Cellular Action of Thyroid Hormone

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INTRODUCTION

The thyroid hormones (THs, thyroxine (T_4) and triiodothyronine (T_3)) have important effects on development, growth, and metabolism (1, 2). Some of the most prominent effects of TH occur during fetal development and early childhood. In humans, the early developmental role of TH is illustrated by the distinctive clinical features of cretinism observed in iodine-deficient areas. In childhood, lack of TH can cause delayed growth. However, in this latter case, many of the effects of TH may be metabolic rather than developmental, as growth is restored rapidly after the institution of TH treatment. In adults, the primary effects of THs are manifested by alterations in metabolism. These effects include changes in oxygen consumption, protein, carbohydrate, lipid, and vitamin metabolism. The clinical features of hypothyroidism and hyperthyroidism emphasize the pleiotropic effects of these hormones on many different pathways and target organs.

At the clinical level, identification of quantitative markers of TH action has been difficult (3). At the extreme ends of the clinical spectrum, which extends from hypothyroidism to hyperthyroidism, the diagnosis of a thyroid abnormality is usually apparent. Clinical suspicion of a thyroid abnormality can be confirmed using laboratory tests for THs and thyroid stimulating hormone (TSH). However, more subtle forms of thyroid dysfunction, such as subclinical hypothyroidism or hyperthyroidism, pose a greater challenge. Although the level of circulating TSH provides a sensitive and quantitative indicator of TH action at the level of the hypothalamic-pituitary axis, there are few reliable peripheral or intracellular markers of TH action (3,4). The effect of TH on basal metabolism has been re-evaluated using measurements of resting energy expenditure (REE). In hypothyroid patients taking varying levels of TH replacement, there is a strong inverse correlation between REE and the TSH level (5). Nevertheless, TSH remains the most sensitive and useful clinical indicator of TH action. As discussed below, tissue-selective metabolism of THs, and variable tissue sensitivity to their effects, underscores the need to develop additional markers of TH activity in peripheral tissues.

Since the initial description of TH effects on metabolic rate more than 100 years ago (6), many theories have been proposed to explain its mechanism of hormone action. The proposed models include: uncoupling oxidative phosphorylation, stimulation of energy expenditure by the activation of Na+-K+ ATPase activity, and direct modulation of TH transporters and enzymes in the plasma membrane and mitochondria (7). Recently, there has been increasing evidence for non-genomic actions (see later

under non-genomic actions of TH) (7); however, the major effects of TH occur via nuclear receptors that mediate changes in gene expression.

In 1966, Tata proposed that TH increased gene expression with attendant increases in protein synthesis and enzyme activity (8). In 1972, high affinity nuclear binding sites for TH were documented (Kd approximately 10-10 M for T3) (9,10). The receptor-binding affinity of various THs and analogues correlated with their biologic potencies, consistent with the view that most biologic effects are mediated via the nuclear receptor (11– 13). Over the past 25 years, there has been a dramatic surge of new information on TH action resulting from the cloning of the TH receptors (14 – 15), the identification of regulatory DNA elements in TH responsive genes (2, 16), and the generation of TR isoform knockout mice (17, 18). In this chapter, we will focus on our current understanding of nuclear TH receptor action.

BINDING OF THs TO NUCLEAR RECEPTORS

In many respects, T_4 can be regarded as a prohormone for the more potent hormone, T_3 . Most of the TH bound to receptors is in the form of T_3 , either secreted into the circulation by the thyroid gland or derived from T_4 to T_3 conversion by 5′ monodeiodinases (see Chapter 3C). There are three distinct deiodinases- type I, type II, and type III (19, 20). The distribution and regulation of these enzymes can have important effects on TH action. For example, Type II deiodinase has high affinity for T_4 (Kd in the nanomolar range) and is found primarily in the pituitary gland, brain, and brown fat where conversion of T_4 to T_3 modulates the intracellular concentration of T3. Thus, tissues that contain type II deiodinase can respond differently to a given circulating concentration of T_4 (by intracellular conversion to T3) than organs that only can respond to T_3 (21, 22). Additionally, it appears that both type 1 and type II deiodinase regulate the circulating T_4 and T_3 levels (23). Recently, MCT8, OATP-1, and System L amino acid transporters have been identified as TH transporters which regulate T_4 and T_3 uptake into cells . Mutations in the former have been involved in a number of syndromes of x-linked mental retardation and neurologic deterioration (24,25).

T_3 binds to its receptors with approximately 10-15 fold higher affinity than T_4 . The dissociation constants for liver nuclear receptors measured in vitro are 2×10^{-9} M for T_4 and 2×10^{-10} M for T_3 . Nuclear receptors are approximately 75% saturated with TH in brain and pituitary and 50% saturated with TH in liver and kidney. It is notable that the extent of TH receptor occupancy varies in different tissues, providing a mechanism for alterations in circulating TH levels to alter receptor activity. In contrast to the related steroid hormone receptors, TRs are mostly nuclear both in the absence and presence of TH (26,157). In fact, TH receptors are tightly associated with chromatin (28 – 30), consistent with their proposed role as DNA-binding proteins that regulate gene expression.

CLONING, STRUCTURE, AND EXPRESSION OF TH RECEPTORS

Cloning of TH receptors

TH receptors (TRs) were first cloned in 1986 and belong to the nuclear hormone receptor superfamily that includes the glucocorticoid, estrogen, progesterone, androgen, aldosterone, vitamin D, retinoic acid (RARs), retinoid X (RXRs) and "orphan" (unknown ligand and/or DNA target) receptors (2, 14-16, 31, 32). TRs are the cellular homologs of v-erbA, a viral oncogene product involved in chick erythroblastosis. TRs are encoded at two genomic loci (α and β) located on human chromosomes 17 and 3, respectively (2,14-16,33, 34) and their gene products result in two major isoforms, TRα and TRβ .

Structure of TH receptors

Like other members of the nuclear receptor superfamily (2, 16, 31, 32), the TRs have a central DNA-binding domain (DBD) and a carboxy-terminal ligand-binding domain (LBD) (Figure 3d-1). The two major TR isoforms have high amino acid sequence homology in their respective DBDs and LBDs. Dimerization domains also are found in both DBDs and LBDs. The amino-terminal regions are more variable between TRα and TRβ(2), and contain ligand-independent activation domains. In contrast, multiple sub-regions are located in the LBD for ligand-dependent transcriptional activation and basal repression of target genes.

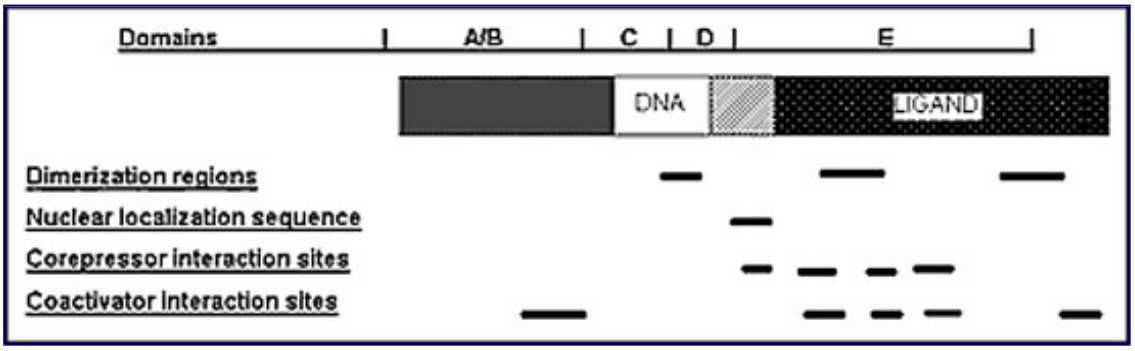

Figure 1. Functional domains of the TH receptor (TR). The TH receptor (TR) is depicted schematically. The zinc finger DNA-binding domain (DBD) is denoted along with the carboxy terminal ligand-binding domain (LBD). Other functional domains and interaction sites are indicated.

