoted cyclical recruitment of SRC2 to the S14 promoter (Fig. 7A, shown) and to the GH and SERCA promoters (not shown). SRC2 recruitment was weaker than obtained with T₃ and delayed, peak SRC2 binding occurred 40 minutes and 3hrs after NH-3 treatment, compared

to 20 minutes and 1 hour with T₃. NH-3 did not promote SRC1 or p300 recruitment to target promoters in this cell type (Fig. 7A and not shown).

By contrast, NH-3 promoted recruitment of all three coactivators to S14 in HTC cells (Fig. 7B). The pattern differed from T₃ in several ways. First, NH-3 only promoted one round of SRC2 recruitment during this time frame. Second, NH-3 dependent SRC1 recruitment was stronger than T₃ at 40 minutes and p300 recruitment was stronger than with T₃ at early times and weaker at later times. Third, recruitment kinetics differed from those of T₃; SRC1 was dismissed at 1hr with NH-3 and returned at 3hrs whereas it persisted at 1hr with T₃ and was dismissed at 3hrs and the second round of p300 recruitment occurred earlier with NH-3.

Together, our results suggest NH-3 induction of S14 in HTC cells correlates with coactivator recruitment. The fact that NH-3 and T₃ induce different patterns of coactivator recruitment may, however, be indicative of different mechanisms of transcriptional activation (see Discussion).

T₃-Dependent Recruitment of N-CoR and Cell Type Specific Effects of NH-3

Finally, we examined ligand effects on corepressor recruitment to TR regulated promoters. Unlike current dogma, T₃ enhanced N-CoR recruitment in both GC and HTC cells. N-CoR was present at low or undetectable levels at the GH, S14 and SERCA promoters without ligand (Fig. 8A). Administration of T₃ led to enhanced N-CoR recruitment, with a peak at 40 minutes that coincided with dismissal of SRC2 and p300 (see Fig. 7A). N-CoR was detectable at the S14 promoter in HTC cells in the absence of ligand (Fig. 8B). Here, T₃ promoted a transient increase in N-CoR recruitment at 40 minutes, coinciding again with SRC2 and p300 dismissal (see Fig. 7B).

NH-3 effects differed from T₃ and varied with cell type. NH-3 promoted weak recruitment of N-CoR to all three promoters in GC cells; this occurred with delayed kinetics relative to T₃ and coincided with loss of SRC2 (compare Fig. 8A to Fig. 7A). By contrast, NH-3 led to dismissal of N-CoR at 40 minutes in HTC cells followed by rebinding at later times (Fig. 8B).

Discussion

In this study, we examined actions of NH-3, which blocks T₃ action in cell free systems (Nguyen, Apriletti et al. 2002; Moore, Galicia et al. 2004; Nguyen, Apriletti et al. 2005), transfected cells (Nguyen, Apriletti et al. 2002; Nguyen, Apriletti et al. 2005), tadpoles (Lim, Nguyen et al. 2002) and rats (Grover, Dunn et al. 2007). Since unliganded TRs bind DNA and are transcriptionally active we suspected that NH-3 may influence TR conformation and activity in a manner that is independent of effects on T₃ binding. This is the case: NH-3 induces S14 in HTC cells, and increases H4 acetylation at the promoter, indicative of a transcriptionally active state. However, it fails to induce this gene, or GH and SERCA, in GC pituitary cells or transfected reporters in either cell type. Thus, NH-3 is a cell-type and promoter-specific agonist.

ChIP analysis confirms that NH-3 alters TR activities. NH-3 induction of S14 in HTC cells is accompanied by enhanced recruitment of three coactivators tested (SRC1, SRC2 and p300) and transient dismissal of N-CoR (opposite to increased N-CoR recruitment with T₃). Lack of NH-3 induction of target genes (including S14) in GC cells is associated with large transient increases in TRβ2 binding and delayed recruitment of one coactivator (SRC2) and N-CoR. Thus, NH-3 alters TR transcription complex formation at target promoters, even when it fails to induce the associated gene, and the fact that the drugs tends to push the balance of coregulator recruitment at S14 in towards coactivators in HTC cells but not GC cells probably explains its cell-specific agonist actions.

It is not clear how NH-3 promotes coactivator recruitment. It does not seem likely that preferential expression of TRβ1 vs. TRβ2 in HTC cells explains cell-specific actions of this compound; transfected TRβ1 did not permit NH-3 to activate a T₃-inducible S14 reporter in GC (not shown). We also do not know which TR surfaces are involved in coactivator recruitment. NH-3 blocks AF-2 in GC or HTC cells (Figs 1–3), as seen in other cell types (Nguyen, Apriletti et al. 2002; Webb, Nguyen et al. 2002; Moore, Galicia et al. 2004), suggesting that NH-3 must promote coactivator recruitment differently from T₃, a potent AF-2 activator. We suspect that NH-3-dependent cofactor recruitment involves AF-1, but have not been able to test this idea directly because TRs paradoxically repress S14 dependent reporters in HTC cells (Ota and Mariash 2003). We note, however, that TR AF-1 mediates T₃ response at a reporter with S14 TREs (Wilkinson and Towle 1997) and is required for optimal T₃ response at standard reporters in HTC cells, confirming it is active (Fig. 3). We also found that NH-3 allows TR AF-1 dependent interactions with SRC1 and SRC2 in pulldowns *in vitro* (not shown). These observations are all consistent with the idea that NH-3 could promote coactivator recruitment through AF-1 (Webb, Nguyen et al. 2002).

