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Ligand-induced recruitment of a histone deacetylase in the negative-feedback regulation of the thyrotropin β gene

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We have investigated ligand-dependent negative regulation of the thyroid-stimulating hormone β (TSHβ) gene. Thyroid hormone (T3) markedly repressed activity of the TSHβ promoter that had been stably integrated into GH_3 pituitary cells, through the conserved negative regulatory element (NRE) in the promoter. By DNA affinity binding assay, we show that the NRE constitutively binds to the histone deacetylase 1 (HDAC1) present in GH_3 cells. Significantly, upon addition of T3, the NRE further recruited the thyroid hormone receptor (TRβ) and another deacetylase, HDAC2. This recruitment coincided with an alteration of *in vivo* chromatin structure, as revealed by changes in restriction site accessibility. Supporting the direct interaction between TR and HDAC, *in vitro* assays showed that TR, through its DNA binding domain, strongly bound to HDAC2. Consistent with the role for HDACs in negative regulation, an inhibitor of the enzymes, trichostatin A, attenuated T3-dependent promoter repression. We suggest that ligand-dependent histone deacetylase recruitment is a mechanism of the negative-feedback regulation, a critical function of the pituitary–thyroid axis.

Keywords: histone deacetylases/negative feedback/thyroid hormone/thyrotropin

Introduction

Thyrotropin (thyroid-stimulating hormone; TSH) is a 28 kDa glycoprotein hormone composed of the α and β chains. While the α chain is shared with other hormones, the β chain is unique to TSH. TSH, produced in the anterior lobe of the pituitary, directs the production of thyroid hormones (T3 and T4) in the thyroid gland. Both TSHα and TSHβ are negatively regulated by T3 at the transcriptional level (Chin *et al*., 1993). The T3-mediated negative regulation is an integral part of the function of the hypothalamic–pituitary–thyroid axis, for which ligand-bound thyroid hormone receptor (TR) is required. Patients with a mutation in the TRβ manifest the thyroid hormone resistance syndrome. In these patients, both negative-feedback regulation and positive regulation by T3 are disrupted (Refetoff *et al*., 1993).

In terms of its strict ligand dependence, the negative regulation of TSHβ differs from the transcriptional repression by unliganded TR noted for TRβA and other promoters (Baniahmad *et al*., 1992; Fondell *et al*., 1993; Wong *et al*., 1995), which may be caused by nuclear receptor co-repressors (Chen and Evans, 1995; Horlein *et al*., 1995). The ligand-dependent negative regulation also differs fundamentally from receptor-mediated repression of transcription activation by CREB/AP-1, caused by various ligands, and can be seen by multiple receptors (Saatcioglu *et al*., 1994). This type of repression is thought to occur as a result of competition between receptors and other activators for CBP/p300 (Chakravarti *et al*., 1996; Kamei *et al*., 1996), or of interference of the JNK kinase pathway (Caelles *et al*., 1997).

Because of the physiological importance of this negative regulation for T3 endocrine function, a number of previous studies sought to identify the element responsible for the negative regulation using TSHβ and other promoters (Chin *et al*., 1993). These studies identified the region downstream from the transcription start site of the TSHβ gene as being involved in T3-mediated repression (Wondisford *et al*., 1989; Carr *et al*., 1992; Chin *et al*., 1993; Carr and Wong, 1994; Cohen *et al*., 1995). This region does not contain a known positive T3-responsive element (TRE), such as identified in the promoters activated by T3. Nevertheless, the involvement of TR has been implicated based on the observed binding of TR to the element (Darling *et al*., 1989; Carr and Wong, 1994). Wondisford *et al.* (1993) suggested that this region might function as a composite site involving receptor and other factors, similar to that originally described for the glucocorticoid receptor (Diamond *et al*., 1990). Despite the delineation of the negative element in the TSHβ promoter of several species, the mechanism by which TR represses transcription in a strictly ligand-dependent manner has been elusive. This is in contrast to the rapid progress made in the area of ligand-dependent activation. Transcription activation by T3 involves binding of retinoid X receptor (RXR)–TR heterodimers to the hormone-responsive element, often composed of a direct repeat spaced by four nucleotides (DR4) (Umesono *et al*., 1991). Similar to

other receptor systems, ligand binding changes the conformation of the receptors, which leads to the recruitment of SRC-1 and related factors (Glass *et al*., 1997) as well as CBP/p300 (Chakravarti *et al*., 1996; Kamei *et al*., 1996). These co-activators carry histone acetylase activity (Ogryzko *et al*., 1996; Spencer *et al*., 1997). Coincidentally, an additional histone acetylase, PCAF, is recruited to a ligand-inducible promoter (Blanco *et al*., 1998). The recruitment of multiple histone acetylases is thought to relax histone–DNA interactions in the chromatin to allow enhanced binding of transcription factors to the promoter and stimulation of transcription (Wade *et al*., 1997). Conversely, unliganded heterodimers have been shown to complex with co-repressors such as SMRT and NcoR which are released from the heterodimer by ligand binding (Chen and Evans, 1995; Horlein *et al*., 1995). Recent studies show that the co-repressors are complexed with Sin3 and histone deacetylases (HDACs), which may be involved in transcriptional repression by unliganded receptors (Alland *et al*., 1997; Heinzel *et al*., 1997; Nagy *et al*., 1997).

While a number of reports link histone acetylases with transcriptional activation (Turner, 1991; Brownell and Allis, 1996; Grunstein, 1997; Struhl, 1998), there is strong evidence that HDACs are associated with transcriptional repression found in yeast as well as in mammalian systems (Taunton *et al*., 1996; Kass *et al*., 1997; Pazin and Kadonaga, 1997; Struhl, 1998). The involvement of HDAC has been documented for repression by specific transcription factors YY1, Mad/Max, as well as by unliganded nuclear receptors (Yang *et al*., 1996; Alland *et al*., 1997; Hassig *et al*., 1997; Heinzel *et al*., 1997; Laherty *et al*., 1997; Nagy *et al*., 1997). Furthermore, transcriptional repression by retinoblastoma protein involves interaction with HDACs (Brehm *et al*., 1998; Magnaghi *et al*., 1998). More recent studies indicate that repressed transcription in methylated promoters is partly due to recruitment of HDACs by MePC2 (Jones *et al*., 1998; Nan *et al*., 1998). In these cases, it is thought that HDACs tighten nucleosome–DNA interactions and thereby reduce transcription factor access to the chromatinated promoter (Kass *et al*., 1997).

In the present study, we wished (i) to identify a negative regulatory element (NRE) that is functional in the chromatinated promoter, (ii) to detect nuclear factors that associate with the NRE and (iii) to assess the role of HDACs in negative regulation and in an alteration of chromatin structure in the promoter. We first established a TSHβ reporter stably integrated into GH_3 pituitary cells that can be activated by cyclic AMP and repressed by T3. The chromatin structure of the integrated TSHβ promoter was found to undergo a dynamic alteration by cAMP and T3, consistent with opening and closing of the promoter by these stimuli. Constitutive and T3-mediated repression of the reporter transcription was mapped to the NRE, a sequence downstream from the start site that shares homology with other promoters negatively regulated by ligands. A major thrust of this paper is the observation that HDACs are recruited to the NRE by both ligand-independent and -dependent mechanisms, and that the latter is associated with the recruitment of TRβ1 to the NRE. Providing a mechanism for ligand-induced HDAC recruitment, we show that TRs interact directly with HDAC2, through the DNA binding domain. These results are consistent with previous evidence linking HDACs and transcriptional repression, and indicate their critical role in negative-feedback regulation of the thyroid hormone system.