The DNA-binding domains of the nuclear receptors are comprised of two distinct zinc fingers that are separated by a 15-17 amino acid linker sequence. The crystal structure of the DNA-binding domains of the TH receptor and its heterodimeric partner, RXR has been determined. The two heterodimer partners interact with a direct repeat of the receptor binding site in a head-to-tail manner (27,36,37).

A small stretch of amino acids at the base of the first finger (referred to as the P-box) dictates the DNA sequence specificity of the receptor (35, 36, 38). The P-box sequence of the TH receptor is shared by other receptors that bind to similar or identical DNA recognition sites (AGGTCA). The underlined amino acids in the P-box (EGCKG) of the TH receptor are also found in the retinoic acid receptors, the

retinoic acid X receptors, the rev-erbA protein, the vitamin D receptor, and NGFI-B. Of note, the steroid hormone receptors have a different P box sequence and bind as homodimers to a different consensus DNA half-site sequence (AGAACA). The region between the DBD and LBD is called the hinge region and contains the nuclear localization signal, typically a basic amino acid rich sequence, first described in viral nuclear proteins.

X ray crystallographic studies of the liganded rat TRα-1 show that TH is embedded in a hydrophobic "pocket" lined by discontinuous stretches of amino acid sequences within the LBD. Additionally, there are several hydrophobic interfaces within the LBD that contribute to the TR homo- and heterodimerization with RXR (39). There are twelve amphipathic helices in the LBD and specific helices among them provide the critical contact surfaces for protein-protein interactions with co-activators and co-repressors (helices 3,5,6,12 and 3,4,5,6, respectively) (40-43). Ligand-binding to TR causes a major conformational change in the LBD, particularly in helix 12. This, in turn, facilitates TR discrimination between co-activators and co-repressors (see below).

Splicing variants of TH receptors

The carboxy-terminal hormone-binding domain of the TRα gene is alternatively-spliced to generate several protein products (Figure 3d-2, below). One variant, referred to as α-2, is identical to TRα-1 through the first 370 amino acids, but then its sequence diverges completely, owing to splicing of alternate exons (44-47). Another splicing variant, referred to as TRvII or α-3, is similar to α-2 except that it lacks the first 39 amino acids found in the unique region of α 2 (45). α 2 cannot bind TH because of the replacement of critical amino acids at the extreme carboxy-terminal end of the protein due to alternative splicing(48), and thus cannot mediate ligand-dependent gene transcription (49– 51). The amino acid replacements in α-2 also alter its dimerization properties and reduce DNA-binding affinity (52-55). The α-2 splicing variant is highly expressed in many tissues such as brain, testis, kidney, and brown fat, but its function remains poorly understood (56). The α-2 isoform has been proposed to be an endogenous inhibitor of TH receptor function as it inhibits TRα and β activity in transient gene expression assays (44,54). The mechanism by which α-2 antagonizes TR action is controversial. Some studies indicate that α-2 competes for active receptor complexes at DNA target sites (57,58). Other studies indicate that α-2 inhibits TR activity independent of DNA-binding (59). It is likely that the inhibitory effects of α-2 involve more than one mechanism. Amino acid substitutions in the carboxy-terminal region of α-2 also prevent its interactions with transcriptional corepressors (see below) (55), and may provide an explanation as to why α-2 is not a more potent inhibitor of TR activity. Additionally, the phosphorylation state of α-2 may modulate its inhibitory activity (60). Given the foregoing features, the TRα 1 and α-2 system represents one of the few examples in mammals whereby multiple mRNAs generated by alternative splicing encode proteins that are antagonistic to each other.

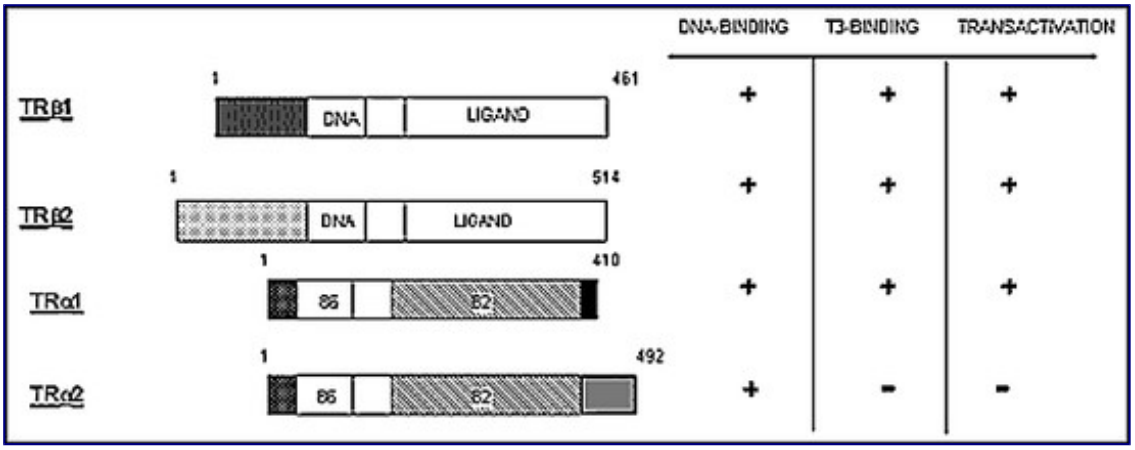

Figure 2. TH receptor isoforms. The TH receptors (TR) β and α are expressed from separate genes. Each TR gene can be expressed as distinct isoforms, reflecting the use of alternate promoters and exons. The central zinc finger DNA-binding region is indicated and unique domains are shown by distinct patterns of shading. The TRβ-2 isoform, which is expressed predominantly in the pituitary and hypothalamus, contains a unique amino-terminus. The TRα-2 isoform contains unique carboxy-terminal sequences that eliminate hormone binding. The DNA- and T 3 -binding properties and transcriptional activity of the various isoforms are shown at the right.

A receptor-like molecule, Rev-erbA, is, surprisingly, encoded on the opposite strand of the TRα gene locus (61, 62). Rev-erbA mRNA contains a 269-nucleotide stretch which is complementary to the α-2 mRNA due to its transcription from the DNA strand opposite of that used to generate TRα-1 and α-2. This protein also is a member of the nuclear hormone receptor superfamily, and is highly expressed in adipocytes and muscle cells. Rev-erbA, contains a DBD that is homologous to the TR DBD. However, Rev-erbA does not bind TH and its putative LBD has minimal homology with other nuclear hormone receptors. Since no cognate ligand has been identified for Rev-erbA, it is categorized as an "orphan nuclear receptor. " It can act as a transcriptional repressor for nuclear hormone receptors and other transcription factors (63-65). Since Rev-erbA shares an exonic segment of the bidirectionally transcribed TRα gene, it is possible that it modulates the expression or splicing of TRα 1 and α 2 (66, 67) as parallel increases in rev-erbA mRNA and TR α-1mRNA expression occur relative to α 2 mRNA expression.

The major variant of the TRβ gene, TRβ 2, has a different amino-terminus than TRβ 1 (Figure 3d-2, above) (68). The distinct amino-terminal region of the TRβ-2 is due to transcription from a tissue-specific promoter. The function of the amino-terminus of the TH receptor is not known, but it likely plays a role in transcriptional control (69,70). The TRβ-1 and TRβ-2 isoforms function similarly in most transient gene expression assays (69, 71), although differences in the transcriptional activities of the TRβ 1 and TRβ 2 isoforms have been noted with respect to certain target genes (69-72). It is likely that tissue-restricted expression of the TRβ-2 isoform contributes to unique patterns of TR expression, which in turn, may modulate target gene regulation.

Recently, short isoforms of TRα and TRβ have been described (73, 74). The novel TRα isoforms arise from translational start sites in the 7th intron and yield shortened TRα 1 and α 2 isoforms that have dominant negative activity on WT TR. Novel short TRβ isoforms arise from alternative splicing of TRβ . It is possible that these isoforms may modulate T_3 -responsiveness in a tissue- and/or developmental stage-specific manner.