Surprisingly, T₃ enhances corepressor recruitment at all target genes in this study. Whereas current coregulator exchange models predict ligand dependent dismissal of corepressors and replacement with coactivators (Glass and Rosenfeld 2000), we find that T₃ promotes transient N-CoR recruitment to S14, GH and SERCA in GC and to S14 in HTC cells. We also expected that NH-3 would act as a partial TR agonist at T₃-activated genes by displacing corepressors. Instead, NH-3 failed to induce target genes in GC cells, acts as an agonist at S14 in HTC cells, and, respectively, promotes transient recruitment of N-CoR and cyclical dismissal and rebinding of N-CoR to S14 in these cell types.

We recognize that much available experimental evidence supports each aspect of the coregulator exchange model, including mutational analysis of TRs (Feng, Ribeiro et al. 1998; Hu and Lazar 1999; Nagy, Kao et al. 1999; Jepsen, Hermanson et al. 2000; Marimuthu, Feng et al. 2002), blockade of N-CoR expression with interfering RNAs (Yoon, Chan et al. 2003) or targeted disruption of the N-CoR gene (Jepsen, Hermanson et al. 2000) and ChIP analysis in frogs (Li, Lin et al. 2002; Lim, Nguyen et al. 2002; Buchholz, Hsia et al. 2003; Tomita, Buchholz et al. 2004; Paul, Buchholz et al. 2005; Paul, Fu et al. 2005) and in cultured cells at stably integrated GAL4-responsive reporters regulated by GAL-TR LBD (Sharma and Fondell 2002; Ishizuka and Lazar 2003; Yoon, Choi et al. 2005; Liu, Xia et al. 2006).

How, then, do we reconcile our findings with these studies? Coactivator vs. corepressor recruitment has not been widely explored at native TR-target genes and we propose that the coregulator exchange model applies could only to subsets of T₃-induced genes, and none investigated here. This agrees with the fact that unliganded peroxisome proliferator activated receptor gamma recruits corepressors to some promoters and not others (Guan, Ishizuka et al. 2005) and could explain why unliganded TRs only repress a subpopulation of T₃ responsive genes in liver in mouse TR knockouts (Yen, Feng et al. 2003). The idea also agrees with observations that steroid receptor agonists sometimes promote corepressor recruitment (Chen, Welsbie et al. 2004; Hodgson, Astapova et al. 2005; Ki, Cho et al. 2005; Wang and Simons 2005; Yoon and Wong 2006; Higgins, Liu et al. 2008). Finally, we note that our findings are exactly consistent with a model of TR action proposed by Lee and coworkers (Sohn, Kim et al. 2003) who envisaged dynamic interplay of coactivator and corepressor complexes at the T₃-liganded TR with corepressors serving to restrict T₃ response.

It will be interesting to determine the function of ligand-dependent corepressor recruitment. We suspect that N-CoR recruitment serves to limit T₃ activation, as proposed by Lee and coworkers (Sohn, Kim et al. 2003), because the timing of this event approximately precedes transient reduction of mRNA levels that are feature of cyclical T₃ dependent transcriptional responses (compare Fig. 4 with results of Fig. 8. We must also consider the possibility that NH-3 dependent blockade of AF-1 and AF-2 transactivation functions in GC cells (Fig. 3) may be related to the fact that this ligand promotes corepressor recruitment to target promoters in GC cells while failing to recruit the complete complement of coactivators in these contexts.

We do not know how T₃ promotes corepressor binding and why NH-3 effects vary with cell type. TR H12 position could alter in the transcription cycle, respectively favoring coactivator and corepressor binding. Evidence against this argument is that both T₃ and NH-3 consistently block TR β -LBD interactions with N-CoR NR interaction domains in all cell types tested. Alternatively, liganded TRs have been shown to bind a coactivator-like LxxLL peptide in N-CoR (Loinder and Soderstrom 2004; Loinder and Soderstrom 2005), and this may mediate T₃-dependent N-CoR recruitment to target genes. This would not explain how NH-3, which blocks TR interactions with LxxLL motifs, promotes N-CoR binding in GC cells. We presently favor the idea that T₃-dependent N-CoR recruitment is mediated by alternate interaction surfaces and suggest that this effect could share features with known mechanisms of transcriptional repression (Ogawa, Lozach et al. 2005; Blaschke, Takata et al. 2006; Pascual and Glass 2006; Ghisletti, Huang et al. 2007), where liganded NRs block gene expression by arresting corepressor complexes at target promoters.