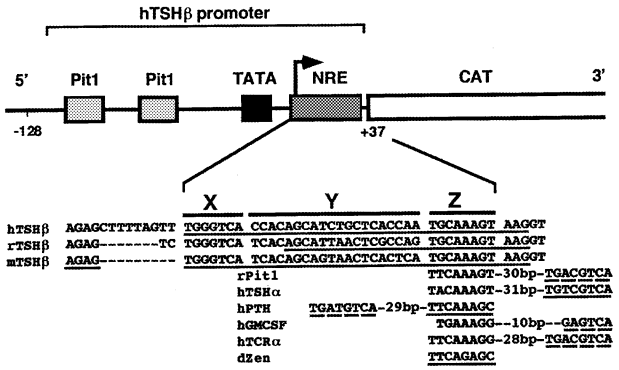

Fig. 1. Structure of the TSHβ promoter: comparison with other negatively regulated promoters. The light gray boxes indicate two Pit 1 sites (activating elements). The dark gray box indicates the NRE. A solid line indicates the regions reported to be involved in negative regulation (see the text). A broken line indicates the region reported to be a cAMP-responsive element.

Results

The human TSHβ reporter stably integrated in GH_3 pituitary cells is activated by cAMP and repressed by T3

With the aim of studying a promoter that is negatively regulated by T3 in a native chromatin context, we first established stable GH_3 clones harboring a human TSHβ promoter connected to the CAT reporter gene (see Figure 1 for a map). The use of transfectants was necessary as tissue culture cells of thyrotroph origin which express the endogenous TSHβ gene have not been available to date. The activity of the TSHβ promoter (from –128 to +37; Figure 1) was tested in 12 clones after treatment with T3 (1 μM) followed by treatment with dibutyryl cyclic AMP (cAMP, 1 mM) for 8 h (see Figure 2A for the time course). Results in Figure 2B show CAT activity observed with clone L017, chosen for further study. Addition of cAMP enhanced CAT activity by ~8-fold over background, which is probably due to Pit1 activated by protein kinase A (Steinfelder *et al*., 1991, 1992a,b). Pre-incubation of cells with T3 (1 μM) repressed reporter activity when cells were treated with or without cAMP. Repression was much more prominent when cells were treated with cAMP (3- to 4-fold repression). T3-mediated repression was maximal at 0.1–1 μM T3, although partial repression was observed even at 1 nM ligand (Figure 2C). Results in Figure 2D show that TSHβ reporter activity was repressed not only when cells were pre-treated with T3 followed by cAMP, but also when they were treated simultaneously with cAMP and T3. However, repression was not seen when T3 was added after cAMP treatment. This reporter was also stimulated by treatment with forskolin (10 μM), another protein kinase A activator (Steinfelder *et al*., 1992a). The forskolin stimulation of reporter activity was also repressed by T3 (not shown). These results establish that the stably integrated TSHβ promoter is negatively regulated by T3 in the GH_3 pituitary cells, mimicking

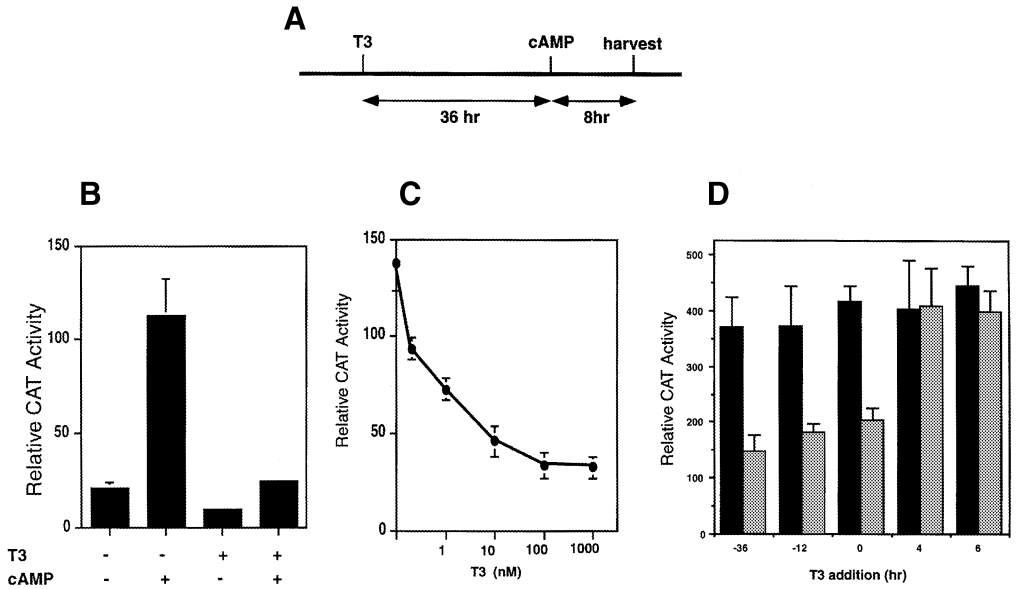

Fig. 2. Characterization of the hTSHβ reporter stably integrated into GH_3 cells: activation by cAMP and repression by T3. (**A**) Timing of T3 and cAMP treatment. L017 cells, a clone stably transfected with the hTSHβ CAT reporter, were treated with T3 (1 μM) for 36 h and with dibutyryl cAMP (1 mM) for 8 h before harvesting. (**B**) T3-mediated repression of CAT activity. L017 cells were then treated with T3 and cAMP as in (A). Relative reporter activity was estimated by normalization with protein concentrations. Values represent the average of three assays ± SD. (**C**) T3 dose dependence. L017 cells were treated with the concentrations indicated of T3 with the time course shown in (A), and reporter activity was measured as in (B). (**D**) Timing of T3 addition. T3 (1 μM) was added to L017 cells at the times indicated before (–) or after addition of cAMP (1 mM), and reporter activity was measured as in (B). Black bars indicate cAMP (1 mM) alone and gray bars indicate T3 (1 μM) and cAMP (1 mM).

the ligand-dependent negative-feedback regulation of the TSHβ gene *in vivo*. T3-dependent repression of the TSHβ reporter activity was observed following transient transfection of the reporter as well (Figure 3).

