Thyroid hormone receptors are ligand-modulated transcription factors that can repress or activate transcription depending upon the absence or presence of thyroid hormone and the nature of the hormone response element to which the receptors are bound. The ability of thyroid hormone receptors to repress transcription in the absence of ligand is thought to be due to associations with nuclear hormone receptor corepressors. Ligand binding by the thyroid hormone receptor is believed to dissociate these corepressors and recruit coactivators to promote transcription from target promoters. We hypothesize that variations in response element architecture may influence both the association and dissociation of corepressors from DNA-bound thyroid hormone receptors. Using a chimeric corepressor, we find that ligand alone does not fully relieve corepressor-mediated repression, particularly in the presence of thyroid hormone receptor and its heterodimerization partner, the retinoid X receptor. Interestingly, the steroid receptor coactivator 1 together with ligand is able to mediate full release of corepression, but this relief is dependent upon the architecture of the response element to which the nuclear receptor dimer-corepressor complex is bound. These studies suggest that other cellular factors in addition to ligand may be required for the release of corepressors from thyroid hormone receptor dimers.

Thyroid hormone receptors (TRs) are ligand-modulated transcription factors in the erbA superfamily that are found constitutively in the nucleus, bound to specific DNA sequences known as thyroid hormone response elements (TREs). TRs can bind TREs as monomers, homodimers, or heterodimers with the retinoid X receptor (RXR) and positively or negatively regulate gene expression depending on the nature of the TRE and the presence of thyroid hormone (T3). TREs are generally composed of two or more TR binding half-sites arranged as direct repeats spaced by four nucleotides, palindromes without a nucleotide spacer, or inverted palindromes spaced by six nucleotides. The optimal TR binding half-site is the octamer TATAAGGTCA, an extended version of the common core response element hexamer AGGTCA. Using Saccharomyces cerevisiae as a system devoid of endogenous RXR, we have shown that TREs composed of optimal octameric half-sites can be activated by TR alone. In contrast, TREs composed of suboptimal hexameric half-sites require the expression of RXR with TR for substantial gene activation.

In mammalian cells, unliganded TRs can function as transcriptional repressors when bound to positive TREs. Upon T3 binding, however, TRs direct the activation of gene expression from target promoters. Recent studies have characterized numerous TR-associated proteins that play important roles in converting unliganded TR transcriptional repression into liganded TR activation. In the absence of bound ligand, TRs associate with the nuclear receptor corepressor (N-CoR) or the silencing mediator of retinoid and thyroid hormone receptors (SMRT), which mediates the repression of basal levels of gene expression from target promoters. TR binding of T3 is thought to induce a conformational change in the receptor, which results in dissociation of the corepressor complex and allows recruitment of coactivators such as the steroid receptor coactivator 1 (SRC-1) and the cAMP response element-binding protein (CREB)-binding protein (8). The association of coactivators with the liganded TR tethers activation domains into the vicinity of target promoters, resulting in transcriptional activation. Gene activation in response to thyroid hormone is, therefore, both a release of repression and a recruitment of activation functions. The molecular mechanisms by which ligand binding modulates these opposed functions are only beginning to be understood.

All mammalian cell lines examined to date contain endogenous corepressors and coactivators, which complicates studies designed to address the roles of these cofactors in TR-dependent gene regulation. To delineate the relative contributions of release of repression and recruitment of coactivation functions in T3-dependent gene activation, we have reconstituted TR-dependent gene expression in the lower eukaryote S. cerevisiae. Since S. cerevisiae lacks endogenous nuclear hormone receptors, nuclear receptor corepressors, and nuclear receptor coactivators, it provides a null background in which the functions of these molecules can be studied independently or in combination.

It has been shown that unliganded TRβ is a transcriptional activator in S. cerevisiae (3), finding likely due to the absence of endogenous nuclear receptor corepressors in yeast. Ligand-dependent gene activation (above this basal activation) from single copy TREs requires the presence of coactivators such as SRC-1 (3). Interestingly, the orientation of the half-sites composing single TREs and the 5′ dinucleotides flanking the hexameric half-sites modulate the ability of SRC-1 to in-
Corepressor Release from TR Dimers

The top strands of the TRE oligonucleotides are shown without the 5’-GATC overhangs. The 6 or 8 preceding the orientation designations indicate sub-optimal hexameric AGGTCA or optimal octameric TA-AGGTCA half-site, respectively. The consensus hexamer is in bold type, and the face, and the 5’-nucleotide TA is underlined in each optimal half-site. The number after the orientation designation (4, 0, or 6) indicates the number of nucleotides separating the consensus hexameric half-sites.