Tissue and developmental expression of TRs

Most studies of TR isoform expression have employed mRNA analyses rather than protein measurement (75). In general, the α and β receptor isoforms are distributed widely and exhibit overlapping patterns of expression (56, 75, 76). TRα 1 mRNA is expressed in skeletal and cardiac muscle whereas TR β-1 mRNA is predominant in liver, kidney, and brain. A-2 mRNA is most prevalent in brain and testis. In contrast, TRβ-2 mRNA has the most tissue-restricted expression, and is present in the anterior pituitary gland, hypothalamus, and cochlea (77-79).

The TRs also are expressed in specific stages during development, and are subject to regulation by hormones and other factors (80, 81). For instance, TRα-1 mRNA is expressed early whereas TRβ 1 mRNA is expressed later during embryonic brain development. In the rat pituitary gland, TH decreases TRβ 2, TRα-1, and α-2 mRNAs while slightly increasing TRβ-1 mRNA. However, in most other tissues, TH decreases TRα-1 and α-2, but not TRβ-1 mRNA (56). Isoform-specific knockout mice of each of the TR isoforms display distinct phenotypes (17,18). However, lack of significant TR isoform-specific gene expression was observed in cDNA microarrays of hepatic genes in TR isoform knockout mice (82). Given the apparent redundancy in TR isoform function, it is possible the different KO phenotypes may be due to absolute TR expression levels in critical tissues and developmental stages.

TRANSCRIPTIONAL REGULATION BY TH RECEPTORS

TH receptors bind to TH response elements (TREs) in specific target genes (Figure 3d-3, below). After binding TH, the receptor induces changes in gene expression by either increasing or decreasing the transcriptional activity of target genes. Examples of the target genes that are positively- and negatively-regulated by TH are summarized in Table 1. Recently, cDNA microarrays have been employed to study TH regulation of hepatic genes in mice, and led to the identification of a large number of novel target genes (both positively- and negatively-regulated) (83,84). These studies demonstrated that TH affected gene expression in a wide range of cellular pathways and functions, including gluconeogenesis, lipogenesis, insulin signaling, adenylate cyclase signaling, cell proliferation, and apoptosis. Although many of the TH-responsive genes were regulated directly by TRs, others were probably regulated indirectly through intermediate genes. Indirect regulation of TH-mediated transcriptionis suggested when the time course for induction is slow (hours) and when it is blocked by protein synthesis inhibitors. Although TH acts mainly at the level of transcription, it also can affect mRNA stability and translational efficiency (84a, 84b. Thus, TH acts at multiple levels to alter protein expression.

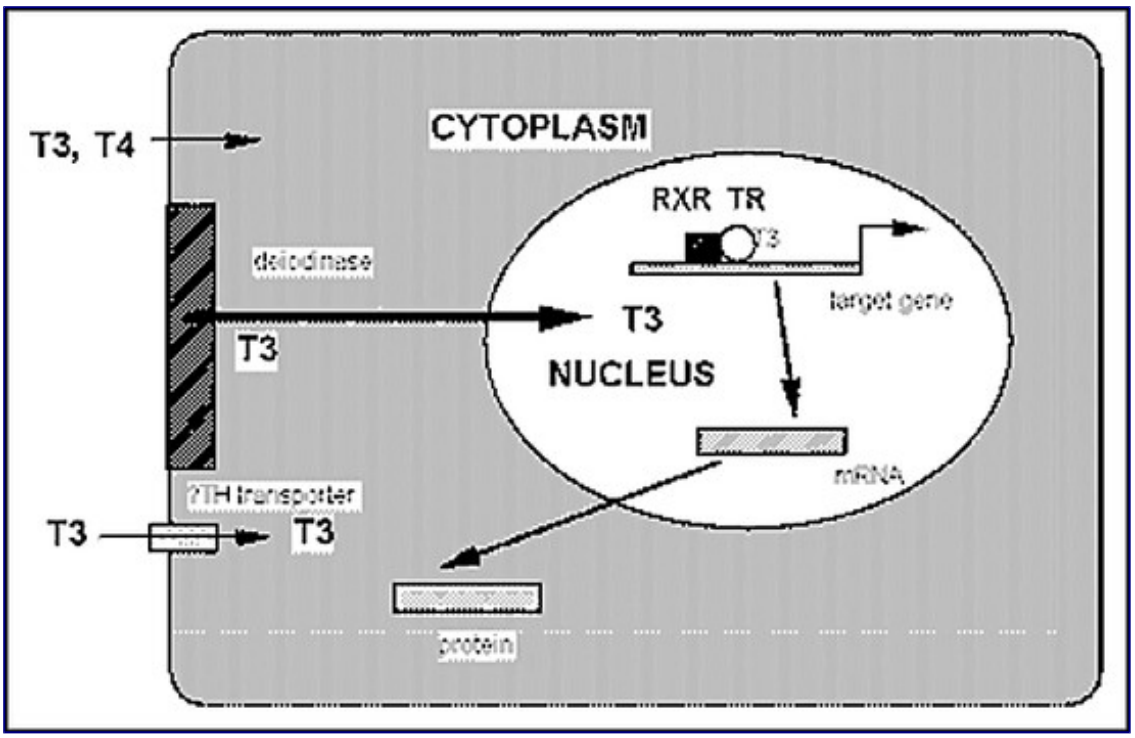

Figure 3. Mechanism of TH action via its nuclear receptor. TH is transported across plasma membrane and likely diffuses through nuclear membrane to bind to its receptor. The TH receptor (open circle) is localized almost exclusively in the nucleus where it associated with DNA as a homodimer or as a heterodimer with RXR (stippled box). The hormone-activated receptor binds to TH response elements (TREs) to alter rates of gene transcription and consequently levels of mRNA.

Table 1. Examples of Genes Positively-regulated by T_3 **.**

1. Fatty acid synthetase

2. Growth hormone

3. Lysozyme silencer

4. Malic enzyme

5. Moloney leukemia virus enhancer

6. Myelin basic protein

7. Myosin heavy chain α

8. Phosphoenolpyruvate carboxykinase

9. RC3

10. Spot 14 lipogenic enzyme

11. Type I 5′-deiodinase

12. Uncoupling protein

13. A large number of other genes (not shown) are positively and negatively-regulated by TH (refs 83-85).

Table 2. Examples of Genes Negatively-regulated by T_3 **.**

1. Epidermal growth factor receptor

2. Myosin heavy chain β

3. Prolactin

4. Thyroid-stimulating hormone α

5. Thyroid-stimulating hormone β

6. Thyrotropin-releasing hormone

7. Type II 5'-deiodinase

TR binding to TH response elements (TREs)

Detailed analyses of thyroid response elements (TREs) have led to the identification of a canonical TRE half-site sequence (2,16, 85) (Figure 3d-4). The TRE half-site is generally considered to be a hexamer (AGGTCA), but TR binding is optimal with a more extended binding site (86,87). Specifically, the sequence TAAGGTCA is optimal for TR binding and T_3 -responsiveness. However, inspection of TREs from many different target genes reveals there is a relatively low degree of sequence conservation among these elements. This finding suggests the possibility that naturally-occurring TREs may have diverged from an ideal consensus element during evolution as a means to modulate the degree of TH responsiveness.

TR interactions with DNA are quite different from those observed with steroid receptors, which bind to palindromic DNA sequences as homodimers. Although TR also can bind to certain TREs *in vitro* as a homodimer, it binds preferentially to most TREs as a heterodimer with the retinoid X receptors (RXRs) (2,16,31,32). The TR-RXR heterodimer binds to half-sites that are arranged in several different configurations. These include palindromic arrangements (head-to-head), direct repeats (head-to-tail), and inverted repeats (tail-to-tail). Most naturally occurring TREs are direct repeats (Figure 3d-4), typically separated by four nucleotides. The ability of TR dimers to bind to TRE's in different configurations suggests a flexible protein structure, or the possibility that distinct protein surfaces are involved in the formation of dimers (32, 86-89). Taken together, the specificity and affinity for the TR-RXR heterodimer is primarily determined by sequences within the half-site, the length of the spacer region between the half sites, and the sequence context within the spacer region.