Our findings have broad implications for NR selective modulator design. Effects of ligands that inhibit NR AF-2 were established with steroid receptors, where ligands also promote receptor translocation into the nucleus (Webb, Nguyen et al. 2002). This is different from TRs, which bind to nuclear chromatin without ligand. Although NH-3 is a general TR antagonist, it clearly has potential for complex effects on coregulator recruitment and this is also likely to be the case with similar compounds that bind other NRs that interact with chromatin in the absence of ligand. Our results also address potential for TR drugs that

inhibit coactivator *and* corepressor recruitment. Phenotypes of TR α / β -/- knockout mice are mild compared to hypothyroid animals, suggesting that unopposed actions of unliganded TRs mediate harmful effects of this disease and we proposed that TR drugs (such as NH-3) that inhibit corepressor and coactivator recruitment would reverse hypothyroidism without inducing symptoms of thyroid hormone excess (Baxter, Dillmann et al. 2001; Webb, Nguyen et al. 2002). Since TR gene regulation does not always follow the classic coregulator exchange model, we suggest that simple predictions about actions of this class of NR ligand require reevaluation.

Finally, we emphasize that our experiments focus on nuclear TH signaling pathways. It is important to note that TR ligands also elicit rapid responses through binding to the same receptor in the cytoplasm, and that there are alternative TH signaling pathways that do not involve the traditional NRs (Davis, Leonard et al. 2008). It will be interesting to determine how NH-3 affects these alternative signaling pathways and define contributions of classical nuclear actions and alternative actions of such ligands to observed responses in animal models.

Materials and Methods

Cell Culture

HTC cells and GC cells were maintained in DMEM medium containing 10% FBS and streptomycin. Serum was withdrawn 24 hrs prior to the addition of ligand T₃ or NH-3 for various periods before harvesting the cells for transcript analysis or ChIP assays. T₃ was obtained from Sigma; NH-3 was synthesized as previously described (Nguyen, Apriletti et al. 2002).

Reverse Transcriptase Polymerase Chain Reaction (RT-PCR)

GC or HTC cells were treated with T₃ or NH-3 (10⁻⁷ M) for various times after serum withdrawal. Total RNA was prepared from the cells using TRIZOL (GIBCO-BRL). The cDNA were prepared with Super Script™ first-strand synthesis system for RT-PCR (Invitrogen) from 1 μ g of total RNA.

The primers were:

S14:	forward 5'-GTCATGGATCGGTACTIONCGGC-3' reverse 5'-CAGCTCCTCCGAAAGCCTGTC-3'
SERCA1:	forward 5'-GTGAGCGAGACCACAGGCCTTACC-3' reverse 5'-CCTTCAGCGCCTCGATGGCATTCT-3'.
GH:	forward 5'-GCCTACATTCCTCCGAGGGACAGCGC-3' reverse 5'-GAGCAGAGCGTCATCGCTGCGCAT-3'
GAPDH	forward 5'-GCACAGTCAAGGCTGAGAATGGGA-3' reverse 5'-CATGGACTGTGGTCATGAGCCCTT-3'.

Chromatin Immunoprecipitation (ChIP)

Antibodies against TR (06-539 and 06-540), acetyl-Histone H4 (06-866), SRC1 (05-522) were obtained for Upstate Biotechnology, Lake Placid NY. Antibodies against SRC2 (C-20), p300 (C-20), N-CoR (C-20) were obtained from Santa Cruz Biotechnology, Inc.

ChIP assays were performed essentially as previously described in Orlando *et al.*, (Orlando, Strutt *et al.* 1997) with slight modifications. Approximately 6×10^6 cells were cross linked with 1% formaldehyde for 15 min at room temperature. Cross linking was stopped by addition of 0.125 M glycine. Cells were washed twice with ice-cold PBS and collected by scraping in 5 ml ice-cold PBS. The pellet was washed with (10 mM Tris, pH 8.0, 10mM EDTA, 0.5mM EGTA, 200 mM NaCl and 1 mM phenylmethylsulfonyl fluoride). Cells were lysed in 0.25% triton buffer (10 mM Tris, pH 8.0, 10mM EDTA, 0.5mM EGTA, 0.25% Triton X-100, and 1 mM phenylmethylsulfonyl fluoride) with rotation at 4°C for 15 minutes. and lysed cell extract resuspended in immunoprecipitation (IP) buffer (20mM Tris HCl, pH 8.0, 0.05% Deoxycholic acid, 0.5% Triton X-100, 0.5% NP-40, 200 mM NaCl, 1mM phenylmethylsulfonyl fluoride) and sonicated 3–5 times for 10 sec to reduce DNA fragment length to approximately 500–2000 bp, as verified by Agarose Gel electrophoresis. Supernatants were collected and immunocleared with salmon sperm DNA/protein A agarose slurry (upstate) for 1 hr at 4°C. One tenth of the supernatant was saved to quantitate the amount of input DNA present in each sample before immunoprecipitation.

Immunoprecipitation was performed overnight with agitation at 4°C with specific antibodies 5µg/500 µg of total lysate. Precipitates then were washed sequentially in low salt IP buffer, IP buffer supplemented with 0.5M NaCl and then final LiCl wash buffer (Orlando, Strutt *et al.* 1997). Beads were then washed twice in Tris-EDTA buffer and extracted twice with 1% sodium dodecyl sulfate buffer. Pooled eluates as well as saved chromatin solution for quantitating the amount of input DNA from above were heated at 65°C for overnight in 0.2 M NaCl solution to reverse the formaldehyde cross-linking. Diluted sample with TE to lower the concentration of SDS to 0.5% incubated at 45 C for 2 h with 20 µg proteinase K. DNA fragments were purified with phenol/chloroform/isoamyl alcohol (25:24:1) and ethanol precipitation and analyzed by PCR.

