Physiological and Molecular Basis of Thyroid Hormone Action

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I. Introduction

Thyroid hormones (THs) play critical roles in differentiation, growth, metabolism, and physiological function of virtually all tissues. Indeed, TH is required for the normal function of nearly all tissues, with major effects on oxygen consumption and metabolic rate (375). Disorders of the thyroid gland are among the most common endocrine maladies. Furthermore, endemic cretinism due to iodine deficiency remains a public health problem in developing countries at the advent of the third millennium. Thus the study of TH action has important biological and medical implications.

The story of TH action is interwoven with many of the major advances in biomedical science during the past century. Contributions from clinical medicine, physiology, biochemistry, and molecular genetics have had major...
impacts on our understanding of TH action (376, 551). The following outline sketches only some of the many early contributions to our knowledge.

In 1888, the Clinical Society of London published the definitive report that first linked cretinism and adult hypothyroidism to the destruction of the thyroid gland (97a). Soon afterward, thyroid extracts from sheep were used for the treatment of hypothyroidism. Around this time, Emil Kocher performed some of his pioneering studies on the pathology and surgery of the thyroid gland for which he was awarded the Nobel prize in medicine in 1909. In 1914, Kendall (241) isolated 3,5,3',5'-tetraiodo-L-thyronine (T₃) from thyroid extracts, and almost 40 years later, Gross and Pitt-Rivers (178) synthesized 3,5,3'-triiodo-L-thyronine (T₄) and demonstrated its presence in human plasma and its ability to prevent goiter in thiourea-treated rats. Over the ensuing years, the metabolic and oxygen consumption effects of THs as well as its effects on development, particularly in amphibians, were appreciated (59, 79, 463).

In the 1960s, Tata and co-workers (509, 510) first suggested that THs might be involved in the transcriptional regulation of target genes. These investigators observed that T₃ treatment of hypothyroid rats induced a rapid increase in RNA synthesis in the liver which preceded new protein formation and mitochondrial oxidation (509, 510). The groups of Oppenheimer (372) and Samuels (441) then used radiolabeled TH to demonstrate specific nuclear binding sites in different T₃-sensitive tissues, and thus provided the first evidence for TH receptors (TRs). Moreover, T₃ binding was observed in almost all tissues (377). Attempts to purify these receptors biochemically were only partially successful; however, photoaffinity labeling of nuclear extracts demonstrated different-sized receptors and raised the possibility of multiple TR isoforms (123, 384). Studies on T₃ induction of the rat growth hormone (GH) gene transcription suggested that TRs recognized enhancer sequences or TH response elements (TREs), similar to steroid hormone receptors (101, 281, 282, 440). Thus TRs behaved similar to steroid hormone receptors with respect to nuclear site of action, recognition of specific DNA sequences, and ligand-dependent regulation of transcription. In 1985, the glucocorticoid receptor was cloned and, surprisingly, had homology with a known viral oncogene product, v-erbA, that in conjunction with v-erbB, can cause erythroblastosis in chicks (209). Subsequent cloning of the estrogen receptor suggested that there was a family of nuclear hormone receptors (174). A year later, the laboratories of Evans (545) and Vennstrom (444) ushered in the molecular era of TH action when they cloned two different TR isoforms and showed they were the cellular homologs of v-erbA.

Since the molecular cloning of TRs 15 years ago, there has been an explosion of information on the molecular mechanisms of TR action. The power of molecular genetics has greatly aided our understanding of the roles of unliganded and liganded TRs in regulating target genes. We have learned that there are multiple TR isoforms that bind to TREs with variable orientation, spacing, and sequences for TRE half-sites. TRs also interact with other nuclear proteins such as corepressors or coactivators to form complexes that regulate local histone acetylation and interact with the basal transcriptional machinery. Additionally, the solution of the crystal structures of the TR ligand-binding domain (LBD) and other nuclear hormone receptors have provided insight into some of these complex interactions at the molecular level. The development of transgenic and knockout mouse models have shed light on the roles of TRs in the regulation of specific target genes and development. These findings have greatly aided our understanding of the molecular mechanisms of TH action in normal and disease states. In particular, much has been learned about the pathogenesis of the human genetic disorder of resistance to thyroid hormone (RTH). We review some of the major advances in these areas by initially focusing on what is known about the molecular mechanisms of TH action and then discuss their implications for TH action in specific tissues, RTH, and genetically engineered mouse models.

