Thyroid hormone receptors (TRs) mediate hormone action by binding to DNA response elements (TREs) and either activating or repressing gene expression in the presence of ligand, T₃. Coactivator recruitment to the AF-2 region of TR in the presence of T₃ is central to this process. The different TR isoforms, TR-β₁, TR-β₂, and TR-α₁, share strong homology in their DNA- and ligand-binding domains but differ in their amino-terminal domains. Because TR-β₂ exhibits greater T₃-independent activation on TREs than other TR isoforms, we wanted to determine whether coactivators bound to TR-β₂ in the absence of ligand. Our results show that TR-β₂, unlike TR-β₁ or TR-α₁, is able to bind certain coactivators (CBP, SRC-1, and pCIP) in the absence of T₃ through a domain which maps to the amino-terminal half of its A/B domain. This interaction is specific for certain coactivators, as TR-β₂ does not interact with other co-factors (p120 or the CBP-associated factor (pCAF)) in the absence of T₃. The minimal TR-β₂ domain for coactivator binding is aa 21–50, although aa 1–50 are required for the full functional response. Thus, isoform-specific regulation by TRs may involve T₃-independent coactivator recruitment to the transcription complex via the AF-1 domain.

Thyroid hormone receptors (TRs) belong to the superfamily of nuclear receptors and contain at least five discrete domains: 1) the amino-terminal A/B domain containing AF-1 function; 2) the DNA-binding or C domain, which is highly conserved among nuclear receptors; 3) the hinge region or D domain, where corepressors bind; 4) the ligand-binding or E domain; and 5) the carboxyl-terminal AF-2 or F domain (1). TR acts as a transcription factor on thyroid hormone response elements (TREs) in the absence and presence of its ligand, triiodothyronine (T₃) (Ref. 2, and for review, see Refs. 3–5). On positively acting TREs (e.g. growth hormone, malic enzyme, myosin heavy chain-α), gene expression is repressed in the absence of T₃ and stimulated when T₃ binds to the TR (6–8). In contrast, on negatively regulated genes (e.g. TSH α and β subunits, myosin heavy chain-β), gene expression is activated in the absence of ligand and repressed in the presence of ligand (9–11).

There are three known TR isoforms: TR-α₁, TR-β₁, and TR-β₂. A fourth isoform, α₂, does not bind T₃ and may inhibit the function of other TRs. The different isoforms of the TR are derived from two different genes, c-erbA-α and c-erbA-β, found on different mammalian chromosomes. TR-α₁ and α₂ are generated from the c-erbA-α locus by alternative RNA splicing of carboxyl-terminal exons (12–14), whereas the TR-β isoforms are derived from differential exon utilization of the c-erbA-ß locus (15, 16). TR-β₁ and TR-β₂ therefore differ only in their amino-terminal domains (A/B domains). Whereas TR-α₁ and TR-β₁ are expressed ubiquitously (19), TR-β₂ is expressed almost exclusively in hypothalamus (20) and pituitary (15) and, therefore, could play an important role in controlling the thyroid axis centrally (16). Within the TR-β₂ amino terminus are two distinct domains (n-terminal and c-terminal) that have been shown to mediate ligand-independent activation on positive and negative TREs, respectively (18, 19).

Transcriptional regulation by TRs is modulated by coactivating and corepressing proteins. Two corepressors, Nuclear receptor CoRepressor (NCoR) and Silencing Mediator of Retinoic and Thyroid hormone receptors (SMRT), have been shown to bind to the hinge region of the TR in the absence of ligand (3, 4). These corepressors mediate ligand-independent repression on positive TREs, probably through deacetylation of histones (23–25). Binding of the ligand results in release of the corepressor and recruitment of coactivators.