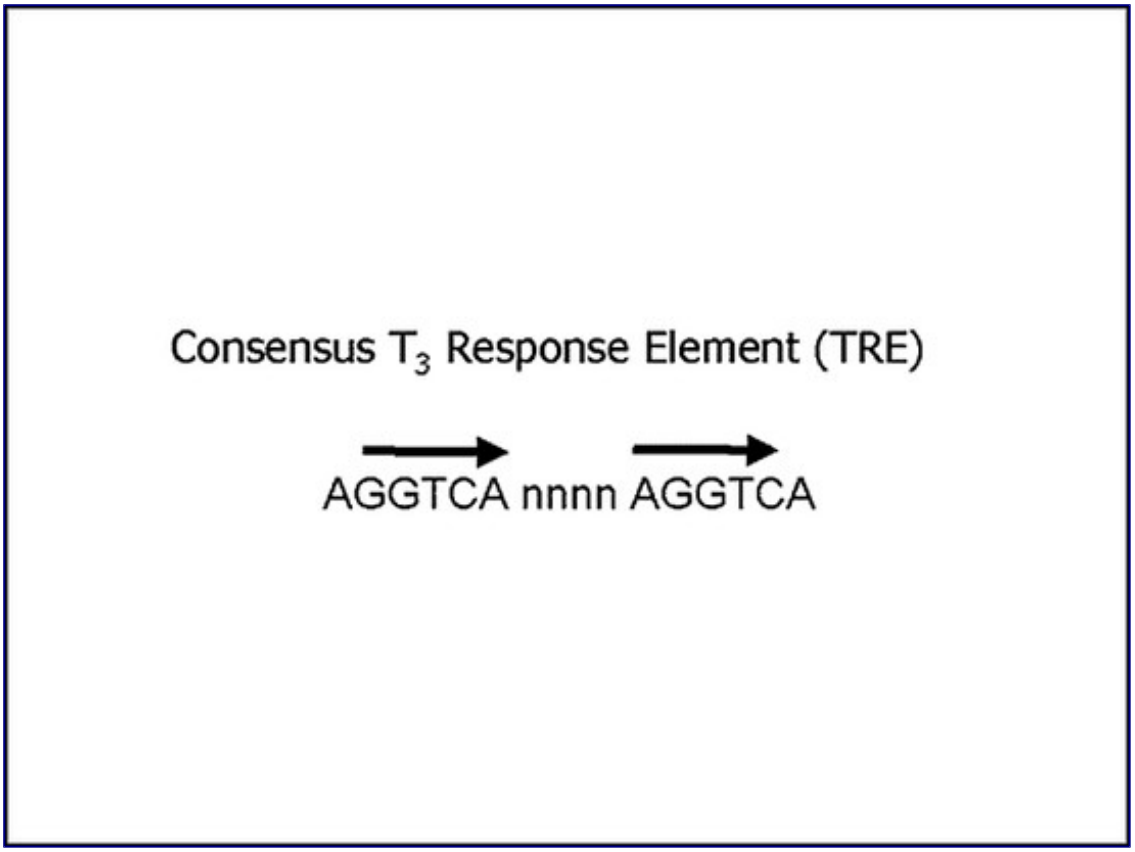

Figure 4. Consensus thyroid response element (TRE). Studies of TRE's in many different promoters has allowed the derivation of a

Although TR can interact with a wide variety of other nuclear receptors and transcriptional adaptor proteins (see below), the RXR proteins (α, β , and γ) represent its most important heterodimeric partners (90). The RXR proteins enhance TR binding to DNA and reduce the rate of receptor dissociation from DNA (91). RXR binds to the 5' sequence and TR binds to the 3' sequence of TREs in which half-sites are arranged as direct repeats (93, 94). The DNA-binding domains interact with the major grooves of the half-sites on the same face of the DNA (39). The carboxy-terminal end of the TR DNA-binding domain forms an α -helical structure that interacts with the spacer region in the DNA minor groove between the TRE half-sites. Although protein-protein contacts between the RXR and TR DNA-binding domains are important for dimerization, the major sub-regions involved in dimerization reside in the carboxy-terminii of the receptors (31,32). The dimerization surface of the TR appears to involve residues that lie along the surfaces of helices 10 and 11. T_3 binding enhances the formation of TR-RXR heterodimers (95). On the other hand, T_3 dissociates TR-TR homodimers (96). These findings raise the possibility that T_3 binding might induce disruption of TR homodimers and induce the formation of TR-RXR heterodimers. The RXRs bind a stereoisomer of all trans retinoic acid, 9-cis retinoic acid (97,98). which variably alters transcriptional activity depending on the nature of the TH responsive gene (2, 16,99). Additional studies are required to clarify the functional roles of RXRs and their ligands in TH action and interaction with other transcription factors. It also appears that TRs can have cross-talk with other nuclear hormone receptors owing to their common abilities to heterocimerize with RXRs. TR crosstalk with peroxisome proliferator-activated receptor (PPAR) and LXR signaling via heterodimerization with RXR is a prominent example. PPARγ regulates the expression of its target genes by binding to the PPAR response element (direct repeat 1; DR1) as a heterodimer with RXR. Recently, it was shown that TRβ1 competes with PPARγ for binding to DR1 as a heterodimer with

RXR *in vitro* and *in vivo* to repress the transcriptional activity of PPARγ (99a). Since PPARγ plays a key role in lipid metabolism, carcinogenesis, and cardiovascular diseases (99b, 99c), this mode of TR may exert some of its effects by crosstalk with PPARs. Recently, cell-based studies indicate that TRβinhibits the activity of LXR-αtranscription activity of the CYP7A1 promoter which shares a common DR-4 element with TR (99d). These studies show that TR cross-talk with other nuclear hormone receptor-mediated signaling expands TR effects beyond those target genes directly regulated by TRs.

Basal repression/Transcriptional corepressors

After binding to DNA, TR alters transcriptional activity by interacting directly or indirectly with a complex array of transcriptional cofactors. These proteins include corepressors (CoRs), coactivators (CoAs), integrators like CREB-binding protein (CBP), and general transcription factors (GTFs) (reviewed in 2, 16,31,32,99, 100). Many of these factors have been identified by protein-protein interaction assays such as the yeast two-hybrid and glutathione-s-transferase pull down assays.

In the absence of TH, TR represses basal transcription in proportion to the amount of receptor and the affinity of receptor binding sites in positively-regulated target genes (101). This phenomenon also is referred to as transcriptional silencing (102, 103). (Figure 3d-5, below). The addition of TH reverses basal repression and increases transcriptional activation above basal levels seen in the absence of receptor. Our understanding of the molecular mechanism for basal repression of transcription by unliganded receptor was advanced significantly by the discovery of a family of repressor proteins that bind selectively to unliganded TRs and RARs. This corepressor family includes silencing mediator for retinoid and TH receptors (SMRT) and nuclear receptor corepressor (NCoR) (32, 33, 104,105). These corepressors are 270 kD proteins that contain three transferable repression domains and two carboxy-terminal α -helical interaction domains. They are able to mediate basal repression by TR and RAR, as well as orphan members of the nuclear hormone receptor family such as rev-erbA α and chicken ovalbumin upstream transcription factor (COUP-TF). They have little or no interaction with steroid hormone receptors and therefore do not mediate basal repression by these receptors. Another protein, small ubiquitous nuclear co-repressor (SUN-CoR) enhances basal repression by TR and rev-erbA (65). This 16kD protein may form part of a co-repressor complex as it interacts with NCoR.

Within the interaction domains of NCoR and SMRT are consensus LXXI/HIXXXI/L sequences which resemble the LXXLL sequences that enable co-activators to interact with nuclear hormone receptors (40-43)(see below). Interestingly, these motifs allow both corepressors and co-activators to interact with similar amino acid residues on helices 3, 5, and 6 which are part of the ligand-binding pocket of TR. Differences in the length and specific sequences of the co-repressor and co-activator interaction sites coupled with the conformational changes in the LBD upon ligand binding, determine whether corepressor or coactivator binds to TR (32, 42).