II. BACKGROUND: THYROID HORMONE SYNTHESIS

TH synthesis and secretion is exquisitely regulated by a negative-feedback system that involves the hypothalamus, pituitary, and thyroid gland [hypothalamic/pituitary/thyroid (HPT) axis] (467). Thyrotropin releasing hormone (TRH) is a tripeptide (PyroGlu-His-Pro) synthesized in the paraventricular nucleus of the hypothalamus. It is transported via axons to the median eminence and then to the anterior pituitary via the portal capillary plexus. TRH binds to TRH receptors in pituitary thyrotropes, a subpopulation of pituitary cells that secrete thyroid stimulating hormone (TSH). TRH receptors are members of the seven-transmembrane spanning receptor family and are coupled to Gₛ₁₁. TRH stimulation leads to release and synthesis of new TSH in thyrotropes. TSH is a 28-kDa glycoprotein composed of α- and β-subunits designated as glycoprotein hormone α- and TSH β-subunits. The α-subunit also is shared with other hormones such as luteinizing hormone, follicle stimulating hormone, and chorionic gonadotropin. Both TRH and TSH secretion are negatively regulated by TH. An important mechanism for the negative regulation of TSH may be the intrapituitary conversion of circulating T₄ to T₃ by type II deiodinase. Additionally, somatostatin and dopamine from the hypothalamus can negatively regulate TSH secretion.

TSH is the primary regulator of TH release and se-
cretion. It also has a critical role in thyroid growth and development. TSH binds to the TSH receptor (TSHr), which also is a seven-transmembrane spanning receptor coupled to $G_s$ (255, 382). Activation of TSHr by TSH or autoantibodies in Graves’ disease leads to an increase in intracellular cAMP and stimulation of protein kinase A-mediated pathways. A number of thyroid genes, including Na$^+/I^-$ symporter (NIS), thyroglobulin (Tg), and thyroid peroxidase (TPO), are stimulated by TSH and promote the synthesis of TH. Of note, activating mutations in TSHr and $G_s$ have been described in autonomously functioning thyroid nodules and familial congenital hyperthyroidism (381, 383).

The THs, $T_4$ and the more potent $T_3$, are synthesized in the thyroid gland (Fig. 1). Iodide is actively transported and concentrated into the thyroid by NIS (102, 475). The trapped iodide is oxidized by TPO in the presence of hydrogen peroxide and incorporated into the tyrosine residues of a 660-kDa glycoprotein, Tg. This iodination of specific tyrosines located on Tg yields monooiodinated and diiodinated residues (MIT, monoiodo-tyrosines; DIT, diiodo-tyrosines) that are enzymatically coupled to form $T_4$ and $T_3$. The iodinated Tg containing MIT, DIT, $T_4$, and $T_3$, then is stored as an extracellular storage polypeptide in the colloid within the lumen of thyroid follicular cells. Genetic defects along the synthetic pathway of THs have been described in humans and are major causes of congenital hypothyroidism in iodine-replete environments (117, 261).

The secretion of THs requires endocytosis of the stored iodinated Tg from the apical surface of the thyroid follicular cell (511). The internalized Tg is incorporated in phagolysosomes and undergoes proteolytic digestion, re-capture of MIT and DIT, and release of $T_4$ and $T_3$ into the circulation via the basal surface. The majority of released TH is in the form of $T_4$, as total serum $T_4$ is 40-fold higher than serum $T_3$ (90 vs. 2 nM). Only 0.03% of the total serum $T_4$ is free (unbound), with the remainder bound to carrier proteins such as thyroxine binding globulin (TBG), albumin, and thyroid binding prealbumin. Approximately 0.3% of the total serum $T_3$ is free, with the remainder bound to TBG and albumin. It is the free TH that enters target cells and generates a biological response.