**TABLE I**

**Synthesized oligonucleotide response elements**

| Response elements | DNA sequence |
|-------------------|--------------|
| 6DR4 (sub-optimal direct repeat) | AGCAGGTCAATACTGTGCG |
| 8DR4 (optimal direct repeat) | CTAGGTCAATACTGTGCG |
| SPAl0 (optimal palindrom) | CTAAGGTCAATACTGTGCG |
| 8IP6 (optimal inverted palindrome) | GAGGTCAATACTGTGCG |

**EXPERIMENTAL PROCEDURES**

**Yeast Reporter Strains and Expression Vectors**—All yeast strains were derived from the parental strain SEY 6210 (MATa; ura3-52; leu2–3,112; his3–Δ200; trp1–Δ901; lys2–801; suc2–Δ9; β-Galactosidase reporter constructs containing octameric (optimal) or hexameric (sub-optimal) direct repeats or palindromes (Table I) were inserted into the yeast chromosome at the leu2 locus by homologous recombination (3). Strains bearing single insertions were identified by Southern blot analysis (data not shown). TRp1 (12) and RXRα (13) were subcloned into pRS-GalH on opposite sides of a bi-directional galactose-inducible promoter (3). SRC-1 (7) was subcloned into the plasmid p413-TEF, and T-CoR (see below) was subcloned into the plasmid p424-GPD (14). Yeast transformations were performed using a high efficiency lithium acetate protocol (15), and single transformants were isolated on agar plates containing appropriate selective synthetic medium.

**Construction of the Fusion Protein T-CoR**—The chimeric corepressor protein T-CoR was created containing the repression domain of the yeast repressor protein Tup1 fused to the TR interaction domains of N-CoR. The SV40 nuclear localization sequence, hemagglutinin epitope tag, and the coding sequence of amino acids 1–200 of Tup1 were amplified by the polymerase chain reaction using YCP91-Tup1 (16) as the template. The 5’ oligonucleotide primer encoded a 5’ EcoRI restriction site, start codon, and 18 nucleotides of the SV40 nuclear localization sequence; the 3’ oligonucleotide primer contained an Apol site and the reverse complement of Tup1 codons 195–200. The amplified fragment was digested with EcoRI and Apol, purified by agarose gel electrophoresis, and then ligated to the gel-purified Apol-ClaI fragment of pRS-S-N-CoR (5), which contains N-CoR codons 1819–2453 with two copies of FLAG epitope sequences attached. This 2.6 kilobase pair fragment, designated T-CoR, was purified by agarose gel electrophoresis and then ligated into the multiple cloning site of the expression vector p424-GPD using the EcoRI and ClaI sites.

**Hormone Inductions and Reporter Gene Assays**—Yeast strains were grown in selective synthetic media containing a nonrepressive carbon source (3% glycerol, 3% ethanol) to limit the levels of nuclear receptor expression before hormone inductions. Yeast cultures were diluted to an absorbance at 650 nm (A560) of 0.2, nuclear receptor expression was induced by the addition of 3% galactose, and the cultures were grown overnight at 30 °C in the presence or absence of 1 μM 3,5,3’-triiodothyroacetic acid (triac), a T3 analog. The next day the A560 of each culture was determined. 1.5 ml of each culture was pelleted by microcentrifugation, the supernatants were removed, and the pellets were frozen at −20 °C. The β-galactosidase activity of each pellet was measured as described previously (3). β-Galactosidase activities were normalized to the level of activity present in each yeast reporter strain containing an empty nuclear receptor expression vector and the indicated p413-TEF and p424-GPD constructs. Data represent the means of at least five independent experiments and are expressed as the mean ± S.E.

Percent repression was calculated as the difference between the normalized β-galactosidase activities in the presence of nuclear recep-
direct repeat TRE in the context of TR alone or TR with RXR, and that ligand largely relieves this repression. In addition, T-CoR functioned more effectively as a coressor of constitutive TR-TR homodimer activity than of RXR + TR activity (76% repression versus 55%; p = 0.003), suggesting the possibility of different functional interactions of corepressor with TR-TR homodimers versus RXR-TR heterodimers.