Over the last years a number of coactivators have been described that interact with the TR, including the CREB Binding Protein, CBP (26), Steroid Receptor Coactivator-1, SRC-1 (5), the CBP Interacting Protein, p300 (27–29), p120 (30), and P300/CBP Associated Factor, pCAF (28). These coactivators contain LXXLL motifs that bind to the AF2 domain of liganded TR (31). The majority of these proteins have been shown to contain intrinsic histone acetyltransferase activity (28, 32–34) and probably function as activators through this mechanism. The ligand-independent activation of transcription by the TR-β₂ isoform could be mediated by binding of some corefactors to the amino terminus (AF-1 domain) of the receptor. This could explain the greater ligand-independent activation of TR-β₂ compared with TR-β₁ in some transfection systems. We therefore investigated the interaction of coactivators with the A/B (AF-1) domain of TR isoforms.

EXPERIMENTAL PROCEDURES

Constructs Used in Transfection Assays—The TRE constructs contain two copies of an idealized TRE (DR × 4, LYS, PAL) upstream of a minimal thymidine kinase promoter (109 bp of 5′-flanking DNA of the herpes simplex TK promoter fused to the luciferase reporter gene (35)). The cDNAs encoding the full-length coactivators CBP, SRC-1, p300 (generous gift from Dr. W. Chin, Lilly Corp., Indianapolis, IN), p120, and pCAF (generous gift from Dr. Y. Nakatani, National Institutes of Health) were placed into the pSG5 expression vector. The cDNAs en-
coding human TR-α, TR-β1, and TR-β2 were inserted into the expression vector pSG5, which employs the SV40 early promoter (36). The human TR amino termini were obtained by polymerase chain reaction amplification of human full-length TR cDNAs (TR-α1 and TR-β1) or genomic DNA (TR-β2) and ligated in-frame into an expression vector containing five copies of the GAL4 DNA binding domain. The TR-β2 amino-terminal deletion constructs were made using polymerase chain reaction to introduce an EcoRI site at the 5′-end and an XbaI site at the 3′-end of the constructs. The amino-terminal cDNAs were cloned in-frame with the GAL4 DNA as an EcoRI-XbaI fragment in the GAL4 vector (37). The reporter used for heterologous expression systems was UAS-TK fused upstream of the luciferase gene (38). The integrity of all constructs was confirmed by restriction endonuclease digestion and dideoxy sequencing.

Transfection Assays—Transient transfection studies were performed in JEG cells. Transfections were performed in 12-well plates on subconfluent cells, using the calcium-phosphate technique without glycerol shock. In the 12-well format, 1 μg of reporter with 0.2 μg of the isolated TR amino termini fused to the GAL4 DNA binding domain (N-α1, N-β1, and N-β2) or an "empty" GAL4 vector control (vector) along with 1 μg of a UAS-TK luciferase reporter. The data are expressed as -fold activity, where 1 represents the luciferase activity of the GAL4 empty vector alone.

RESULTS

In the absence of ligand (T₃), TR-β2 has an increased capacity to activate gene expression on negative TREs, when compared with the other TR isoforms (19). We first wanted to determine whether this ability of TR-β2 is mediated by its unique amino terminus. As shown in Fig. 1A, transient transfection studies of the isolated amino terminus of TR-β2 fused to the GAL4 DNA binding domain exhibits greater ligand-independent activation than the amino termini of TR-α1 or TR-β1 (7-fold versus 0.5- or 2.0-fold, respectively) on a GAL4 reporter (UAS-TK). Thus, the TR-β2 amino terminus contains a T₃-

![Fig. 1](image-url)
performed transfection assays and GST-interaction assays with the full-length receptors. Fig. 3A demonstrated that co-transfection of the full-length receptors with either a CBP or SRC-1 expression vector results in a specific 7–12-fold stimulation by TR-β2 in the absence of T₃. Deletion of the amino-terminal amino acids 1–50 (construct TR-β2Δ1–50) resulted in complete loss of the ligand-independent activation of TR-β2. In contrast, CBP or SRC-1 cotransfection yielded a 20-fold T₃-dependent stimulation of reporter gene expression regardless of the isoform tested, indicating that all three TR isoforms and the TR-β2 deletion construct showed similar functional interaction with these coactivators and the AF-2 domain in the presence of T₃. Specific ligand-independent activation of TR-β2 in the presence of CBP or SRC-1 was also observed on other response elements, Lys and Pal element, respectively (Fig. 3B). Fig. 3C supports these findings by demonstrating that full-length TR-β2 in the absence of T₃ bound to CBP, SRC-1, and pCIP 25-, 15-, and 21-fold above background, respectively, as quantified by densitometry. In the presence of T₃, there was only a small increase in binding of CBP, SRC-1, and pCIP to TR-β2 (26-fold, 27-fold, and 29-fold, respectively). In contrast, full-length TR-β1 interacted much less well with the same coactivators in the absence of T₃ (0.5-, 3.1-, and 6.1-fold, respectively) versus in the presence of T₃ (25-fold, 11-fold, and 25-fold, respectively). This structural assay also showed specificity, as p120 and pCAF did not exhibit T₃-independent interaction with TR-β2.