The major pathway for the production of $T_3$ is via 5’-deiodination of the outer ring of $T_4$ by deiodinases and accounts for the majority of the circulating $T_3$ (50, 256). Type I deiodinase is found in peripheral tissues such as liver and kidney and is responsible for the conversion of the majority of $T_4$ to $T_3$ in circulation. Type II deiodinase is found in brain, pituitary, and brown adipose tissue and primarily converts $T_4$ to $T_3$ for intracellular use. These deiodinases recently have been cloned and demonstrated to be selenoproteins (280). 5’-Deiodination by type I deiodinase and type III deiodinase, which is found primarily in placenta, brain, and skin, leads to the generation of rT$_3$, the key step in the inactivation of TH. rT$_3$ and $T_3$ can be further deiodinated in the liver and are sulfo- and glucuronide-conjugated before excretion in the bile (124). There also is an enterohepatic circulation of TH as intestinal flora deconjugates some of these compounds and promotes the reuptake of TH.

Although THs may exert their effects on a number of intracellular loci, their primary effect is on the transcriptional regulation of target genes. Early studies showed that the effects of THs at the genomic level are mediated by nuclear TRs, which are intimately associated with chromatin and bind TH with high affinity and specificity (375, 440). Similar to steroid hormones that also bind to nuclear receptors, TH enters the cell and proceeds to the nucleus (Fig. 2). It then binds to TRs, which may already be prebound to TREs located in promoter regions of target genes. The formation of ligand-bound TR complexes that are also bound to TREs is the critical first step in the positive or negative regulation of target genes and the subsequent regulation of protein synthesis. Given their abilities to bind both ligand and DNA as well as their ability to regulate transcription, TRs can be regarded as ligand-regulatable transcription factors.

### III. MULTIPLE THYROID HORMONE RECEPTOR ISOFORMS

In 1986, the laboratories of Vennstrom (444) and Evans (545) independently cloned cDNAs encoding two different TRs from embryonal chicken and human placental cDNA libraries. Several unexpected findings stemmed from their landmark work. First, they demonstrated by amino acid sequence comparison that TRs are the cellular homologs of the viral oncogene product v-erbA. Second, TRs were shown to have amino sequence homology with steroid hormone receptors. This was initially surprising.
since T₃ and cholesterol-derived steroids are structurally different ligands. However, in the ensuing years, TRs have been shown to belong to a large superfamily of nuclear hormone receptors that include the steroid, vitamin D, and retinoic acid receptors as well as “orphan” receptors for which there are no known ligand or function (35, 285). TRs share a similar domain organization with other family members as they have a central DNA-binding domain containing two “zinc fingers” and a carboxy-terminal LBD. These initial studies also suggested that there were multiple TR isoforms. Subsequent work by many groups has confirmed that there are two major TR isoforms encoded on separate genes, designated as TRα and TRβ, encoded on human chromosomes 17 and 3, respectively (284). Moreover, these multiple isoforms exist in different species such as amphibians, chick, mouse, rat, and human (284). Both TR isoforms bind T₃ (reported dissociation constant values between 10⁻⁹ and 10⁻¹⁰ M) and mediate TH-regulated gene expression (148, 340, 449). In mammalian species, TRα-1 and TRβ-1s range from 400 to slightly over 500 amino acids in size (284, 289) and contain highly homologous DNA-binding domains and LBD (Fig. 3).

In addition to two separate genes that encode TRs, there is additional heterogeneity of TRs due to alternative splicing (220, 292, 330, 349). Alternative splicing of the initial RNA transcript of the TRα gene generates two mature mRNAs that each encode two proteins: TRα-1 and c-erbAα-2. In the rat, these proteins are identical from amino acid residues 1–370, but their respective sequences diverge markedly thereafter (Fig. 3). Consequently,
c-erbAα-2 cannot bind T₃ because it contains a 122-amino acid carboxy terminus that replaces a region in TRα-1 that is critical for TH binding. Additionally, c-erbAα-2 binds TREs weakly but cannot transactivate TH-responsive genes. Thus TRα-1, but not c-erbAα-2, is an authentic TR. Indeed, c-erbAα-2 may act as an inhibitor of TH action possibly by competing for binding to TREs (253, 291). The TRα-1 and c-erbAα-2 system, then, represents one of the first examples in which multiple mRNAs generated by alternative splicing encode proteins that may be antagonistic to each other. Mitsushashi et al. (330) also have described a second TRα variant, c-erbAα-2V, in which the first 39 amino acids of the divergent sequence are missing (330). Its function currently is unknown. Yet another interesting feature of the TRα gene is the employment of the opposite strand to encode a gene product, rev-erbA. Rev-erbA mRNA contains a 269-nucleotide stretch which is complementary to the c-erbAα-2 mRNA due to its transcription from the DNA strand opposite of that used to generate TRα-1 and c-erbAα-2 (293, 331). This protein also is a member of the nuclear hormone receptor superfamily. It is expressed in adipocytes and muscle cells, and can bind to TREs and retinoic acid response elements (RAREs) and repress gene transcription (190, 477, 597). However, rev-erbA should be considered an orphan receptor since its cognate ligand and function are not known. One potential role for rev-erbA may be to regulate the splicing that generates c-erbAα-2 as increased levels of rev-erbA mRNA correlate with increased TRα-1 mRNA relative to c-erbAα-2 (80, 224, 290).