Deletion analysis: role for the NRE in negative regulation of TSHβ promoter activity

Previous reporter analyses performed in transient assays indicated that the sequence downstream from the transcription start site is involved in T3-dependent repression of the rat and human TSHβ promoters (Wondisford *et al*., 1989; Carr *et al*., 1992; Chin *et al*., 1993; Carr and Wong, 1994; Cohen *et al*., 1995). As depicted in Figure 1, this element, the NRE, includes the sequence designated Z, which is conserved in the human, rat and mouse TSHβ gene as well as in the Pit1 and TSHα genes (McCormick *et al*., 1990; Pennathur *et al*., 1993). These genes are known to be negatively regulated by T3 (Chin *et al*., 1993; Sanchez *et al*., 1995). A sequence similar to Z is also present within the negative regulatory element of granulocyte-macrophage colony-stimulating factor (GM-CSF) and parathyroid hormone (PTH) gene is negatively regulated by vitamin D_3 (Demay *et al*., 1992; Towers and Freedman, 1998). Immediately upstream from Z, there is the sequence XY (Figure 1) that resembles a cAMP-responsive element, which is also conserved among TSHβ genes. To study the role of the NRE, particularly of Z, in detail, deletion constructs lacking the Z region or the Z plus XY regions were tested by both stable and transient transfection assays (Figure 3A). Stable promoters were analyzed with pooled GH_3 cells after cells were co-transfected with the reporters and the neomycin resistance gene and selected under G418 (Materials and methods). Results are shown in Figure 3B. Results of transient assays are shown in Figure 3C. We found that constitutive CAT activities of these deletion constructs were significantly higher than that of the wild-type reporter both in transient and stable assays. These deletions nevertheless responded to cAMP to increase CAT activity further. However, importantly, these deletion constructs exhibited less repression when cells were treated with T3 and cAMP, as compared with repression seen by the wild-type reporter, both in stable and transient assays (see fold repression in Figure 3D). These results indicate that TSHβ promoter activity is repressed constitutively as well as following T3 addition, and that repression is mediated by the Z region in both cases. It is of note that these deletions did not completely relieve the T3-dependent effect. The residual T3-dependent repression is likely to be attributable to competition between liganded TR and activators for CBP/p300 (Chakravarti *et al*., 1996; Kamei *et al*., 1996), and/or to ligand-induced inhibition of the Jun N-terminal kinase (JNK) cascade (Caelles *et al*., 1997).

Binding of HDACs to the NRE: ligand-dependent recruitment of HDAC2 and TRβ1

Although T3-mediated repression is localized to the NRE, the mechanism by which this element exerts transcriptional repression in a ligand-dependent manner has been obscure. This problem is partly attributed to the lack of information on factors that are assembled on the NRE during repression. To address this issue, we employed a DNA affinity binding assay in which factors that bind to the NRE were detected on a solid bead matrix (see Figure 4A for a diagram). Four copies of the NRE (containing XYZ) were conjugated to the beads and incubated with nuclear extracts from GH_3 cells in the presence or absence of T3. Bound materials were eluted and tested by immunoblot analysis.

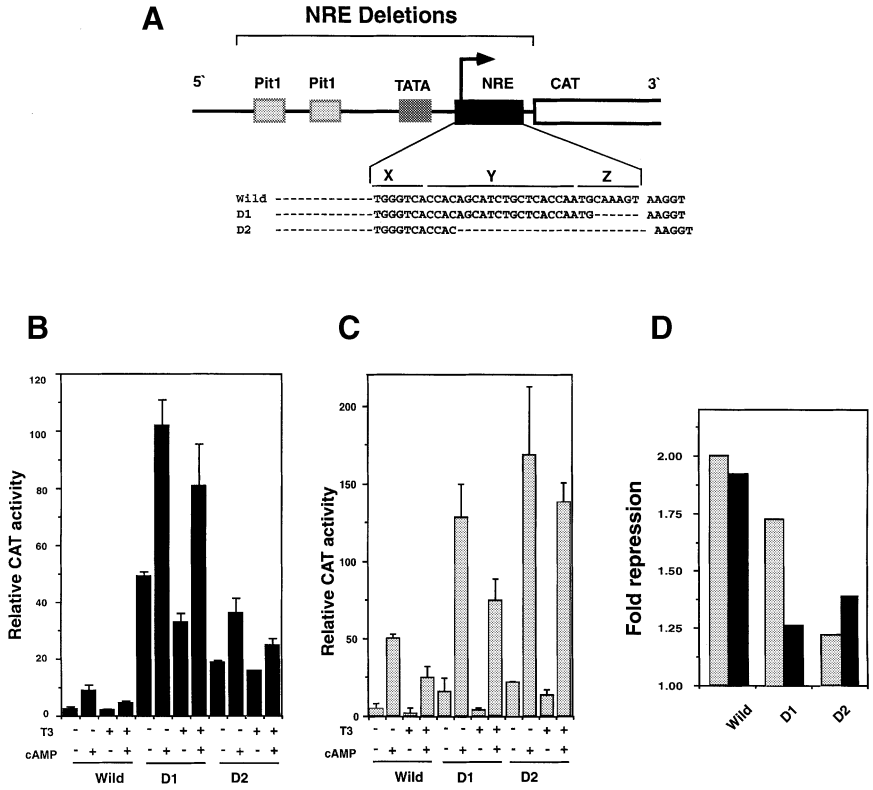

Fig. 3. NRE deletion analysis. (**A**) Deleted nucleotides in constructs D1 and D2 are shown by '–'. (**B**) CAT activity by stable transfection. GH_3 cells were stably transfected with the wild-type or deletion CAT reporters. G418-resistant cells were pooled and treated with 1 μM T3 for 36 h and 1 mM cAMP for 8 h, and reporter activity was normalized by protein concentration. (**C**) GH_3 cells were transiently transfected with the same reporters and treated with T3 and cAMP as in (B). Relative CAT activity was normalized with β-galactosidase activity. (**D**) Attenuated negative regulation as fold repression. Black bars indicate values obtained from stable transfectants and gray bars indicate values from transient transfection.

As a control for TR binding, the positive T3-responsive element DR4 DNA (three copies) (Umesono *et al*., 1991) was conjugated to the beads and tested in parallel. As shown in Figure 4B, the NRE–beads recruited the histone deacetylase, HDAC1, both in the presence and absence of ligand. Significantly, another histone deacetylase, HDAC2, was recruited to the NRE–beads only upon T3 addition. These HDACs were not detected on the DR4–beads under these conditions, indicating that the NRE, but not DR4, associates with HDACs with high affinity. It should be noted here that using extracts from other cells and recombinant (r) TR, binding of HDACs to DR4 was observed, indicating that recruitment of HDACs to the positive element can be seen depending on the cell type and sensitivity (S.Sasaki and K.Ozato, unpublished) (see below; Figure 5).

Results in Figure 4B also show that TRβ1 was recruited to the NRE–beads in a ligand-dependent manner. On the other hand, the DR4–beads recruited TRβ1 in a ligand-independent manner, as would have been expected (Blanco *et al*., 1998). Also expected and observed was the binding of co-activator, CBP, to the DR4–beads in a T3-dependent manner (Chakravarti *et al*., 1996; Kamei *et al*., 1996; Blanco *et al*., 1998) (Figure 4B). Contrary to the results with DR4, CBP binding to the NRE–beads was weak and almost undetectable. Thus, T3 stimulates recruitment of a complex that contains TRβ1 and HDAC2 to the NRE. Confirming the specificity of recruitment, TRβ1 binding to the NRE was competed by free NRE (Figure 4C). As shown in Figure 4D, binding of HDAC2 and TRβ1 occurred in a nuclear extract dose-dependent manner. It is interesting that while the level of TRβ1 recruited to the NRE–beads was comparable to or slightly less than that to the DR4–beads, HDAC2 recruitment was detected only with the NRE–beads.