Recently, it has been shown that corepressors can form a complex with other repressors such as Sin 3 and histone deacetylases that are mammalian homologs of well-characterized yeast transcriptional repressors RPD1 and RPD3 (2,16,32,99,100). Thus, local histone deacetylation likely plays a critical role in the basal repression by unliganded TR/corepressor complex by maintaining local chromatin structure in a state that decreases basal transcription. Upon T_3 binding, TR undergoes a conformational change that dissociates CoRs and recruits an array of coactivators (CoAs). Thus, hormone binding relieves repression and stimulates transcription by altering receptor binding to distinct classes of cofactors. Additionally, DNA-methylation may play a role in basal repression as methyl-CpG-binding proteins can associate with a co-repressor complex containing Sin3 and histone deacetylases (106,107). This repression was relieved by the deacetylase inhibitor, trichostatin A. These findings suggest that

two repression processes, DNA methylation and histone deacetylation, may be linked via methyl-CpG-binding proteins.

The fact that TR alters the level of gene transcription in both the absence and presence of T_3 has important implications for TH physiology. At low hormone concentrations, such as hypothyroidism, the unliganded receptor is predicted to repress transcription rather than function as an inactive, passive receptor. In some respects, this model is borne out by targeted inactivation of the TRα and TRβ genes. The phenotype of these double knockout mice are, for the most part, much less pronounced than the clinical features of congenital hypothyroidism (108,109). Thus, basal repression of transcription may explain why absence of receptor has less deleterious effects than absence of hormone (82,108,109).

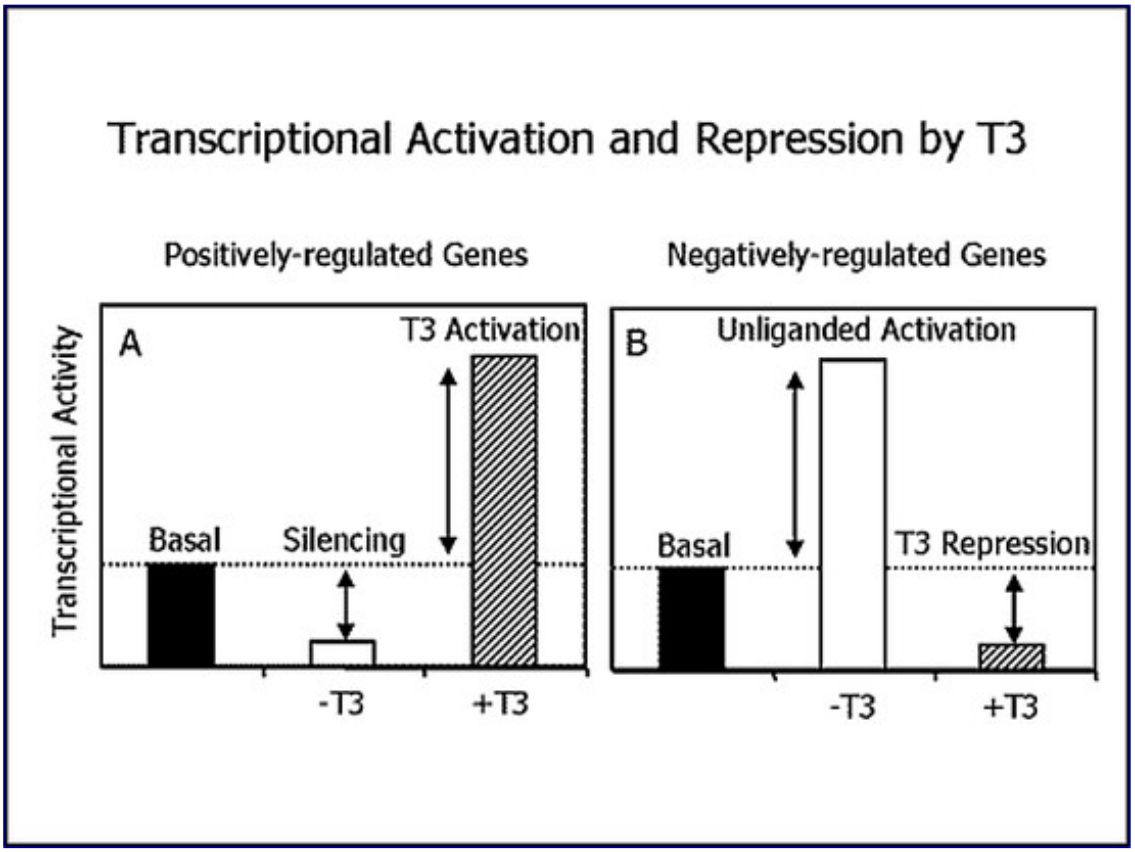

Figure 5. TH receptor-mediated transcriptional silencing and activation. (A) Positively regulated genes. In the absence of hormone, the unliganded TH receptor represses or

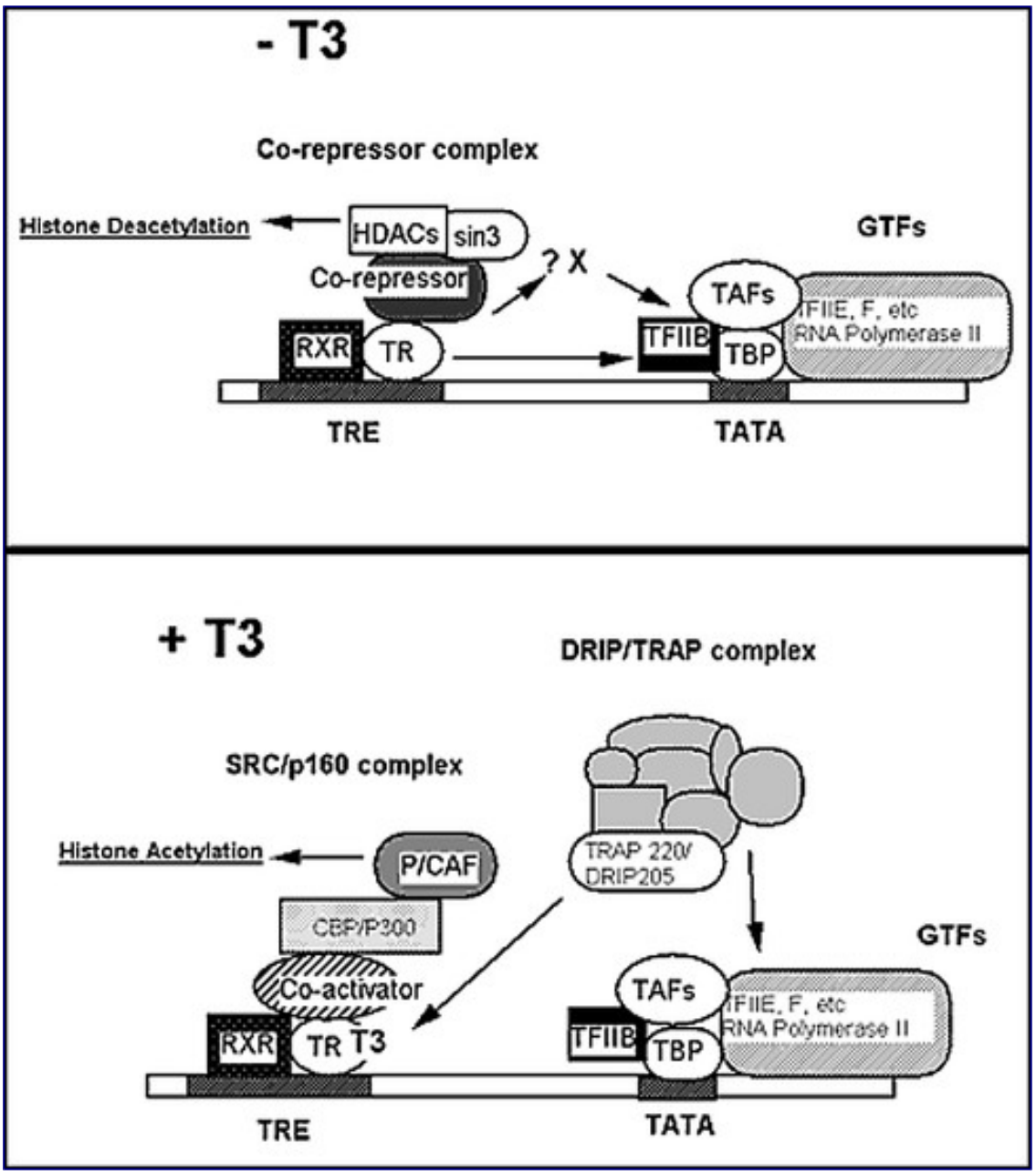

Figure 6. Role of corepressors and coactivators in the control of T 3 -regulated genes. In the absence of T3, the RXR-TR heterodimer recruits corepressors (CoR), which in turn, assemble additional components of a repressor complex that includes histone deacetylase (HDAC). Deacetylation of histones induce transcriptional repression. In the presence of T 3 , the corepressor complex dissociates and coactivators (CoA) bind to TR. The coactivator complex can include steroid receptor co-activators (SRCs)/p160,CREB-binding protein (CBP), p300/CBP associated factor (P/CAF), and proteins with histone acetyltransferase (HAT) activity. Vitamin D receptor interacting protein/TR associated protein (DRIP/TRAP) complex can also interact with liganded TR, and may cycle with SRC/p160 complex. The General transcription factors (GTFs) are also indicated.