All of the ChIP experiments were repeated several times.

S14:	Forward: 5'-CCAGAGGAAGTGGGGTCAAGGGCC-3' Reverse: 5'-CAGCCCTGACGTAGCGGAGGATAG-3'
SERCA1:	Forward: 5'-GGCCTAAGGGCAAGAGGGCTTACG-3' Reverse: 5' CACCTGCCTGTAACTGGGCTCC-3'
GH:	Forward: 5'-CTCCTTGGAGAGGCTCTGTGCCCC-3' Reverse: 5'-GGCTGGAGCCACTGACAGCTTGTG-3'
GAPDH:	Forward: 5' GTCAAGCTCCTACCATTTCATGCTG-3' Reverse: 5'CGGTCACCTCACACGGTGGGGTATC-3'

PCR was carried out in standard conditions with Taq polymerase. PCR primers used for analysis of ChIP experiments were:

Transfections

The following plasmids have been described previously. Mammalian expression vectors for TRs, GAL4-TR β LBD, GAL4-N-CoR and Vp16-TR β LBD (Feng, Ribeiro et al. 1998; Webb, Anderson et al. 2000; Velasco, Togashi et al. 2007). Reporter genes with two copies of IP-6, DR-4 and palindromic TREs upstream of a minimal promoter driving luciferase expression and the GAL responsive reporter comprising five GAL response elements linked upstream of a minimal promoter driving luciferase expression.

Cells were transfected by electroporation with 5 μ g of the respective reporter gene, 1 μ g of CMV- β -galactosidase internal control, and 1 μ g expression vectors for TR or various fusion proteins or empty vector control. Transfected cells were plated and induced overnight with ligand. Luciferase and β -galactosidase assays were performed as previously described (Webb, Anderson et al. 2000; Velasco, Togashi et al. 2007).

Gel Shifts

Binding of TR to TREs were assayed by mixing 20 fmols of ³⁵S labeled TRs produced in a reticulocyte lysate, (TNT T7; Promega) with 10ng oligonucleotide and 1 μ g of poly(dI-dC) (Amersham Pharmacia Biotech) in a final volume of 20 μ l 1X binding buffer (c25mM HEPES, 50mM KCl, 1mM DTT, 10 μ M ZnSO₄, 0.1% NP-40, 5% glycerol). After 30' incubation, the mixture was loaded onto a 5% nondenaturing polyacrylamide gel that was pre-run for 30 min at 200 V and run at 4°C for 120 min at 240 V, in a running buffer of 6.7 mM Tris (pH 7.5), 1 mM EDTA, and 3.3 mM sodium acetate. The gel was then fixed, treated with Amplify (Amersham Pharmacia Biotech), dried and exposed for autoradiography. TRs used in assay were quantified with ¹²⁵I-T₃ binding assay and SDS-PAGE analysis of ³⁵S-TRs.

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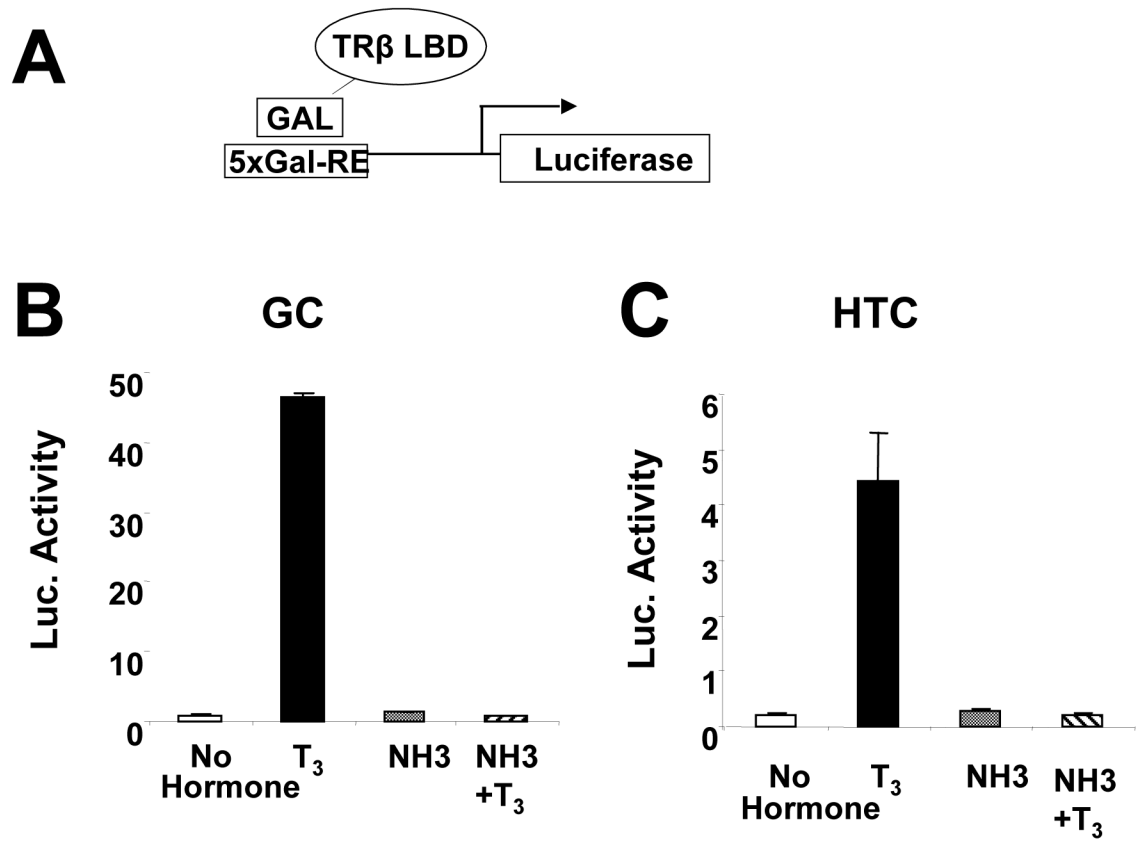