In order to assess whether binding of TRβ1 to the NRE is dependent on the formation of a heterodimer with RXR, electrophoretic mobility shift assay (EMSA) was performed with rTRβ1 and rRXRβ. As presented in Figure 4E, rTRβ1 alone could bind to the NRE probe in a ligand-independent manner. In contrast, rRXRβ failed to bind to this sequence. Co-addition of rTRβ1 and rRXRβ did not enhance binding activity for the NRE, but rather appeared to decrease it. Binding of rTRβ1 was competed by excess NRE as well as DR4 oligomers, but not by control unrelated oligomers (NS in Figure 4E). In contrast, rTRβ1 and rRXRβ bound to DR4 as a heterodimer, as expected (not shown). Thus, TRβ1 is capable of binding to the NRE without RXR, indicating that this binding does not involve the formation of heterodimer with RXR. Taken together, these results show that T3 induces recruitment of HDAC2 as well as TRβ1 to the NRE. Combined with constitutive binding of HDAC1, multiple HDACs are assembled on the NRE, a feature not found with the positive DR4 element.

Direct interaction of TRβ1 with HDAC2

Data in Figure 4 raised the possibility that TRβ1 recruits HDAC2 to the NRE by protein–protein interaction. Here we tested whether TR is capable of interacting directly

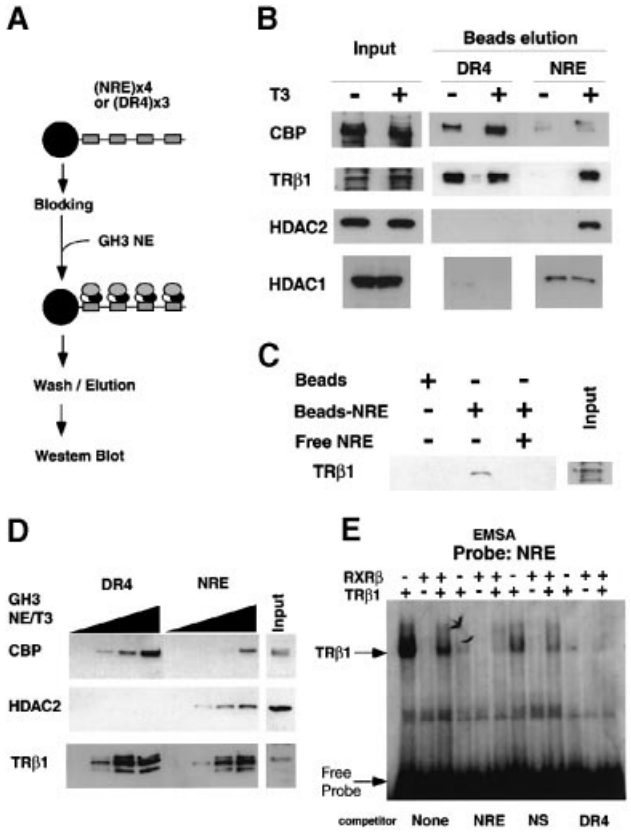

Fig. 4. Recruitment of HDACs to the NRE. (**A**) Diagram of the DNA affinity binding assay. Four copies of the NRE (XYZ) or three copies of the positive T3-responsive elements (DR4) were conjugated to the beads and incubated with nuclear extracts from GH_3 cells in the presence or absence of T3 (1 μM). (**B**) Immunoblot analysis of materials eluted from the NRE– or DR4–beads. Input represents unfractionated nuclear extracts from GH_3 cells. (**C**) Competition by free NRE. Nuclear extracts were incubated with unconjugated beads (–) or NRE-conjugated beads in the presence of 100-fold molar excess of NRE (+) or sermon sperm DNA (–) with T3 (1 μM). (**D**) Nuclear extract dose dependence. NRE– or DR4–beads were incubated with increasing amounts of GH_3 extracts (from 300 to 1800 μg) in the presence of T3. The amounts of bound materials were detected by immunoblot assays. (**E**) Binding of rTRβ1 and rRXRβ to the X + Y: EMSA analysis. ^{32}P-labeled X + Y probe (5′-TTTGGGTCACCA-CAGCAT-3′) was mixed with recombinant receptors in the presence of the oligonucleotide competitors indicated at 100-fold molar excess.

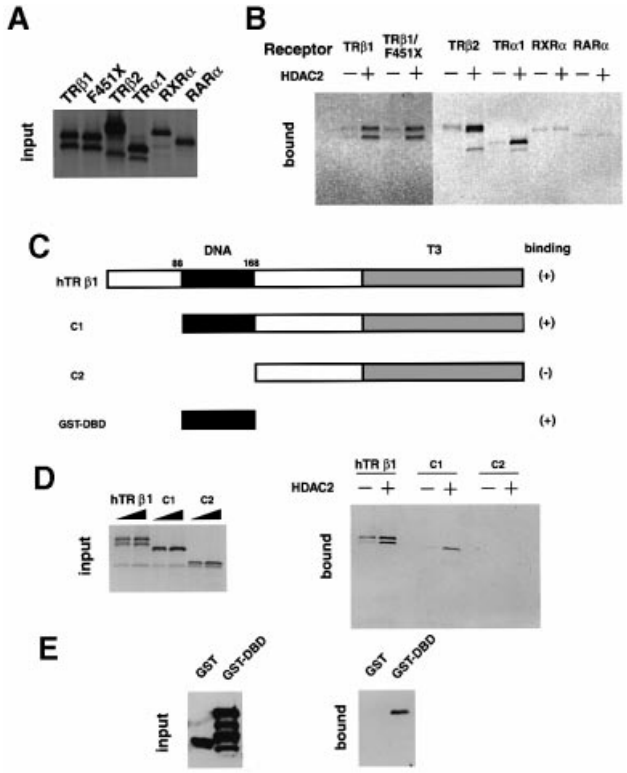

Fig. 5. Interaction between TRs and HDAC2. (**A**) Input ^{35}S-labeled TRs, RXRβ and RARα were prepared by *in vitro* translation of appropriate receptor plasmids. (**B**) Ni–NTA beads bound to rHDAC2 (+) or control extracts from uninfected cells (–) were incubated with ^{35}S-labeled receptors in (A). Bound materials were fractionated in 10% SDS–PAGE and visualized by autoradiography. (**C**) Diagram of TRβ deletions. (**D**) ^{35}S-labeled TRβ deletions (input shown in the left panel) were tested for binding to Ni–NTA beads with or without HDAC2. (**E**) GST–TRβ DBD or control GST peptide (input in the left panel) was tested for binding to Ni–NTA beads conjugated to HDAC2. GST peptides were detected by immunoblot assay using anti-GST antibody.

with HDAC2. Results in Figure 5 show binding of ^{35}S-labeled TRs to baculovirus rHDAC2 immobilized on Ni–agarose beads. In addition to TRβ1, ^{35}S-labeled TRβ2, TRα and a mutant TRβ1 from a thyroid hormone resistance syndrome patient (F451X) (Miyoshi *et al.*, 1998), as well as RXRβ and RARα, were tested in these assays (Figure 5A). All TRs, including the mutant F451X that lacks the AF-2 domain (Miyoshi *et al.*, 1998), bound avidly to HDAC2. In contrast, RXRβ and RARα failed to bind to HDAC2 (Figure 5B). None of these receptors bound to the control beads without HDAC2, as expected. Binding of TRs was not affected by addition of T3. Similarly, binding of RXR and RAR was not induced by 9cis-RA, a ligand for the receptors (not shown). These results show that thyroid hormone receptors can interact directly with HDAC2, and this ability is not shared with other receptors tested here.