There also are two TRs derived from the TRβ gene (205, 284). This gene contains two promoter regions each of which is vital for the transcription of an mRNA coding for a distinctive protein. By the use of alternate promoter choice, one or both of the coding mRNAs are generated (566). The resultant TRβ isoforms are designated as TRβ-1 and TRβ-2. The amino acid sequences of the DNA binding, hinge region, and LBDs of these two TRβs are identical, but the amino-terminal regions bear no homology (Fig. 3). Both are authentic receptors as they bind TREs and TH with high affinity and specificity and can mediate TH-dependent transcription. The expression of the two TRβ isoforms may be regulated by pituitary-specific transcription factors such as Pit-1 (566).

Both TRα-1 and TRβ-1 mRNAs and proteins are ubiquitously expressed in rat tissues (204). However, TRα-1 mRNA has highest expression in skeletal muscle and brown fat, whereas TRβ-1 mRNA has highest expression in brain, liver, and kidney. In contrast to the other TR isoforms, TRβ-2 mRNA and protein have tissue-specific expression in the anterior pituitary gland and specific areas of the hypothalamus as well as the developing brain and inner ear (47, 48, 98, 204, 590). In the chick, TRβ-2 mRNA also is expressed in the developing retina (473).

Careful low-stringency hybridization studies so far have not yielded any additional TR isoforms. Double TRα and TRβ knockout mice are viable, and these mice did not have detectable [¹²⁵I]T₃ binding in nuclear extracts of several tissues (171, 316a). However, a number of short forms of TRα and TRβ generated by alternative splicing of mRNA or by use of internal translational start sites have been found in embryonic stem cells and in fetal bone cells and may have biological significance (41, 75, 553, 567). The identification of a novel estrogen receptor isoform (ERβ) 10 years after the discovery of ERα serves as a cautionary warning to remain open to the possibility of novel TR isoforms, particularly in restricted tissues or during transient periods in fetal development (265).

The regulation of the TR mRNAs is isoform and cell type dependent. In the intact rat pituitary, T₃ decreases TRβ-2 mRNA, modestly decreases TRα-1 mRNA, and slightly increases rat TRβ-1 mRNA (204). Despite these opposing effects, the total T₃ binding decreases by 30% in the T₃-treated rat pituitary. Similar findings also were observed in GH₃ cells, a somatolactotropitc rat cell line (205). In other tissues, T₃ slightly decreases TRα-1 and c-erbAα-2 mRNA except in the brain where c-erbAα-2 levels are unaffected. TRβ-1 mRNA is minimally affected in nonpituitary tissues. Additionally, the hypothalamic tripeptide TRH decreases TRβ-2 mRNA, slightly decreases TRα-1 mRNA, and minimally affects TRβ-1 mRNA in GH₃ cells (230). Retinoic acid blunts the negative regulation by T₃ in these cells (114, 231). Additionally, in patients with nontyroidal illness in which their circulating free T₃ and T₄ levels were decreased, TRα and TRβ mRNAs were increased in peripheral mononuclear cells and liver biopsy specimens (556). Thus induction of TR expression may compensate for decreased circulating TH levels in these patients.