Fig. 1. NH-3 does not activate TR β AF-2 in GC and HTC cells. **(A)** Transfection components in schematic. **(B)** Results of transcription readout assays performed in GC cells or **(C)** HTC cells.

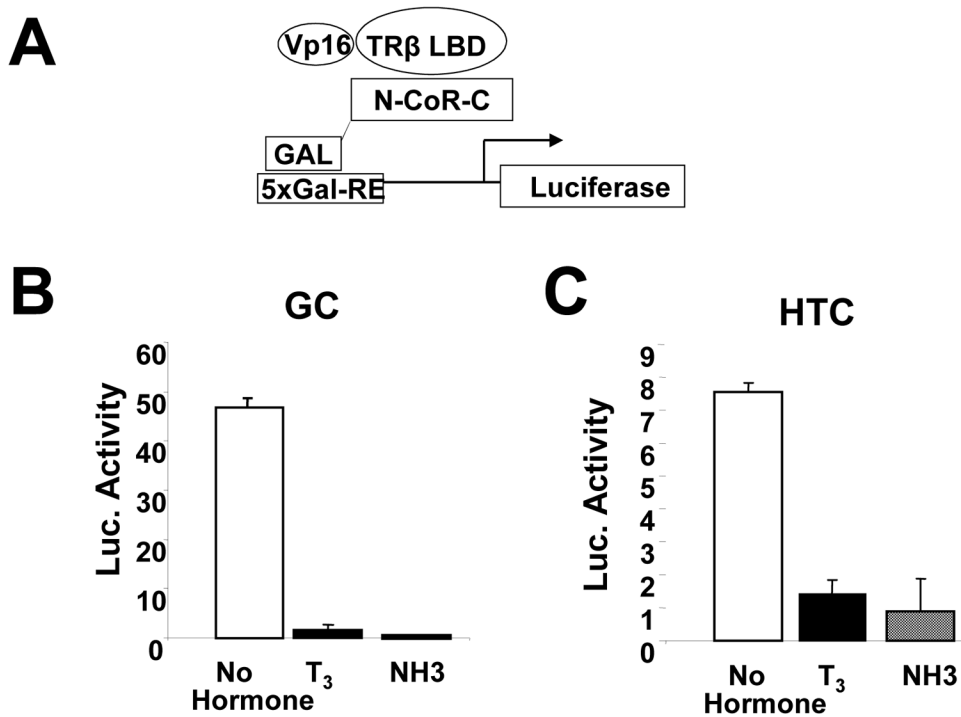


Fig. 2. NH-3 and T₃ promote corepressor release from the TR β LBD in GC and HTC cells in mammalian 2-hybrid assays. **(A)** Transfection components in schematic. **(B)** Results of transcription readout assays performed in GC cells and **(C)** HTC cells.