In the light of strong HDAC2 binding observed by the F451X mutant, it was of interest to investigate which domain in TRβ1 binds to HDAC2. Two deletion constructs C1 and C2, lacking the N-terminal domain and DNA binding domain (DBD), were tested for their ability to bind to immobilized HDAC2 (diagram in Figure 5C). As shown in Figure 5D, the wild-type TRβ1 and C1 lacking the N-terminal domain only bound to HDAC2. In contrast, C2, lacking both the N-terminal domain and DBD, failed to bind to HDAC2, indicating involvement of the DBD in HDAC binding. To assess the role of DBD further, we tested binding of the glutathione *S*-transferase (GST) fusion protein containing the DBD of TRβ1. As seen in Figure 5E, this GST–DBD fusion, but not a control GST peptide, strongly bound to HDAC2. These results indicate that TRβ1 interacts with HDAC2 primarily through the DBD.

Histone deacetylase enzymatic activity on the NRE and its effect on T3-mediated transcriptional repression

To ascertain whether protein complexes formed on the NRE in Figure 4 possess HDAC enzymatic activity, GH_3 extract proteins bound to the NRE–beads were eluted and tested for HDAC activity. As a comparison, enzymatic activity was also measured for samples eluted from the DR4 beads.

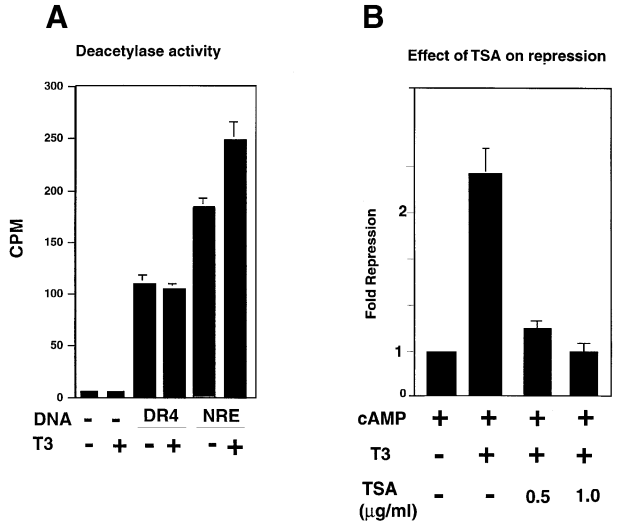

Fig. 6. HDAC activity and the effect of specific deacetylase inhibitor. (**A**) HDAC activity. Beads without DNA (–), DR4- or NRE-conjugated beads were incubated with GH_3 extracts in the presence or absence of T3 as in Figure 4B. Bound and eluted materials were tested for HDAC activity using *in vivo* ^{3}H-labeled histone. Values represent the average of three assays ± SD. (**B**) The effect of TSA on TSHβ reporter activity. L017 cells were treated with T3 and cAMP as in Figure 2A. TSA was added at the concentrations indicated 24 h before harvest. Values represent the average of three experiments ± SD.

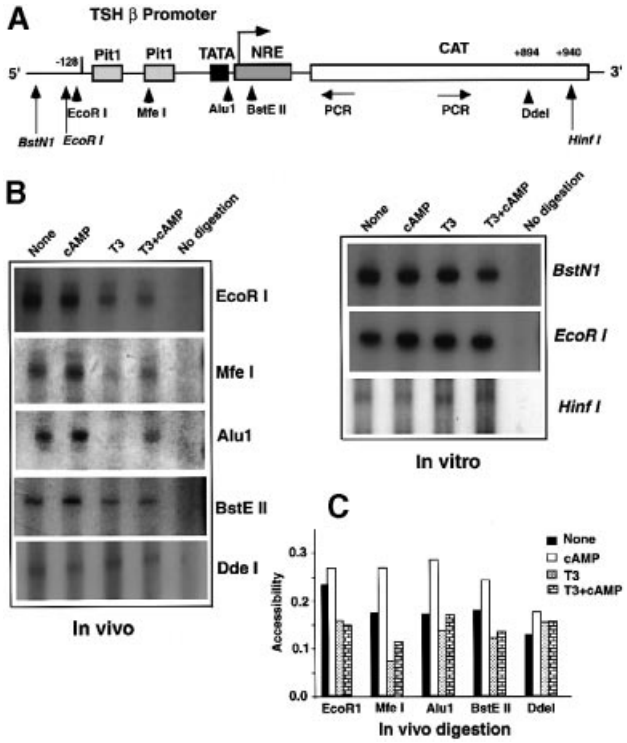

Fig. 7. Restriction site accessibility assay. (**A**) Diagram of restriction sites. Arrowheads indicate the sites for restriction enzymes used for digestion *in vivo*. Thin arrows indicate sites for digestion *in vitro* (used as controls, in italics). Digested materials are detected by linear PCR using primers corresponding to the indicated positions. (**B**) Nuclei from L017 cells treated with T3 and cAMP were partially digested with the indicated enzymes (*Eco*RI, *Mfe*I, *Alu*I, *Bst*EII, *Dde*I) *in vivo*. Purified DNA was then digested to completion with the second enzymes *Bst*NI (or *Eco*RI) and *Hin*fI. Digestion products were detected by linear PCR. Undigested nuclei were tested as a control for endogenous nuclease activity. The restriction site for each enzyme is shown on the top. PCR products generated by *in vivo* digestion were compared with those generated by *in vitro* digestion (shown in italics). (**C**) PCR products were quantified using the NIH Image Program (Version 1.61). Products of *in vivo* digestion were normalized by those of *in vitro* digestion.

As shown in Figure 6A, while samples from the control beads (which had no DNA) contained a background level of enzymatic activity, those recovered from the NRE beads showed high levels of HDAC activity. On the NRE–beads, T3-treated samples gave greater enzymatic activity than untreated samples, consistent with the ligand-induced recruitment of HDAC2 seen in Figure 4. The enzymatic activity recovered from DR4 was substantially lower than that from the NRE, and the former was not affected by addition of T3. Nevertheless, samples recovered from DR4 did exhibit enzymatic activity significantly higher than background, suggesting that, albeit to a lesser degree, HDACs in GH_3 cells were recruited to the DR4 element as well.

To assess whether HDAC activity accounts for T3-mediated repression of TSHβ transcription, we tested the effect of a HDAC inhibitor, trichostatin A (TSA) (Yoshida *et al*., 1995; Minucci *et al*., 1997), on TSHβ promoter activity in L017 cells (Figure 2). L017cells were treated with T3 and TSA, followed by cAMP. As shown in Figure 6B, in the absence of TSA treatment, T3 addition repressed transcription by ~3 fold. However, T3-dependent repression was markedly reduced in the presence of TSA. The repression was reduced at both doses of TSA tested, even though TSA treatment increased CAT activity in the absence of T3 as well, as would have been expected (Minucci and Ozato, 1996). These results further support the involvement of HDACs in T3-mediated repression.