Each of the TR isoforms found in human, rat, and mouse are highly homologous with respect to their amino acid sequences (284). This conservation among species suggests that there may be important specialized functions for each isoform (109). However, the evidence for isoform-specific functions has been scant since most co-transfection experiments have failed to show important functional differences. Nonetheless, recent studies have suggested that TRβ-1 may exhibit isoform-specific regulation of the TRH and myelin basic protein genes, and TRβ-2 may play an important role in the regulation of the GH and TSHβ gene expression in the pituitary (4, 129, 208, 283, 305). Future studies with TR knockout mice, antisense oligonucleotides in tissue culture, and isoform-specific ligands, perhaps in conjunction with cDNA microarrays, should shed more light on the respective roles of TR isoforms in regulating specific target genes (24, 86, 136, 151).
IV. THYROID HORMONE RECEPTOR
FUNCTIONAL DOMAINS

Mutational analyses of TRs and comparisons with other members of the nuclear hormone receptor superfamily have yielded much information on the structural features of TRs (284, 583). All TRs have a similar domain organization as that found in all nuclear hormone receptors: an amino-terminal A/B domain, a central DNA-binding domain containing two “zinc fingers” (DBD), a hinge region containing the nuclear localization signal, and a carboxy-terminal LBD (Fig. 4). It should be noted that each of these domains and regions may subserve multiple functions, and thus their names may only reflect the first function ascribed to them.

A. DNA-Binding Domain

The DBD is located in the central portion of TR and has two zinc fingers, each composed of four cysteines coordinated with a zinc ion (Fig. 5). The integrity of each zinc finger is critical, as deletion of zinc fingers or amino acid substitution of these cysteine residues abrogates DNA-binding and transcriptional activity of steroid hormone receptors and TRs (173, 345, 460, 592). Within the first zinc finger, there is a “P box,” comprised of amino acids located between and just distal to the third and fourth cysteines, which is similar to that of estrogen receptors (ERs), retinoic acid receptors (RARs), retinoid X receptors (RXRs), and vitamin D receptors (VDRs) (106, 317, 531). This critical region has been shown to be important in sequence-specific recognition of hormone response elements by different members of the nuclear hormone superfamily and contacts nucleic acids and phosphate groups within the major groove of the TRE (353, 413). Additionally, there are other important contact points within the minor groove of the TRE just downstream from the second zinc finger (A-box region). Also, as discussed below, TRs can heterodimerize with RXRs and can bind to TREs that are arranged as direct repeats separated by a four nucleotide gap. These TR/RXR heterodimers bind to TREs with a 5’ to 3’ polarity with TR in the downstream position (268, 391, 586). The ability to heterodimerize with RXR is critical for TR binding to the asymmetric TRE, as dimerization contacts stabilize the DNA binding and determine the spacing between half-sites. Within the DBD, there are dimerization interfaces in the TR just upstream of the first zinc finger, within the first zinc finger, and in a subregion distal to the second zinc finger (T box). The RXR dimerization surfaces are located in the second zinc finger including an arginine located in the D box, a region which previously has been shown to be important for distinguishing spacing between half-sites of hormone response elements (315, 531).

B. Ligand-Binding Domain

The LBD not only is necessary for TH binding but also plays critical roles for dimerization, transactivation,
and basal repression by unliganded TR. The recent solutions of the crystal structures of the liganded TRα-1, unliganded RXRa, and RARγ LBDs have greatly aided our understanding of its role on these functions and the attendant conformational changes that occur when T3 binds to the receptor (Fig. 6) (46, 417, 544). Ligand is buried deep within a hydrophobic pocket in the LBD formed by discontinuous stretches that span almost the entire LBD. In particular, the most carboxy-terminal region (Helix 12) contributes its hydrophobic surface as part of the ligand-binding cavity. The hydrophobic residues face inward, whereas the conserved glutamate faces outward. The cavity also is bounded by hydrophobic surfaces from helices 3, 4, and 5. Although the crystal structure of unliganded TR has yet to be solved, the crystal structure of unliganded RXRa shows that helix 12 projects into the solvent. Thus it is likely that helix 12 undergoes major conformational changes upon ligand binding, from a more open conformation to a closed one, which has been likened to a “mouse trap” mechanism. In an analogous manner, estrogen-bound LBD shows a similar structure as liganded-TR LBD with helix 12 facing inward (61). However, helix 12 of raloxifene-bound ER LBD is in a different position, lying in a groove between helices 3 and 5. Thus the relative positions of helix 12 and the boundary helices may determine whether coactivators can interact with TR. Indeed, studies using TR-LBD mutants based on the TR-LBD crystal structure have confirmed these regions for interacting with the coactivator GRIP-1 (107, 134).