To isolate the region of the TR-β2 amino terminus that is important for ligand-independent interaction with coactivators, we constructed a number of deletion constructs of the TR-β2 amino terminus (shown in Fig. 4A) fused downstream and in-frame with the GAL4 DNA-binding domain. Shown in Fig. 4B are results with GAL4 fusion constructs tested on the UAS-TK reporter. When co-transfected with CBP or SRC-1, only constructs which contain amino acids 1–50 (N-β2, 1–75, and 1–50) were completely sufficient to mediate reporter gene activation. Constructs containing only amino acids 21 to 50 (21–120 and 21–87) stimulated reporter gene expression about 60–80% of the full-length TR-β2 amino terminus, whereas constructs lacking amino acids 1–50 (51–120 and 89–120) were unable to stimulate reporter gene activity.

We next expressed these deletion constructs as GST fusion proteins and then employed them in GST-interaction assays. As shown in Fig. 4C, the fusion proteins containing amino acids 1 to 50 (N-β2, 1–75, and 1–50) were able to bind 35S-labeled CBP, SRC-1, and p-CIP as efficiently as the full-length TR-β2 amino terminus. In contrast, proteins with a deletion of the first 50 amino acids were unable to bind to these coactivators (51–120 and 87–120), and proteins retaining amino acids 21–50 showed significant but reduced coactivator binding. Fig. 4D demonstrates that equal amounts of GST fusion proteins were used in the GST-interaction assays.

**Discussion**

Members of the nuclear hormone receptor superfamily activate gene transcription by binding to their cognate response elements in the regulatory regions of target genes either as monomers, homodimers, or heterodimers with the retinoid X receptor (RXR). Depending on the target gene and nuclear receptor, transcriptional activity is either activated or repressed in the presence of ligand. Ligand-dependent activation of NRs occurs principally through the AF-2 domain of the ligand-binding domain. In the presence of ligand, amino acid residues in helices 3 and 12 allow for the formation of a groove which binds to the LXXLL motifs of coactivator molecules (38). Coactivator molecules include members of the p160 family (SRC-1, TIF II, and ACTR), RIP 140, TRIP100, p300/CBP, and p120 as well as members of the DRIP complex and a number of
FIG. 3. Ligand-independent interaction of the full-length TR-β2 with certain coactivators. A, JEG 3 cells were transiently transfected in the absence or presence of T3 (±T3) with 0.2 μg of the indicated TR fused to the GAL4 DNA binding domain (TR-α1, TR-β1, TR-β2, and TR-β2 Δ 1–50) or an empty GAL4 vector control (vector) with 0.33 μg of coactivator in pSG5 and 1 μg of a DR+4 luciferase reporter. The data are expressed as fold activity ± S.E., where 1 represents the luciferase activity of the GAL4 empty vector alone. B, JEG 3 cells were transiently transfected as in Fig. 3A. Instead of a DR+4 luciferase reporter, a Lys or a Pal luciferase reporter, respectively, was employed. C, full-length TR-β1 and TR-β2 were expressed as GST fusion proteins and used to pull down S35-labeled coactivators (CBP, SRC-1, pCIP, p120, and pCAF) in the absence and presence of ligand (±T3).
other proteins (39, 40). Although the mechanism of action of the coactivator complex has not been fully ascertained, it is believed that transcriptional activation is mediated, at least in part, by histone acetylation by the coactivator complex, which is formed in response to ligand.