Chromatin structure analysis: T3-dependent changes in restriction site accessibility

In the hope of studying a possible chromatin structure alteration that would correlate with T3-induced repression of TSHβ promoter activity, several methodologies were investigated. One of them was DMS-based genomic footprinting, a widely used approach to detect factor-occupied G-residues (Dey and Ozato, 1997). However, with this technique, no discernible footprint could be observed on this promoter (not shown). On the other hand, restriction site accessibility assay has allowed us to delineate chromatin changes in the TSHβ promoter in response to T3. This assay utilizes endonuclease sensitivity as a measure of chromatin structure alterations, and has been used for analysis of ligand-responsive promoters (Archer *et al*., 1991; Bhattacharyya *et al*., 1997). Nuclei from clone L017 treated with cMP and/or T3 were digested *in vivo* with *Eco*RI, *Mfe*I, *Alu*I or *Bst*EII, which cut within the promoter (see the diagram in Figure 7A, arrowheads). *Dde*I, which cuts outside the promoter, was also used for *in vivo* digestion as an internal control. DNA was then purified and digested *in vitro* with the second enzymes *Bst*NI (or *Eco*RI) or *Hin*fI (italics in Figure 7A). Digestion products were detected by linear PCR using primers shown in Figure 7A and quantified by densitometry scanning (Figure 7B and C). Samples treated with cAMP gave increased sensitivity to all four enzymes that cut in the promoter (*Eco*RI, *Mfe*I, *Alu*I, *Bst*EII), albeit to a variable degree. Furthermore, samples treated with T3 (alone or with cAMP) showed markedly reduced sensitivity to these

enzymes. Sensitivity to *Dde*I remained unchanged by the treatments, indicating that changes occurred within, but not outside, the promoter. Verifying comparable PCR reactions and equal sample loading, PCR products generated by *in vitro* digested DNA (*Bst*NI, *Eco*RI, *Hin*fI; see the top in Figure 7B) were comparable among samples of different treatments. In addition, undigested DNA gave no PCR products, confirming that the PCR products are a result of digestion by these enzymes, rather than spurious *in vivo* DNA cleavage by endogenous nucleases. Similar results were observed with six separate preparations of nuclei. Thus, restriction site accessibility in the TSHβ promoter is increased by cAMP and decreased by T3, coinciding with promoter activation and repression. These changes probably reflect alterations of chromatin structure in the promoter.

Discussion

The present paper shows that T3 induces recruitment of HDAC2 and TRβ1 to the NRE of the TSHβ promoter. Combined with the constitutive association of another enzyme, HDAC1, the NRE is thus a site at which high levels of HDAC activity can accumulate. Importantly, the ligand-induced recruitment of these proteins to the NRE coincided with reduced nuclease accessibility in the promoter *in vivo* and with repression of promoter activity. In line with the involvement of HDAC in negative regulation, TSA alleviated T3-dependent repression of TSHβ promoter activity.

Ligand-dependent HDAC recruitment

These observations lend credence to a model (Figure 8) in which HDAC recruitment at least partly accounts for ligand-mediated negative-feedback regulation of the TSHβ gene. This model, unlike other models put forward previously, offers a basis for ligand specificity, a key feature of negative-feedback regulation. However, additional mechanisms, proposed earlier, are also likely to have a role in this negative regulation, i.e. competition for co-activators p300/CBP (Kamei *et al*., 1996) and inhibition of the JNK kinase pathway by nuclear hormone receptors (Caelles *et al*., 1997).

There is strong evidence indicating that HDACs are involved in transcriptional repression at a number of eukaryotic genes (Grunstein, 1997; Kass *et al*., 1997; Pazin and Kadonaga, 1997; Brehm *et al*., 1998; Jones *et al*., 1998; Magnaghi *et al*., 1998; Nan *et al*., 1998). Deacetylases are often complexed with Sin3, and nuclear receptor co-repressors, which themselves possess repressive activity, as well as RbAp48 (Taunton *et al*., 1996; Caelles *et al*., 1997; Hassig *et al*., 1997; Heinzel *et al*., 1997; Laherty *et al*., 1997; Nagy *et al*., 1997). In this study, HDAC2 and TRβ1 were co-recruited to the NRE after T3 addition (Figure 4). The observations that TRβ1 and other TRs bind directly to HDAC2 (Figure 5), and that TRβ1 binds to the NRE *in vitro* (Figure 4), strongly indicate that the liganded TR complexes with HDAC2 on the NRE.

The ligand-dependent HDAC recruitment observed in the present work would not have been anticipated based on previous studies with TR bound to DR4, where only unliganded RXR/TR complexes with HDACs (through co-repressors SMRT and NCoR) (Heinzel *et al*., 1997; Nagy *et al*., 1997). Upon ligand addition, HDACs are thought to be dissociated, along with the release of co-repressors from the heterodimer. The present work shows that, contrary to the expectation based on DR4-derived data, liganded TR can interact with and recruit HDAC2 to the NRE. These results raise the possibility that co-repressors are not involved in HDAC recruitment to the NRE. Several studies (Olson and Koenig, 1997; Zamir *et al*., 1997; Olson *et al*., 1998) indicate that interactions with co-repressors require heterodimer formation. Our data in Figure 4E, along with the previous work, suggest that RXR is not involved in NRE binding (Carr and Wong, 1994; Cohen *et al*., 1995; Hallenbeck *et al*., 1996). Supporting this view, excess co-repressors appear to interfere with T3-dependent negative regulation by squelching TR (Tagami *et al*., 1997).

In our domain analysis (Figure 5), binding of TRβ1 to HDAC2 did not require the AF-2 domain, but occurred through the DBD. This result poses an interesting implication for the mechanism of HDAC action, in that this interaction may facilitate HDAC access to chromatinated promoter. The role of DBD in recruiting HDAC is also intriguing in view of the fact that the histone acetylases PCAF and GCN5 are also recruited to nuclear receptors through the DBD (Blanco *et al*., 1998). The fact that both acetylases and deacetylases interact with the DBD of a nuclear receptor strengthens the idea that these interactions represent a shared mechanism by which the two enzymes gain access to a ligand-responsive promoter and affect chromatin environment.

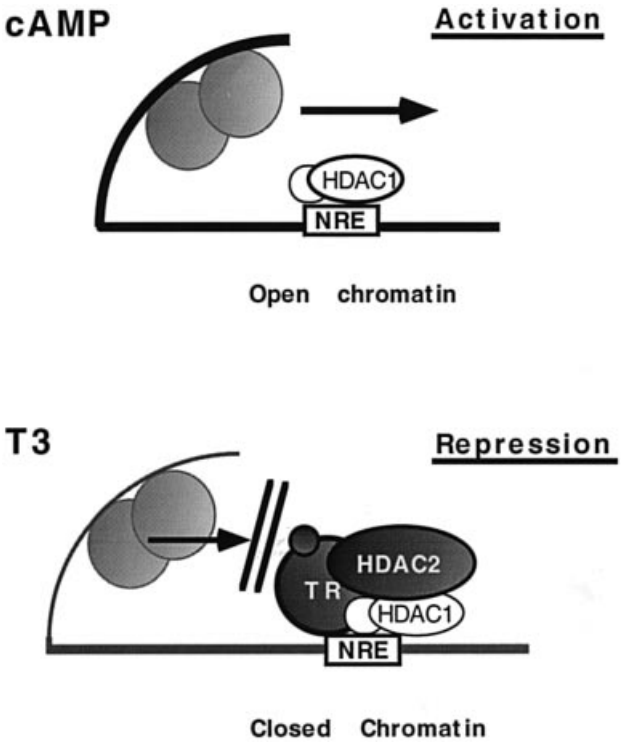

Fig. 8. A model for T3-mediated recruitment of HDAC to the NRE in the TSHβ promoter. cAMP activates transcription of the TSHβ promoter (even though HDAC1 is bound to the NRE), coinciding with the opening of chromatin as demonstrated by increased restriction site accessibility. T3 addition recruits TR and HDAC2 to the NRE, resulting in the closing of chromatin and transcriptional repression.