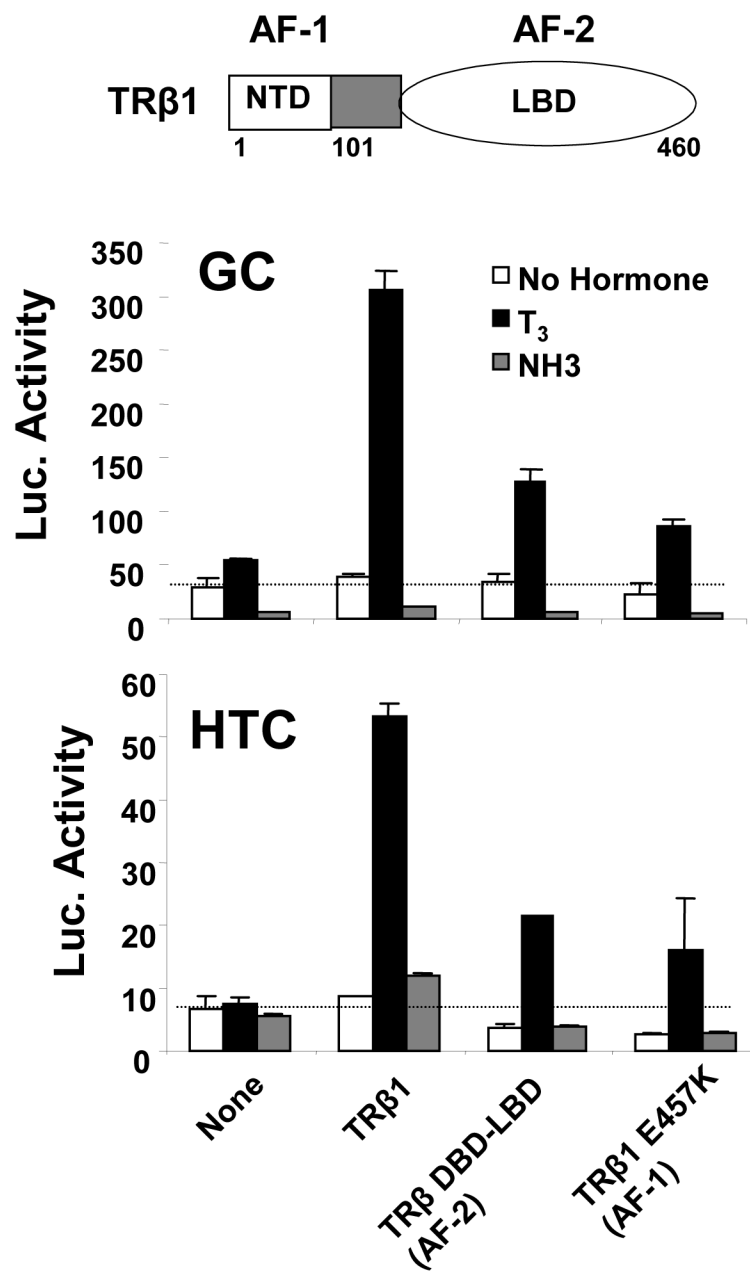


Fig. 3. TR AF-1 and AF-2 cooperate in T₃ response at TRE-regulated reporters in GC and HTC cells, but NH-3 lacks agonist activity. The panels shows transfection assays in GC and HTC cells performed with an IP-6 responsive reporter +/- cotransfected expression vectors for TRβ1, TRβ1 DBD-LBD (AF-2 only) or TRβ1 E457K (AF-1 only) and induced with T₃ (100nM) or NH-3 (1μM).

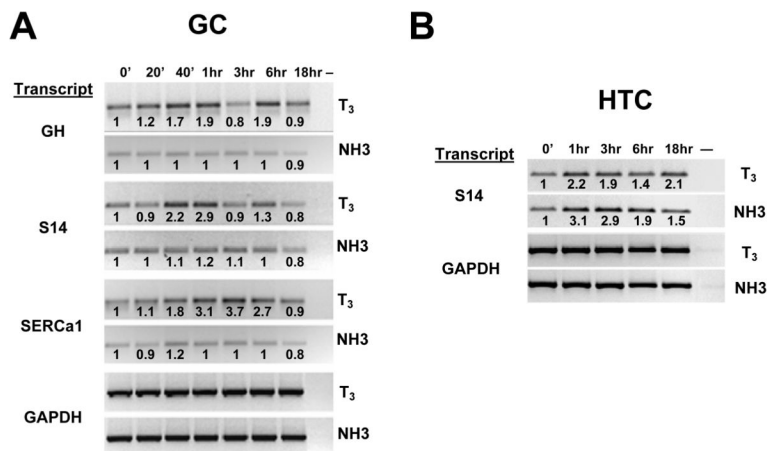


Fig. 4. Effects of T₃ and NH-3 on endogenous gene transcription in (A) GC and (B) HTC cells. GC or HTC cells were treated with T₃ or NH-3 for different length of times and mRNA expression was analyzed by RT-PCR. The image shows PCR products obtained after amplification of mRNA samples prepared at various times after addition of ligand and separated on a 1% agarose gel. Fold inductions relative to t=0 (estimated by image scanning) are shown at top.