TRα and TRβ-1 isoforms can bind T3 and various TH analogs with subtle differences in affinity. TRα binds T3 with slightly higher affinity than TRβ-1 (449). Triac (3,5,3‘-triiodothyroacetic acid) binds TRα with similar affinity as T3 and binds TRβ-1 with two- to threefold higher affinity than T3. Several novel thyromimetics have been designed which bind TRβ-1 (GC-1 and CGS 23425) with 10- to 50-fold higher affinity (86, 512). The transcriptional activities of these isoform-specific compounds parallel their binding affinities and may offer novel therapeutic treatments of diseases such as hypercholesterolemia while sparing the heart (which contains mostly TRα) from side effects. The crystal structures of hTRα and hTRβ LBDs have been solved and may provide important information for designing even more selective thyromimetics in the future (419).

The LBD also is involved in several other important receptor functions. Scattered throughout the LBD are discontinuous heptad repeats that have been proposed to form hydrophobic interfaces for TR homo- and heterodimerization (145). Mutations in the ninth heptad repeat region have selectively decreased TR homo- and heterodimer formation, suggesting that there may be different subregions of the LBD that are important for TR dimerization (21, 140, 344, 592). Indeed, the TRα-1 LBD crystal structure demonstrates that there is a hydrophobic surface in the ninth heptad repeat region that could serve as a potential dimerization interface (544). A natural TRβ mutation from a patient with resistance to TH at amino acid 316 also displayed decreased homodimer formation, suggesting that additional regions of the LBD may be important for dimerization (189, 360, 592). The relative contributions to dimerization by the LBD and DBD interfaces may depend on the receptor. A recent study suggests that a region that contains the ninth heptad region called the “I box” may be important for RAR heterodimerization with RXR in solution and for binding to direct repeats of variable spacing (392). On the other hand, the DBD dimerization interface may be important for dictating binding to direct repeats of a specific spacing (in this case, a 5-nucleotide gap). Recent studies suggest that the ninth heptad region may be more important for heterodimerization of TRα-1, whereas the DBD may play the dominant role for c-erbAα-2 because it lacks a complete ninth heptad region due to alternative splicing (416, 568).

Baniahmad et al. (27) used a GAL4-fusion system to identify at least three transcriptional activation regions in the LBD and designated them as τ2, τ3, and τ4 (27). Uppalari and Towle (534) also have used a yeast trans-
fection system to describe several activation regions in TRβ-1 LBD as well as in the hinge region (534). In particular, τ1 located near the carboxy terminus has high homology with LBD sequences found in other nuclear hormone receptors previously designated as the activation function-2 (AF-2) domain (Fig. 7). This sequence located within helix 12 has been shown to be important for ligand-dependent transcriptional activation by other nuclear hormone receptors (31, 105, 278). Recently, Chatterjee and co-workers (521) have made point mutations in this region and have observed normal T3 binding and DNA binding, but no transcriptional activation, using a GAL4/TRβ LBD fusion protein system (521). As discussed earlier, helix 12 likely undergoes major conformational changes upon ligand binding (419). Studies with steroid hormone receptors and TRs have demonstrated that the AF-2 domain is important for interactions with coactivators such as SRC-1 and related family members (419). Interestingly, mutations in the AF-2 region of the TR LBD had modest effects on T3-dependent interaction of the coactivator TRAM-1 (SRC-3), whereas a mutation in helix 3 of the TR LBD severely impaired T3-dependent interaction with TRAM-1 but had little effect on interaction with SRC-1 (503). Ligand-dependent GRIP-1 (SRC-2) interactions with TR involve helices 3, 5, 6, and 12 (134). These findings suggest that different subregions of TR may differentially contribute to interaction with specific coactivators. Additionally, several groups reported that corepressors may interact with sequences on helices 3, 5, and 6 that overlap sequences involved in interacting with coactivators (214, 348, 390). Several mutations from patients with resistance to TH and artificial mutations in helices 3 and 5 do not interact with coactivators or corepressors (97, 348, 390).

C. Hinge Region

The hinge region between the DBD and T3-binding domain likely contains an amino acid sequence that is associated with nuclear localization (126). This lysine-rich sequence is highly conserved among nuclear hormone receptors and bears homology with the simian virus 40 T antigen nuclear localization sequence. TRs are likely imported into the nucleus shortly after synthesis as they are predominantly found in the nucleus and can bind DNA, even in the absence of hormone. Furthermore, unlike some steroid hormone receptors, TRs do not associate with cytoplasmic heat shock proteins (103). Recent studies using green fluorescent fusion proteins of wild-type TRβ and TRβ hinge region mutants demonstrated this region may be important for T3-mediated translocation of TR into the nucleus (605).