T-CoR Represses RXR+TR Activity on the Sub-optimal Direct Repeat TRE 6DR4—T-CoR function also was studied in the context of the sub-optimal direct repeat 6DR4, which requires the coexpression of RXR with TR for substantial gene activation (3). Thus, although expression of TR or RXR alone had little or no effect on reporter gene activity (Fig. 2, A and B), coexpression of TR and RXR led to a 5.7-fold induction of β-galactosidase in the absence of triac (Fig. 2C). This 5.7-fold RXR + TR induction was repressed to 2.3-fold (73% repression) upon expression of T-CoR. The addition of triac, however, was unable to relieve this repression, as reporter gene induction remained essentially unchanged at 2.4-fold (Fig. 2C). Thus T-CoR repressed gene activation mediated by an obligate RXR-TR heterodimer from the sub-optimal TRE 6DR4. However, in contrast to the situation with TR-TR homodimers on 8DR4, this T-CoR-mediated repression of RXR-TR heterodimers on 6DR4 was unaltered by the addition of ligand. This suggests that other factors in addition to ligand may be required to relieve corepressor-mediated repression, particularly in the context of the RXR-TR heterodimer.

T-CoR Represses TR and RXR + TR Activity on the Optimal Palindrome—To assess the importance of half-site orientation on corepressor function, we expressed various combinations of T-CoR, TR, and RXR in a yeast strain containing a reporter construct driven by an optimal palindromic TRE (8Pal0) or an optimal inverted palindromic TRE (8IP6). T-CoR had no effect on reporter gene activity from 8IP6 in the presence of TR alone, RXR alone, or TR with RXR (data not shown). However, on the palindrome 8Pal0, T-CoR mediated a 73% repression of TR-TR homodimer activity and a 74% repression of RXR + TR activity in the absence of triac (Fig. 3, A and C). Thus, in contrast to the results with the optimal direct repeat 8DR4, T-CoR was equally effective in mediating repression in the presence of TR alone and TR with RXR. The addition of triac modestly relieved T-CoR-mediated repression from 8Pal0 in the context of TR alone (from 73 to 49% repression; p = 0.01), but did not significantly do so in the presence of TR with RXR (p = 0.2). Taken together, these data would suggest that corepressor-mediated repression may be influenced by TRE half-site orientation, and the release of corepressors from TR may require other events in addition to ligand binding, particularly in the context of RXR-TR heterodimers.

SRC-1 Does Not Augment the Release of T-CoR-mediated Repression of RXR+TR Activity on Direct Repeat TRES—Bani-ahmad et al. (17) demonstrated that disruption of the AF-2 activation domain in the extreme C terminus of TRβ1 did not destroy the ability of the receptor to bind ligand but did abolish the ability of ligand to relieve the silencing function of TRβ1. These authors suggested the potential importance of ligand-dependent trans-activating factors, which interact with the AF-2 domain, in the release of receptor-associated corepressors. In yeast, since no endogenous nuclear receptor coactivators exist to associate with the liganded nuclear receptor complex, it might be predicted that ligand alone would fail to relieve corepressor activity fully. We hypothesized that the presence of a nuclear receptor coactivator in addition to T-CoR might be sufficient to restore complete ligand-dependent relief of T-CoR-mediated repression in the yeast reporter strains. To test this hypothesis, we coexpressed the mammalian coactivator SRC-1 with T-CoR and examined its ability to augment the release of T-CoR-mediated corepression in response to the addition of ligand.

If SRC-1 functions to enhance the release of T-CoR-mediated repression, we would predict that the induction of reporter gene activity in strains expressing both T-CoR and SRC-1 would approximate the levels seen in strains expressing SRC-1 alone. Reporter gene inductions lower than this level would suggest an incomplete release of T-CoR-mediated repression. Since triac was particularly ineffective at relieving T-CoR-mediated repression of RXR-TR heterodimers on 6DR4 (Fig. 2C), we first examined the effect of SRC-1 in this context. The 4.2-fold induction of reporter gene expression seen in the presence of TR, RXR, and triac dropped to 2.4-fold when T-CoR was added (Fig. 2C). Contrary to our hypothesis, coexpression of SRC-1 was not effective at relieving this repression; β-galactosidase induction remained unchanged at 2.6-fold, whereas full release of the T-CoR effect should have led to a 5.3-fold induction (i.e., the fold induction seen in the presence of TR, RXR, triac, and SRC-1 without T-CoR) (Fig. 4C). Similarly, studies with T-CoR-mediated repression of RXR-TR heterodimers on 8DR4 demonstrated that SRC-1 fails to enhance the relief of repression by triac (data not shown).
**DISCUSSION**

Thyroid hormone receptors are found constitutively bound to DNA at response element sequences. In general, unliganded TRs function as transcriptional repressors of nearby target promoters. Thyroid hormone binding serves to convert the TR from a repressor to an activator of transcription.