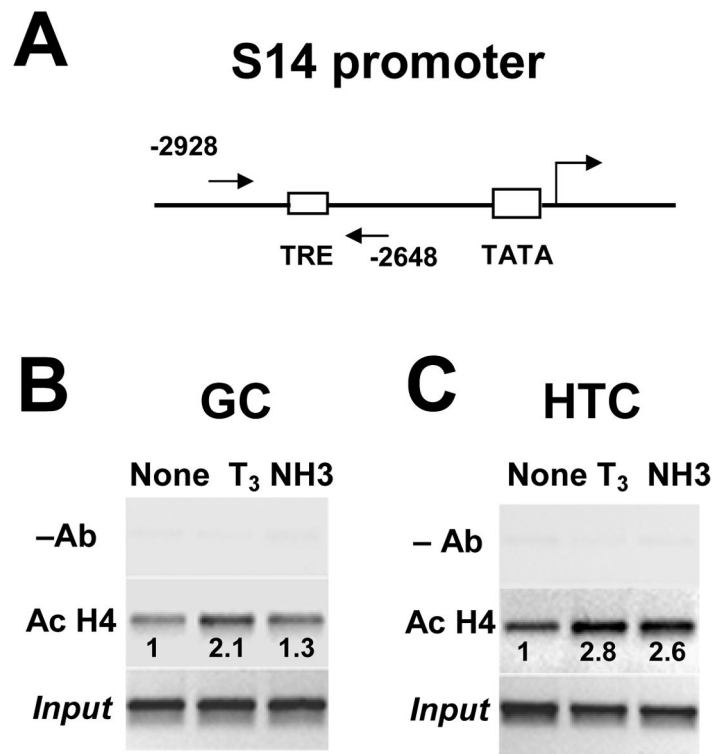


Fig. 5. Effect of T₃ and NH-3 on histone acetylation at the S14 promoter in GC and HTC cells. (A) Schematic of the S14 promoter with positions of RT primers marked. Immunoprecipitated chromatin from GC (B) and HTC cells (C) was analyzed for the binding of acetylated histone by quantitative PCR in the TRE promoter region of S14 in presence of T₃ and NH-3. Products were separated on a 1% agarose gel. Note that expression of S14 transcript in presence of T₃ or NH-3 in GC (Fig. 1A) and HTC (Fig. 1B) cells correlates with acetylation of histone. Fold inductions (estimated by image scanning) at top.

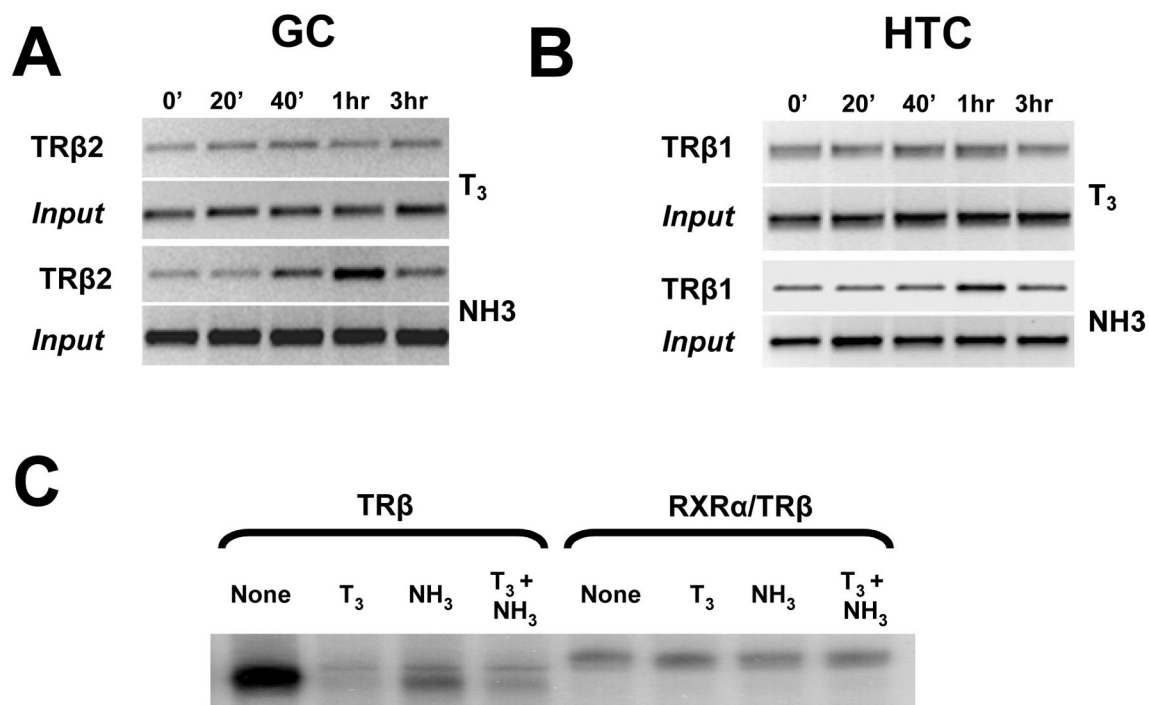


Fig. 6. Effect of T₃ and NH-3 on TR binding to the S14 promoter. **(A)** ChIP analysis performed on GC cells using anti-TRβ2 antibody. **(B)** As above, in HTC cells with anti-TRβ1 antibody. **(C)** Gel shift assay performed with labeled IP-6 oligonucleotide and *in vitro* translated TRβ1 or a mixture of RXRα and TRβ1.