Despite the fact that the endogenous TRβ and HDAC2 were recruited to the NRE in a ligand-dependent manner, interactions by the recombinant counterparts were found to be ligand independent (Figures 4 and 5). This seeming discrepancy may be attributable to another factor postulated to be present in the extracts that can confer ligand-dependent binding of TRβ to the NRE. Although this hypothetical factor has yet to be identified, involvement of an additional factor in conferring ligand dependence has been demonstrated for nuclear receptor–PCAF interaction (Blanco *et al*., 1998). It is possible that this hypothetical factor, together with the NRE itself, dictates ligand-dependent binding of TRβ1 and recruitment of HDAC2.

Role for HADCs in chromatin alterations

HDACs are thought to repress transcription by increasing the affinity of nucleosomal histones for DNA, this in turn decreasing the accessibility of transcription factors to a promoter (Grunstein, 1997; Kass *et al*., 1997; Pazin and Kadonaga, 1997). Thus, HDACs are implicated in alterations of chromatin structure that are associated with repression. We found that endonuclease sensitivity within the TSHβ promoter is markedly decreased by T3, which would suggest closing of chromatin that is associated with transcriptional repression (Figure 8). These results are in accordance with other studies in which alterations in restriction site accessibility correlate with ligand-mediated changes in transcription (Archer *et al*., 1991; Bhattacharyya *et al*., 1997). Given that ligand-dependent repression is mediated through the NRE, the site of repression, T3-dependent chromatin alterations may well be a direct consequence of HDAC recruitment.

Similarity of the NRE to other negative regulatory elements

Similarly to the TSHβ gene, the TSHα and Pit1 genes involved in thyroid hormone regulation in the pituitary are also negatively regulated by T3 (McCormick *et al*., 1990; Pennathur *et al*., 1993; Sanchez *et al*., 1995). Significantly, the previously reported negative regulatory regions of these two genes share a high level of sequence homology with the Z region of the TSHβ NRE (Figure 1). As in the TSHβ gene, the negative regulatory regions of these genes do not contain a known TRE such as identified in promoters positively regulated by T3. In addition to the similarity with Z, both genes carry a sequence resembling X in close proximity with the Z-like motif. Furthermore, the GM-CSF and PTH genes, both negatively regulated by vitamin D_3 (Demay *et al*., 1992; Towers and Freedman, 1998), have a sequence homologous to Z. Considering that the Z sequence plays a key role in repression (Figure 3), ligand-dependent negative regulation of these genes may share a common mechanism, involving HDAC recruitment. The Z sequence has homology with a high-mobility-group-protein binding site of the T-cell receptor α gene (Mayall *et al*., 1997) and the negative regulatory element of the *Drosophila* Zen promoter (Lehming *et al*., 1994). In conclusion, the present study demonstrates that liganded TR plays a role in recruiting HDAC activity to the NRE to exert negative-feedback regulation on the TSHβ gene. A similar mechanism may be postulated for other genes repressed by ligands.

Materials and methods

Plasmids

The CAT reporter gene connected to the human TSHβ promoter (–128/+37) has been reported (Wondisford *et al*., 1989). In this study, a modified CAT construct, hTSHβCAT, was used from which the AP-1 site present in the original vector was deleted. NRE deletion reporters D1 and D2 were constructed by PCR using the following primers: 5′ primer for D1 (5′-GTTTGGGTCACCACAGCATAAGAGCTTGG-CGAGATTTTCAG-3′) or D2 (5′-GTTTGGGTCACCAAGAGCTTG-GCGAGATTTTCAG-3′) and a single 3′ primer, (5′-GAGCGATGA-AAACGTTTCAGTTTG-3′). PCR fragments were digested with *Bst*EII and *Eco*RI, and inserted into the hTSHβCAT digested with the above enzymes. The human TRβ1 expression vector, pCMX-humanTRβ1, is gift from R.Evans. We digested this plasmid with *Kpn*I and *Bam*HI, and ligated it into the pAC-HLT-C baculovirus transfer vector (Pharmingen), resulting in pAC-HLT-C-humanTRβ1. The plasmid for mouse RXRβ has been described elsewhere (Blanco *et al*., 1998). The mouse HDAC2 (formerly RPD3) cDNA was excised from pcDNA I/Amp-mouse HDAC2 (Yang *et al*., 1996) and inserted into pAC-HLT-A.

Stable and transient transfection of hTSHβCAT in GH_3 cells

GH_3 cells, a rat pituitary-derived cell line, were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS). A total of 5×10^5 cells were transfected with hTSHβCAT (6.7 μg) and pSV2-neo (6.7 μg) using the lipofectamine reagent (Gibco-BRL). Cells were cultured in the complete medium for 48 h prior to selection by G418 (700 μg/ml) for 3–5 weeks. Of 12 clones tested for T3-dependent repression in reporter activity, clone L017 was chosen for further study. To prepare pooled stable transfectants (Figure 3), GH_3 cells were transfected with 6.7 μg of hTSHβCAT, NRE deletion constructs D1 or D2, along with 6.7 μg of pSV2-neo. Three to four weeks after selection, ~1000 colonies resistant to G418 were pooled and tested for CAT activity within 3–6 weeks of harvest. For transient assay, 1×10^5 cells were transfected with 2 μg of hTSHβCAT or NRE deletion reporter and 1 μg of pCH110 (Pharmacia Biotech) containing the β-galactosidase gene using the lipofectamine reagent for 5 h and then cultured in phenol red-free DMEM supplemented with 10% resin triple-split calf serum with or without T3 for 36 h. Cells were then treated with dibutyryl cAMP or forskolin (both from Sigma) for an additional 8 h before harvesting. CAT activity in stable transfection was normalized according to protein concentrations. CAT activity in transient assays was normalized by β-galactosidase activity or protein concentrations, which gave similar outcome.

DNA affinity binding assay

Nuclear extracts from GH_3 cells were prepared as follows. Cells were suspended in one packed cell volume of buffer A (10 mM HEPES pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl) and sheared by a 26 G needle five times on ice. They were centrifuged at 14 000 r.p.m. for 10 min and resuspended in elution buffer (20 mM HEPES pH 7.9, 25% glycerol, 420 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA) for 30 min at 4°C. The samples were dialyzed against buffer D [20 mM HEPES pH 7.9, 20% glycerol, 0.2 EDTA, 0.5 mM dithiothreitol (DTT), 42 mM $(NH_4)_2SO_4$] at 4°C for 2 h, and stored in liquid nitrogen.