Transcriptional activation/Coactivators

A large and growing number of co-factors have been shown to interact with liganded nuclear hormone

receptors and enhance their transcriptional activation. These include: steroid receptor coactivator 1 (SRC1); SRC2/transcriptional intermediary factor 2 (TIF2) / glucocorticoid receptor interacting protein 1 (GRIP1); SRC3/ amplified in breast cancer 1 (AIB1)/ receptor associated coactivator 3 (RAC3)/ p300/CBP cointegrator associated protein (p/CIP)/ nuclear receptor coactivator (ACTR)/ thyroid receptor activator molecule 1 (TRAM 1); peroxisome proliferator activated protein binding protein (PBP); TR accesory proteins (TRAPs) /vitamin D receptor interacting proteins (DRIPs); p300/CBP associated factor (p/CAF), and cAMP response element binding protein (CREB) binding protein (CBP)/ p300. among others (reviewed elsewhere (2,16,31,32,99,100).

At present, the precise roles of all these putative coactivators are not known; however, it appears that there are at least two major complexes involved in ligand-dependent transcriptional activation: the steroid receptor co-activator (SRC) complex and the vitamin D receptor interacting protein/TR associated protein (DRIP/TRAP complex) (Fig. 3d-6). SRCs (SRC-1,SRC-2, and SRC-3) are 160 kD proteins that associate with nuclear hormone receptors, including TRs, and enhance their ligand-dependent transcription (100,110-112). SRCs also interact with the CREB-binding protein (CBP), the co-activator for cAMP-stimulated transcription as well as the related protein, p300, which interacts with the viral co-activator E1A (113-116). Recent studies also have shown that CBP/p300 can interact with PCAF (p300/CBP-associated factor), the mammalian homolog of a yeast transcriptional activator, general control nonrepressed protein 5, GCN5. Like GCN5, PCAF has intrinisic histone acetyltransferase activity (HAT) activity. Both PCAF and CBP interact with TBP associated factors (TAFs) and RNA pol II. Thus, PCAF and CBP possess dual functional roles both as adaptors of nuclear receptors to the basal transcriptional machinery as well as enzymes that can alter chromatin structure by histone acetyl transferase (HAT) activity. SRC 1 and CBP may coordinate with TRs to synergize further the actions of TH, and also allow for the convergence of plasma membrane and nuclear hormone receptor signaling pathways in the cell.

The DRIP/TRAP complex also interacts with liganded VDRs and TRs (117-120). However, none of the subunits are members of the SRC family or their associated proteins. Instead, several DRIP/TRAP components are mammalian homologs of the yeast Mediator complex, which associates with RNA Pol II. Thus, TR recruits DRIP/TRAP complex which, in turn, may recruit or stabilize RNA Pol II holoenzyme via their shared subunits. It is noteworthy that DRIP/TRAP complex does not appear to have intrinisic HAT activity. Recent chromatin immunoprecipitation assays of proteins bound to hormone response elements (HREs), suggest that there may be a sequential, possibly cyclical recruitment, of co-activator complexes to hormone response elements by liganded nuclear hormone receptors (121-123). Studies of co-activator recruitment to TH-regulated genes showed distinct temporal patterns of recruitment. Last, other co-factors such as SW1/Snf and BRG-1 may be involved in early chromatin remodeling before the co-activator complexes are recruited to the TREs .

Negative regulation by TRs

In contrast to positively-regulated target genes, negatively-regulated genes can be stimulated in the absence of TH and repressed by TH (Figure 3d-5, above). Regulation of TRH and the TSH α and β -subunit genes have been studied most extensively as models of negatively-regulated genes. From a physiological perspective, negative-regulation of these genes represents a critical aspect of feedback control of the TH axis. The T_3 -responsive regions of these negatively- regulated genes have been localized to the proximal promoter regions (127-129). However, TR binding to putative TREs in these promoters is relatively weak in comparison to the binding sites in positively-regulated genes.

There are several different potential mechanisms for negative regulation by TH. Negative regulation may involve receptor interference with the actions of other transcription factors or with the basal

transcription apparatus (130, 131). For instance, TR can inhibit the activity of AP-1, a heterodimeric transcription factor composed of Jun and Fos. T_3 -mediated repression of the prolactin promoter has been proposed to occur by preventing AP-1 binding (132). The TR also interacts with other classes of transcription factors, including NF-1, Oct-1, Sp-1, p53, Pit-1, CTCF, and GATA (133-138a). By binding to these, or other positive transcription factors, the TH receptor may be able to inhibit gene expression by protein-protein interactions. Negative regulation may also occur by TR directly binding to DNA. A negative TRE from the TSHβ gene resides in an exon downstream of the start site of transcription (129) raising the possibility that it occludes the formation of a transcription complex. (Figure 3d-6, above) Additionally, liganded TRs may potentially recruit positive cofactors off DNA (squelching), which in turn, could lead to decreased transcription of target genes.

Transcriptional CoRs and CoAs, or even novel co-factors, may be involved in the control of negatively regulated genes. In contrast to the basal repression by unliganded TR in the case of positively regulated genes, CoRs cause basal activation of the TSH and TRH genes (127-129,138b). CoAs also play an apparently paradoxical role in T_3 -dependent repression of negatively regulated genes (139, 140). Moreover, both SRC-1 knockout mice and knockin mice which express a TRβ mutant with a mutation in the helix 12 region (that interacts with CoAs) have defective negative regulation of TSH (141,142). Interestingly, histone acetylation is increasedin the T_3 – mediated negative regulation of TSHa whereas it is decreasedin regulation of TSHb and TRH (138a, 138b, 142a, 142b).

Epigenetic modifications by TRs

Transcriptional regulation by TRs is a multistep process involving: (1) the association of TRs with regulatory sites in the genome (usually within the targe gene promoters) in the context of chromatin, (2) the ligand-dependent recruitment and function of coregulators to modify chromatin and thereby regulating RNA Pol II recruitment to the target genes., and (3)Co-valent modifications of histones to alter chromatin structure, recruit RNA pol II complex, and to mediate transcription. In particular, he site-specific acetylation of histone tails induces local relaxation of chromatin, which enhances the binding of some transcriptional regulators and facilitates the recruitment and functioning of the generaltranscriptional machinery. Recent studies have demonstarted that thyroid hormone-positively regulated target genes may have distinct patterns of coactivator recruitment and histone acetylation that may enable highly specific regulation (124). However the epigenetic changes associated with negetively regulated gene seems to be much more complex. For instance, histone acetylation of H3K9 and H3K18 sites, two modifications usually associated with transcriptional activation, occur in negative regulation of TSHa promoter. T_3 also caused the release of a corepressor complex composed of histone deacetylase 3 (HDAC3), transducin b-like protein 1, and nuclear receptor coprepressor (NCoR)/ silencing mediator for retinoic and thyroid hormone receptor from TSH⋄promoter in chromatin immunoprecipitation assays . These findings demonstrate the critical role of NCoR/HDAC3 complex in negative regulation of TSH⋄gene expression and show that similar complexes and overlapping epigenetic modifications can participate in both negative and positive transcriptional regulation (142a). Of note, histone deacetylation has been observed in T_3 -mediated negative regulation of several target genes (139,142b, 143). Moreover, abberant histone modification at the TRH and TSHa genes has been implicated in the inappropriate TSH secretion observed in resistance to thyroid hormone syndrome (142a,142b)). Other coregulators may be involved in T_3 -mediated regulation as RIP140, a coregulator that can decrease transcription by some nuclear hormone receptors, mediated T_3 repression of Crabp1 gene via chromatin remodeling during adipocyte differentiation (143a).