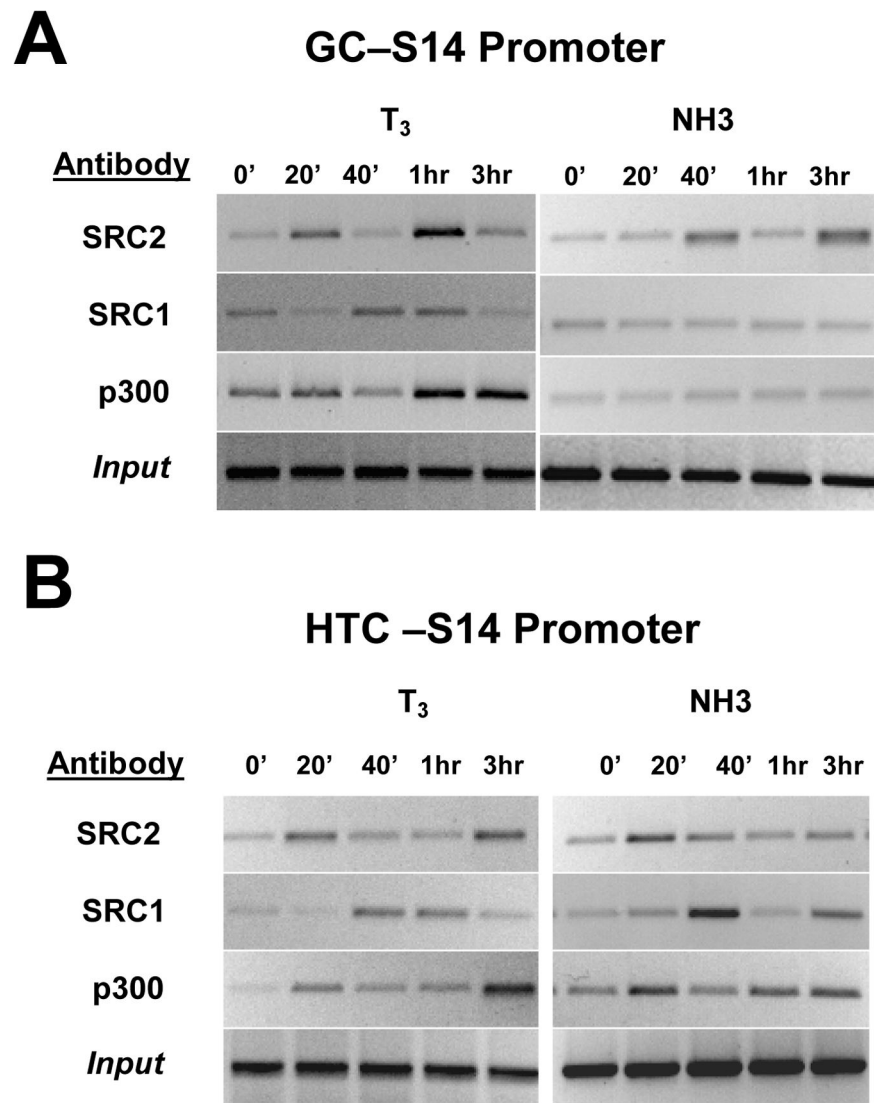


Fig. 7. Recruitment of coactivators. **(A)** Immunoprecipitated chromatin was analyzed for the recruitment of coactivators SRC2, SRC1 and p300 by quantitative PCR in the TRE promoter region of S14 genes in GC cells presence after various times of T₃ and NH-3 treatment. **(B)** As above, using immunoprecipitated chromatin from HTC cells.

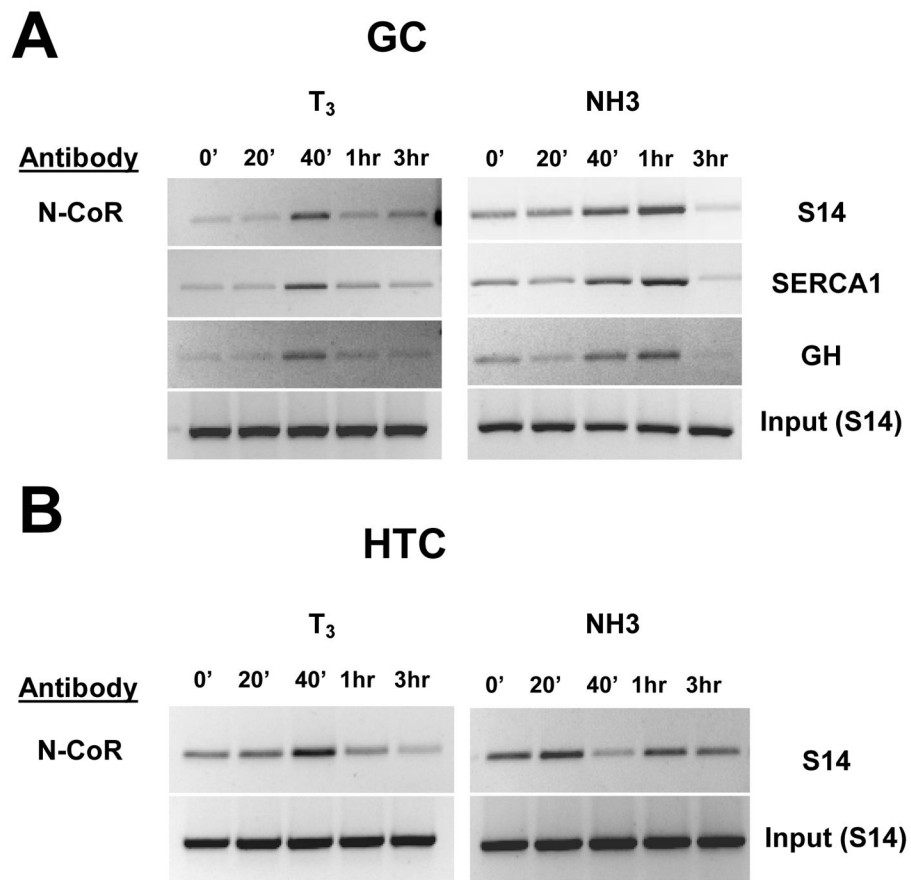


Fig. 8. Recruitment of corepressor N-CoR. Immunoprecipitated chromatin was analyzed by quantitative PCR in the TRE promoter region of (A) S14, SERCA1 and GH genes after various times of T₃ and NH-3 treatment of GC and (B) HTC cells.