Four copies of the NRE DNA were cloned into pUC19 and biotinylated by PCR using a biotin-labeled M13 universal primer and a unique primer corresponding to the 3′ end of the NRE, and purified on a QIAquick column (Qiagen). Three copies of DR4, a positive thyroid-responsive element (Umesono *et al*., 1991), were also cloned in pUC19, and biotinylated in the same manner. Forty micrograms of biotinylated NRE and 42 μg of DR4 DNA were conjugated to 10 mg of M280 magnetic beads (Dynal) in 4 ml of TEN buffer (10 mM Tris–HCl pH 7.5, 1 mM EDTA, 100 mM NaCl) for 1 h at room temperature. Beads were washed in TEN buffer and blocked with 0.5% non-fat milk in binding buffer (20 mM Tris–HCl pH 7.4, 10% glycerol, 2 mM EDTA, 0.02 Triton X-100, 100 mM KCl) for 1 h at room temperature. DNA-conjugated beads (0.4 μg of DNA corresponding to 2 pmol of the NRE corresponding to 40 μl beads) were mixed with 300 μl (~1800 μg protein) of GH_3 nuclear extracts and incubated at 4°C for 4 h with constant rotation. The suspensions were precipitated with the magnetic plate, washed in binding buffer and re-precipitated by centrifugation. The bound materials were eluted with 7 μl of BC500 buffer (500 mM KCl, 20 mM HEPES pH 7.9, 10% glycerol, 0.5 mM EDTA). Materials eluted from the beads were analyzed by immunoblot assay as described (Blanco *et al*., 1998). Rabbit anti-CBP antibody was a gift from H.Serizawa (Kansas University).

Mouse anti-TRβ1 antibody was purchased from Santa Cruz (J52). Rabbit anti-HDAC1 antibody was prepared by using purified recombinant human HDAC1 produced in a baculovirus vector as an immunogen. Rabbit anti-HDAC2 antibody was prepared by using purified recombinant mouse HDAC2 (Yang *et al*., 1996) as immunogen.

Recombinant protein preparation

We co-transfected Sf9 cells with pAC-HLT-C-humanTRβ1 or pAC-HLT-A-mouse HDAC2 plasmid together with Baculogold viral genomic DNA (Pharmingen) using the manufacturer's protocol. Sf9 cells (1×10^8) were incubated with 5 ml of recombinant viral supernatant and kept at 27°C for 2 days (humanTRβ1) or 3 days (mouse HDAC2). The infected cells were washed with phosphate-buffered saline (PBS) twice and suspended with hypotonic buffer (10 mM HEPES pH 7.9, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA) for 10 min on ice. The cells were homogenized using a Teflon homogenizer for 30 strokes on ice. The isolated nuclei were resuspended with extraction buffer (40 mM HEPES pH 7.9, 0.2 mM EDTA, 10% glycerol, 400 mM KCl, 0.1% Triton X-100, 10.08 mM β-mercaptoethanol) and sonicated. The homogenate was centrifuged and supernatants were stored in liquid nitrogen. For mouse RXRβ, we employed the same method except for the salt condition (100 mM KCl) in extraction. We confirmed the expression of these proteins by Western blotting. The purity of rTRβ 1, rHDAC2 and rRXRβ was 10, 70 and 10%, respectively.

Electrophoretic mobility shift assay

Double-stranded DNA probes representing the X + Y region (5′-TTT-GGGTCACCACAGCAT-3′) were radiolabeled with [γ-^{32}P]ATP (Amersham) using T4 polynucleotide kinase (New England Biolabs) and purified with a Sephadex G25 column. Fifteen micrograms of Sf9 cell nuclear extract for rTRβ1 and/or rRXRβ (10% purity for each) were mixed with 1 ng of ^{32}P-labeled double-stranded X + Y oligonucleotide probe and 2 μg of poly(dI–dC) (Pharmacia Biotech) with or without 100-fold excess competitor DNA and incubated in the binding buffer (20 mM HEPES pH 7.9, 50 mM KCl, 10% glycerol, 0.5 mM DTT, 0.1 mM EDTA) for 20 min at room temperature. Reaction mixtures were analyzed in 5% non-denaturing polyacrylamide gel. Using ^{32}P-labeled DR4, we confirmed the enhancement of DNA binding affinity for both receptor proteins by heterodimerization (data not shown).

In vitro binding assay for recombinant HDAC2 and TRs

Ten microliters of Ni–NTA agarose (Qiagen) equilibrated in buffer (20 mM Tris–HCl pH 8.0, 10% glycerol, 1 mM EDTA, 0.02% Triton X-100, 10 mM imidazole, 0.5 mM phenylmethylsulfonyl fluoride, 10 mM β-mercaptoethanol) were incubated with 2.5 μg of rHDAC2 (70% purity) in the presence of 2% bovine serum albumin at 4°C for 1 h with rotation and unbound materials were removed by centrifugation. TRβ1 deletions (C1 and C2) were generated by PCR and cloned into pCI vector (Promega), and translated products were labeled with [^{35}S]methionine. TRβ1 DBD (amino acids 98–218) was cloned into pGST-ag vector and expressed as a GST fusion protein in the BL21 strain of *Escherichia coli*. Beads were incubated with ^{35}S-labeled receptors or GST fusion proteins at 4°C for 1 h and then washed five times. Bound materials were eluted in the above buffer supplemented with 200 mM imidazole. Eluted materials were resolved on a 4–20% gradient SDS–polyacrylamide gel and autoradiographed. Binding of GST or GST–TRβ1DBD was detected by immunoblot analysis using anti-GST antibody

Histone deacetylase assay

Histone deacetylase activity was measured using human core histones radiolabeled with [^{3}H]acetate *in vivo* as substrate (Yoshida *et al*., 1990). Aliquots (2 μl) of elutes from the NRE beads were incubated with 10 μg of [^{3}H]histones in 100 μl assay buffer (20 mM Tris–HCl pH 8.0, 75 mM NaCl, 1 mM DTT) for 20 min at 37°C. The reaction was quenched by addition of 20 μl of 12N HCl and extracted with 0.5 mM ethyl acetate. Acetate release was measured by scintillation counting of ethyl acetate (organic) phase.

Restriction site accessibility assays in vivo

L017 cells were suspended in buffer containing 10 mM Tris–HCl pH 7.4, 15 mM NaCl, 60 mM KCl, 1 mM EDTA, 0.1 mM EGTA, 0.1% NP-40, 5% sucrose, 0.15 mM spermine and 0.5 mM spermidine. After incubation on ice for 2 min, cells were homogenized in the Dounce homogenizer for five strokes. The homogenates were placed on 1 ml of sucrose pad (10 mM Tris–HCl pH 7.4, 15 mM NaCl, 60 mM KCl, 10% sucrose, 0.15 mM spermine, 0.5 mM spermidine) and centrifuged at 2700 r.p.m. for 20 min at 4°C. Nuclear pellets were resuspended in 4 ml of washing buffer (10 mM Tris–HCl pH 7.4, 15 mM NaCl, 60 mM KCl, 0.15 mM spermine, 0.5 mM spermidine). They were digested with 60–200 U of the indicated restriction enzymes (all purchased from New England Biolabs) *in vivo* for 30 min at 30°C with intermittent agitation. DNA was purified by the phenol–chloroform extraction/ethanol precipitation method and digested *in vitro* with the indicated second enzymes (*Bst*NI or *Eco*RI and *Hin*fI) to completion and DNA was repurified. Eight to 12 μg of DNA were subjected to a linear PCR using primer 2 (5′-gcaactgactgaaatgcctcaaaatg-3′) for the promoter region (*Bst*EII, *Mfe*I, *Alu*I and *Eco*RI) or primer 3 (5′-ggcagttattggtgcccta-3′) for the CAT coding region (*Dde*I and *Hin*fI). The PCR procedure was essentially the same as above except for the annealing temperature (57°C for primer 2 and 55°C for primer 3). PCR products were quantified using the NIH Image Program. PCR products of *in vivo* digested samples were normalized by those of *in vitro* digested samples.

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