Another mechanism for T_3 -mediated epigenetic signaling is regulation of small non-coding microRNAs. MicroRNAs act as negative regulators of gene expression by inhibiting the translation or

promoting the degradation of target mRNAs. Since individual microRNAs often regulate the expression of multiple target genes with related functions, modulating the expression of a single microRNA can, in principle, influence an entire gene network and thereby modify complex disease phenotypes (143b). Thyroid hormone have been shown to regulate the levels of microRNA pair miR-206/miR-133b in human skeleton muscles (143c) and miR-208a (84b, 143c) in heart MicroRNAs thus are a noval mechanism for thyroid hormone signaling which may regulate mRNA levels of target genes in which TRs are not recruited to their promoters or directly affect their transcription.

Thyroid hormone receptors and carcinogenesis

There are many reports providing evidence that reduced TR expression and/or alterations in TR genes are common events in human cancer (143d). These alterations include loss of heterozygosity, gene rearrangements, promoter methylation, aberrant splicing and point mutations (143e,f). Tumors, including lung, breast, head and neck, melanoma, renal, uterine, ovarian and testicular tumors, present high frequencies of somatic deletions and mutations in both TR alpha/beta loci (143g,h). Aberrant TRs have also been found in more than 70% of human hepatocellular carcinomas The tendency for TR expression to disappear as malignancies progress suggests that TR can act as a tumor suppressor in human cancers; therefore, loss of expression and/or function of this receptor could result in cell transformation and tumor development (143i). Infact TR overexpression in hepatoma cell lines shows repression of various tumor promoting gene such as PTTG1 (143j), and activation of anti-tumorogenic TGF-beta. However certain mutant TRs like TRbeta $^{PV/PV}$ mey even enhance tumor growth by non-genomicaly activating beta-catenin and PI3K pathways (143k,l). Clarifying the molecular mechanisms by which TRs influence tumor progression and elucidating the epigenetic modifications of T_3 target genes in cancers would perhaps lead to a better understanding of the treatment regime in humans.

Phenotypic EFFECTS OF TR ◇ AND TR KNOCKOUTS

Recently, targeted gene inactivation or knockout (KO) of TR isoforms, and "knockin" of mutant TRs to their native TR genomic locii have provided new information on the mechanisms of TH action (17, 18). The ability to disrupt TR genes by targeted mutagenesis has been particularly challenging given there is more than one gene encoding TRs, multiple splicing variants (TRα-1, α-2, TRβ-1, TRβ-2), and an additional transcript (Rev-erbA) derived from the opposite strand of the TRα gene (2, 16, 18, 99). Two TRα knockout mouse lines have been generated that display different phenotypes (145, 146). It is likely this difference is due to the different sites in the TRα gene locus used for homologous recombination to generate the knockout mice. The TRα gene is complex as it encodes TRα-1, α-2 (which cannot bind T_3), and rev-erbA (generated from the opposite strand encoding TRα) (2, 16, 99). KO mice in which both TRα-1 and α-2 were deleted (TRα $^{-/-}$) had a more severe phenotype with hypothyroidism, intestinal malformation, growth retardation, and early death shortly after weaning (145). T3 injection prevented the early death of pups. KO mice which lacked only TRα-1 (TRα-1 $^{-/-}$) had a milder phenotype with decreased body temperature and prolonged QT intervals on electrocardiograms (146). The phenotypic effects of the loss of TRα 1 are relatively mild (146-148). Unexpectedly, there is no evidence of resistance to TH, as occurs with the TRβ knockout. Disruption of the TRα-1 causes lower heart rates (19% reduced) and prolonged QRS and QT durations. These cardiac effects persist after hormone replacement. No changes were found in the levels of known TH-responsive genes in the heart (e.g., sarcoplasmic Ca^{2+} ATPase, Na^{+}-K^{+} ATPase, β-adrenergic receptors). The bradycardic effect of the TRα-1 knockout may result from alterations in the sympathetic or parasympathetic nervous systems or it could result from an intrinsic defect in cardiac myocytes. The TRα-1-deficient mice also have a 0.5 ^{o}C reduction in body temperature that is independent of TH levels. The mice have normal amounts of brown adipose tissue.

Samarut and co-workers have reported generation of short TRα isoforms from intronic transcriptional start sites which have dominant negative activity on TR function (73), and it is likely these short TRα isoforms are responsible for the more severe phenotype of the TRα $^{-/-}$ mice. In this connection, TRα KO mice which did not express either TRα-1 and α-2 (TRα $^{o/o}$), had a milder phenotype than TRα $^{-/-}$ mice which expressed only the short TRα isoforms (17,18). Interestingly, TH stimulation of some target genes was increased, perhaps due to the absence of α-2 which inhibits normal TR-mediated transcription (149).

Targeted disruption of the TRβ locus created a mouse deficient in both TRβ-1 and TRβ-2 (17, 18). These mice had elevated circulating TSH and T_4 levels, thyroid hyperplasia, as well as hearing defects (150, 151). These findings are similar to the index patients with resistance to TH who were later shown to have homozygous deletion of TRβ (4, 152). Thus, the mouse model appears to faithfully reproduce some of the features seen in humans with resistance to TH who are lack TRβ or express a dominant negative mutant TRβ (4). TRβ-2-selective knockout mice also have been generated and exhibited elevated levels of TH and TSH suggesting TRβ-2 plays the major role in regulating TSH (144). TRβ-2-selective knockout mice also have abnormal color discrimination and suggest TRβ-2 may play a role in cone development of the retina (153).

The relatively mild phenotypes of the TRα 1 and TRβ KO mice suggest the two isoforms have redundant roles in the transcriptional regulation of many target genes. In this connection, microarray studies of TRα and TRβ KO mice showed similar gene regulation profiles in the absence and presence of T_3 in liver (82). Recently a study done using either TR-α or TR-beta receptor over-expressing cell lines have also revealed that both these receptor isoforms mostly share a common gene repertoire but with varying degrees of induction or repression of target genes (143h,i).

When both TR isoforms were abolished, the resultant double knockout mice (TRα 1 $^{-/-}$ TRβ $^{-/-}$) were surprisingly viable (108, 109). Thus, the absence of TRs is compatible with life. These mice had markedly elevated T_4 , T_3 , and TSH as well as large goiters. They also showed decreased growth, fertility, heart rate as well as bone density and development. Interestingly comparison of cDNA microarrays of double KO and hypothyroid mice showed only partial overlap of their gene regulation profiles, confirming the observation that the absence of receptor can give a different phenotype than lack of hormone. It is likely that basal transcription occurs even in the absence of receptor whereas basal repression of target genes occurs in the absence of hormone.

Cheng and colleagues have generated a “knock-in” mouse model in which a mutant TRβ from a patient with RTH was introduced into the endogenous TRβ gene locus (154). These mice have a phenotype similar to patients with RTH, as the heterozygous mice showed elevated serum T_4 and TSH, mild goiter, hypercholesterolemia, impaired weight gain, and abnormal bone development. Homozygous mice had markedly elevated serum T_4 and TSH, and a much more severe phenotype than heterozygous mice. Wondisford and colleagues also have generated a “knock-in” mouse that expresses mutant TRβ (155). These mice had abnormal cerebellar development and function, and learning deficits. These latter studies suggest that expression of mutant TRβ under the control of endogenous TRβ promoter produces many of the clinical features of RTH in mice. This same group also recently developed a knock-in of a mutant TRβ that cannot bind DNA. This model should be useful in distinguishing signaling and developmental patterns due to protein-protein interactions of TRs (as well as non-genomic pathways) from those that require TR binding to TREs of target genes (156).