In addition to the AF-2 domain present in the LBD, NRs also possess an AF-1 domain in their amino-terminal region or A/B domain. The AF-1 function has been shown to be responsive to growth factors in context of the ER and to be a target for phosphorylation by MAP kinase in context of PPARγ (41, 42). Indeed, the down-regulation of PPARγ transcriptional activity by MAP kinase is because of a decrease in ligand-binding by PPARγ because of intermolecular communication between the A/B and LBDs. Thus, the activity of an NR cannot be viewed in context of
the AF-2 domain alone, as the AF-1 domain may influence AF-2 function either through structural alterations or by independently recruiting other proteins. Both of these functions are supported by recent studies which demonstrate that the androgen receptor (AR) AF-1 and AF-2 domains interact in mammalian cells (43) and that the ERα AF-1 domain can bind members of the p160 coactivator family while the ERβ AF-1 domain can also bind p160 members when phosphorylated by MAP kinase (44).

The TR isoforms differ most prominently in their A/B domains, though limited function of these domains has been shown. We and others have demonstrated that the separate TR isoform A/B domains affect DNA binding (35, 45). In addition, a region of the TR-α1 A/B domain directly recruits members of the basal transcriptional machinery (45). Furthermore, the TR-β2 amino terminus appears to possess a function which differentiates its ligand-independent activity from the other TR isoforms. Indeed, the TR-β2 amino terminus has been shown to be constitutively active when fused to a heterologous DNA-binding domain (19), and its separate activity on negative TREs maps to another unique region in the A/B domain (19).

Unlike the majority of nuclear receptors, TR isoforms possess both ligand-independent and dependent functions which are mediated by their ligand-binding domains. In the absence of ligand, TR-α1 and TR-β1 isoforms repress transcription on positive TREs through the recruitment of nuclear corepressors and resulting histone deacetylase containing complexes (39). In contrast, the TR-β2 isoform is a poor repressor on positive TREs, indicating that its unique A/B domain may confer a separate activity. This is further supported by the increased ligand-independent activity of the TR-β2 isoform on negative TREs (19), suggesting that the A/B domain of this isoform may alter its ability to recruit either coactivators or corepressors.

In the present study we have demonstrated that the TR-β2 amino terminus allows for the recruitment of members of the p160 family and CBP through a specific domain located between amino acids 1–50. Indeed, this recruitment can be demonstrated in direct in vitro GST pull-down assays as well as in functional studies in mammalian cells where further activation of this region is seen in the presence of either p160 family members or CBP. This region corresponds to the activation function previously mapped by Sjoberg et al. (18) and suggests that the constitutive function of this isoform may be related to its ability to interact with coactivators in the absence of ligand. Importantly, specificity is also demonstrated in that pCAF, which is known to interact with the DNA-binding domain of the NRs (22), is unable to enhance the activation function of the TR-β2 amino terminus. As well, p20, another NR coactivator, is unable to augment the function of, or bind to, the TR-β2 amino terminus.

To ensure that the interaction of p160 family members and CBP with the TR-β2 amino terminus is not artifactual, we have also demonstrated, using in vitro GST pull-down assays, that the entire TR-β2 isoform selectively binds to these coactivators in the absence of ligand. Furthermore, these binding studies are in agreement with the functional effects of these coactivators on the entire TR-β2 isoform in transfection studies. These data suggest that the recruitment of coactivators by the TR-β2 amino terminus impairs ligand-independent repression by this isoform by either preventing the recruitment of corepressors or more likely by altering the ratio histone acetylation/deacetylation, which ultimately determines the degree of transcriptional activation. In this model, the TR-β2 isoform would recruit both corepressors (through the LBD) and coactivators (through the amino terminus) which would prevent silencing. Tissue-specific expression of coregulators in the pituitary or hypothalamus, where TR-β2 action is paramount, would ultimately affect the ligand-independent activity of this isoform. Further studies in hypothalamic TRH neurons and pituitary thyrotrophs will determine the cofactor profile in these cells.