The interaction of unliganded TRs with corepressor proteins seems a likely explanation for the repression exhibited by unliganded TRs bound to positive TREs. Thyroid hormone binding by TR is thought to induce a conformational change in the receptor, which in some way promotes corepressor dissociation. This ligand-induced destabilization of the TR-corepressor interaction has been demonstrated in vitro (5, 6, 18) and is assumed to occur in vivo. However, the identification of TR mutants that retain the ability to bind ligand but fail to release silencing functions and fail to activate gene expression suggested that ligand alone may not be sufficient to release corepressor activities from TR (17).

Little is known regarding the functional importance of TRE architecture in regulating unliganded TR-mediated repression. Given the influence of TRE architecture on the function of coactivators (3, 19), we hypothesized that variations in the sequence and orientation of TRE half-sites also would modulate corepressor function. *S. cerevisiae* provided an excellent null background in which to examine the regulation of corepressor release from TR-TR homodimers and RXR-TR heterodimers, since, in contrast to mammalian cells, endogenous nuclear receptors, nuclear receptor corepressors, and nuclear receptor coactivators are absent in this organism.

Our initial efforts were directed at studying wild type SMRT or N-CoR in yeast. However, neither SMRT nor N-CoR functioned as a corepressor in our system. Whether this was due to poor expression of these molecules or incompatible differences between mammalian repression domains and yeast basal machinery targets is not clear. To circumvent this problem, we have constructed a fusion protein referred to as T-CoR in which the repression domain of the yeast repressor Tup1 is fused in-frame to the nuclear receptor interaction domains of N-CoR. In yeast, T-CoR functioned as a corepressor of constitutive TR-TR and RXR-TR activity, and this repression could be modulated to varying degrees by the addition of ligand. How relevant is T-CoR to the study of mammalian corepressor function? Recent studies have shown that repression by N-CoR and SMRT involves an interaction with Sin3A and a histone deacetylase (20, 21). Repression by Tup1 does not appear to utilize the yeast homologs of Sin3A and histone deacetylase (22). Therefore, T-CoR is not likely to repress transcription by the identical molecular mechanisms used by N-CoR and SMRT in mammalian cells. However, evidence suggests that interac-

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**FIG. 4. Effect of SRC-1 expression on T-CoR-mediated repression of β-galactosidase inductions from the sub-optimal direct repeat TRE 6DR4.** The 6DR4 strain was transformed with SRC-1 and TR, RXR, or TR plus RXR either without (black bars) or with (striped bars) T-CoR and induced in the absence or presence of 1 μM triac as indicated. Data represent the means of five independent experiments ± S.E. and are presented as -fold inductions over the level of reporter gene expression found in the indicated strain bearing an empty nuclear receptor expression vector. As expected, expression of TR with SRC-1 (A) or RXR with SRC-1 (B) had little effect on β-galactosidase activity. Coexpression of all three proteins resulted in a β-galactosidase induction that was suppressible by T-CoR; however, this T-CoR repression was not relieved by triac (C).
tions with Sin3A and histone deacetylase do not fully account for N-CoR- and SMRT-mediated repression (23). Therefore, it remains possible that the mechanism of repression by these proteins is at least partially related to the mechanism of repression by Tup1. More importantly, the mode of repression by N-CoR is unlikely to alter the mechanisms by which it is recruited and released from TR, as that information appears to be encoded in distinct interaction domains contained within its C terminus (24, 25). Since N-CoR and SMRT do not function in our yeast system, T-CoR is a reasonable tool for dissecting the recruitment and release of corepressors from nuclear receptors bound to the response elements of target genes.

We found that the ability of T-CoR to repress TR-mediated gene activation depended upon the composition of the nuclear receptor dimer (TR-TR or RXR-TR) and the sequence and orientation of the half-sites to which the nuclear receptor dimer was bound. Although T-CoR repressed unliganded TR-TR homodimers on optimal direct repeat and optimal palindromic elements equally well, the ability of ligand alone to relieve this repression was substantially better from the optimal direct repeat than from the optimal palindromic TRE. The functional interaction of mammalian corepressors with nuclear hormone receptors has been shown to require the recruitment and function of coactivator proteins. These multiple layers of integrated regulation may be important in determining the complexity of responses to a seemingly simple thyroid hormone signal.

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FIG. 5. Effect of SRC-1 expression on T-CoR-mediated repression of β-galactosidase inductions from the optimal palindromic TRE 8Pal0. The 8Pal0 strain was transformed with SRC-1 and TR, RXR, or TR plus RXR either without (black bars) or with (striped bars) T-CoR and induced in the absence or presence of 1 μM triac as indicated. Data represent the means of five independent experiments ± S.E. and are presented as fold inductions over the level of reporter gene expression found in the indicated strain bearing an empty nuclear receptor expression vector.
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