Nongenomic pathways regulated by TH

There is increasing evidence for non-genomic effects by TH (2,7) in addition to the transcriptional effects mediated by nuclear TRs. Although there is continuous shuttling of a small amount of TRs between the cytoplasm and nucleus (157), these non-genomic effects are most likely mediated by cellular binding proteins other than TRs. Evidence supporting this notion comes from the rapid time course of some TH effects (thus precluding transcription and protein synthesis), utilization of membrane-signaling pathways such as kinases or calmodulin, lack of dependence on the presence of nuclear TRs, and structure-activity correlations by TH analogs that are different than those observed for nuclear TRs (7, 158). Several non-nuclear sites for TH binding have been identified in various cell systems although their functional significances are not well characterized. Some of these include: plasma membrane associated T_3 transporters, calcium ATPase, adenylate cyclase, and glucose transporters ; an endoplasmic reticulum associated protein, prolyl hydroxylase; and monomeric pyruvate kinase (7,158-161).

TH also has profound effects on mitochondrial activity and cellular energy state . A 43 kD protein has been described in mitochondria which also could bind to TREs and could be recognized by antibodies against the TRα ligand-binding domain (162). Recently, it has been shown that TRβ can interact with the p85 subunit of PI3K and activate the PI3K-Akt/PKB signaling cascade; thus, the small subpopulation of cytosolic TRβ may be involved in cell signaling (163,164). This PI3K activation by T_3 leads to both direct and indirect effects on the transcription of several genes involved in glucose metabolism and provides a mechanism for cross-talk between TH and cell signaling pathways.

Recently, integrin α-Vβ-3, has been identified as a plasma membrane TH-binding site (7, 165). Previously, T_4 , but not T_3 , was shown to promote actin polymerization and integrin interaction with laminin in neural cells (166). Additionally, both T4 and T_3 activated mitogen-activated protein kinase (MAPK) activity, and led, among other events, to phosphorylation of TRβ (167). Using a chick chorioallantoic membrane (CAM) system, Davis et al. showed that both T4 and T_3 stimulated angiogenesis (165,167). Since integrin α-Vβ-3 is involved in angiogenesis, T_4 and T_3 binding to it was examined, and T_4 was found to bind to integrin α-Vβ-3 with high affinity. Tetraiodothyroacetic acid (tetrac) and antibodies against laminin blocked T_4 binding. Moreover, siRNAs against the integrin α-V or β-3 subunits blocked MAPK activation by TH. These findings suggest that TH activates the MAPK cascade and stimulates angiogenesis via TH binding to integrin α Vβ 3. Additionally Thyroid hormone non-genomically suppresses Src thereby stimulating osteocalcin expression in primary mouse calvarial osteoblasts (167a). A direct physical interaction of TR PV with cellular proteins, namely the regulatory subunit of the phosphatidylinositol 3-kinase (p85alpha), the pituitary tumor transforming gene (PTTG) and beta-catenin, that are critically involved in cell proliferation, motility, migration, angiogenesis and metastasis suggest a novel mode of non-genomic action, whereby mutant TR isoform acts as an oncogene in thyroid carcinogenesis (167b). In this connection, mutant TR and TR have been reported in hepatic carcinomas (143i).

TH analogs, metabolites, and antagonists

Several tissue-specific and TR isoform-specific compounds have been developed as potential treatments for hypercholesterolemia, obesity, and heart failure. An early prototypical compound was 3,5-dibromo-3-pyridazinone-L-thyronine (L-940901) which bound preferentially to the TRs in the liver

over those in the heart (168). Although the relative affinity of this compound for the respective TR isoforms has not been reported, the selective action of L-940901 is likely due to tissue-specific uptake of the compound. Interestingly, mice treated with L-940901 had decreased serum cholesterol levels without cardiotoxicity. Recently, several other TH analogs have been described that have isoform-selective affinity for TRβ compared to TRα (169-173). Since TRs in the liver are approximately 90% TRβ whereas those in the heart are mostly TRα, these isoform-selective compounds may serve as novel agents to lower serum cholesterol with minimal cardiotoxicity. N-[3,5-dimethyl-4-(4'-hydroxy-3'isopropylphenoxy)-phenyl]-oxamic acid (CGS 23425), 3,5-dimethyl-4(4'-hydroxy-3'-isopropylbenzyl)-phenoxy) acetic acid (GC-1), and 3,5-dichloro-4[(4-hydroxy-3-isoopropylphenoxy)phenyl] acetic acid (KB-141) all have been reported to lower total serum cholesterol and LDL-cholesterol (169-173). CGS 23425 also increases LDL receptor expression in HepG2 cells. Additionally, these compounds can increase serum apoA1 levels; however, the total serum high density lipoprotein (HDL) cholesterol level does not changes or may even decrease. In this connection, GC-1 decreased serum HDL; increased expression of HDL receptor, SR-B1; stimulated the activity of cholesterol 7α hydroxylase; and increased fecal excretion of bile acids in treated mice (172). Thus, GC-1 regulates important steps in the reverse cholesterol transport pathway. Recently, KB141 was shown to be a potential treatment for obesity by decreasing body weight via stimulation of metabolic rate and oxygen consumption (173). The TR agonist MB07811, which is converted to an active metabolite in the liver, has proven to be effective in reducing hepatic steatosis in rodents (C173a). The TRb-specific compound, KB215 recently was shown to be effective in decreasing LDL cholesterol, apoliprotein B, triglycerides, and Lp(a) in humans (174) suggesting that this may be a new therapy for lipid abnormalities that may be able to be used in combination with stations.TH analogs and derivatives also bind specifically to proteins other than TRs, and are involved in non-genomic cell signaling pathways.

Recently, Scanlan and colleagues identified 3-iodothyronamine (T1AM), which is a naturally occurring byproduct of TH, as a potent agonist of the G protein-coupled trace amine receptor, TAR1(175). Significantly, this compound bound poorly to nuclear TRs. T1AM has interesting physiological actions as it produced a rapid drop in body temperature and heart rate when injected intraperitoneally in mice. T1AM also decreased cardiac output in an ex vivo working heart model. These physiological actions of T1AM are opposite of those observed for T_3, and may provide a counter-regulation to the transcriptional effects of TH by nuclear TRs. As of this date their physiological importance is entirely unknown.

TH can increase cardiac performance by increasing cardiac contractility and decreasing systemic vascular resistance (2); however, TH excess also can cause cardiotoxicity. 3,5-diiodothyropropionic acid (DITPA) is a TH-related compound with low metabolic activity and low affinity for nuclear TRs (Kd 10^{-7} M). DITPA was able to increase cardiac contractility and peripheral circulation without significant effects on heart rate in animal studies (176). Moreover, DITPA improved hemodynamic performance in animal models of congestive heart failure after myocardial infarction. Patients with heart failure treated with DITPA showed significant improvement in systolic cardiac index and systemic vascular resistance in preliminary studies (176). Thus DITPA or similar compounds may represent a novel class of drugs for the treatment of heart failure.

Recently, the naturally occurring analogs, 3,5,3′-triiodothyroacetic (TRIAC) and 3,5,3′,5′-tetraiodothyroacetic TETRAC) acids decreased heat-induced albumin fibrillation suggesting they may have a protective effect against amyloid formation (177). Additionally, tetraiodothyroacetic acid (tetrac) caused radiosensitization of GL261 glioma cells (178). The mechanisms for these effects are not understood at this time but likely involve non-genomic effects as TRIAC and TETRAC have weak binding affinity for TRs.

SUMMARY

We have learned much about molecular mechanisms of nuclear TH action during the past 20 years. In particular, the identification and characterization of TRs, their heterodimeric partners, corepressors, coactivators, and TREs, generation of TR knockout mice, and discovering non-genomic pathways have provided new insight into TH action. It is expected that new information will be obtained from microarray and proteomic studies, structural biology approaches, and in vitro transcriptional systems. Such information should provide an even better understanding of the mechanisms of disease caused by abnormal levels of circulating TH, and could lead to the development agonists and antagonists that may serve as useful therapeutic agents.

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