The hinge region also has additional properties. The laboratories of Evans (83) and Rosenfeld and co-workers (211) identified corepressor proteins that can interact with unliganded TRs, RARs, and v-erbA and mediate repression of basal transcription by these receptors and v-erbA (see sect. viii). Mutations in the TRβ-1 hinge region abrogate the basal repression by corepressor. Additionally, a v-erbA mutation in the hinge region that abrogates its oncogenic potential also failed to interact with a corepressor, silencing mediator for RAR and TR (SMRT) (83). These findings suggest that the hinge region of unliganded TRs located on helix 1 may serve as a contact surface with corepressors or have allosteric effects on their interaction. Recent work by several groups also suggest that sequences within helices 3, 5, and 6 of the LBD contribute to corepressor binding (214, 348, 390).

D. Amino-Terminal (A/B) Domain

The amino-terminal regions have variable lengths and divergent sequences among the TR isoforms. Even among different species, this region is less well conserved for a given TR isoform, because the rat and human TRs are 97 and 99% identical in their DBDs and LBDs, respectively, but only 85% identical in their amino-terminal domains (254). The role(s) of the amino-terminal domain is poorly understood. Studies of the glucocorticoid receptor have suggested that there is a major activation function domain, τ1, which has structural similarities with viral acidic activator proteins such as VP16 (209). Previous work by Tora and co-workers (524, 525) with progesterone and truncated estrogen receptors also have suggested that the amino-terminal domain may modulate cell-specific and promoter-specific transcription. Cotransfection studies generally have demonstrated only a few examples of isoform-specific transcriptional activation by TRs. Fassetti et al. (129) have shown that TRβ-1 has higher transcriptional activity than TRα-1 via a myelin basic protein TRE reporter in the context of the native promoter, but not with the viral thymidine kinase promoter (129). Jeannin et al. (224) observed similar TRβ-1-specific effects on

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Fig. 7. Comparison of AF-2 regions of nuclear hormone receptors. Conserved ⍺-ω sequences are underlined.
myelin basic protein, but not on malic enzyme, gene expression suggesting that isoform-specific gene regulation may occur in a subset of target genes (224). Similar TRβ-1-specific effects on the regulation of the TRH promoter also have been observed (181, 305). TRβ-2 may have a more potent role in the negative regulation of glycoprotein α-subunit and TSHβ than other TR isoforms (208, 275). In particular, TRβ-2 mediates strong ligand-independent activation of negatively regulated target genes such as glycoprotein hormone α-subunit and TSHβ (275). Indeed, recent studies of TR knockout mice have implicated TRβ-2 as the major TR isofom in negative regulation of TSH (3).

The role of the amino-terminal domain in transcriptional activation is still controversial. Some studies have shown that deletion of the amino-terminal domain of TRβ-1 had no effect on T3-dependent transcriptional activation by TRβ-1 (516, 592), suggesting that it does not contain a major activation function domain like the glucocorticoid receptor. On the other hand, studies of TRα-1 and TRβ-1 from several species have shown that the amino-terminal domain may be important for transcriptional activation and interactions with the general transcription factor TFIIB (25, 183, 520). Additionally, it has been shown that the chick TRβ-2 amino-terminal region may have two activation domains (472). A minimal subregion of amino acids 21–50 allows human TRβ-2 to interact with coactivators in the absence of ligand and may account for the ligand-independent activation of some positively regulated target genes (358). However, one study of the amino-terminal region suggested that rat TRβ-2 does not appear to have a strong activation domain in the amino-terminal region (520), whereas other studies have shown that TRβ-2 transactivates similar to TRβ-1 (205, 478). It is possible that differences in species, cell types, TREs, and minimal promoters may account for these observations (129, 491, 580). The amino-terminal domain also may modulate ligand-independent repression via positively regulated TREs because one study showed that TRα-1 is more potent than TRβ-1 or TRβ-2 in mediating basal repression in the absence of T3 (208). Recent studies showed that the amino terminus of TRβ-2 may interact with the silencing domain of the corepressor, SMRT, and thereby block the recruitment of other components of the corepressor complex (575). Finally, the amino-terminal domain of TR also may influence the conformation of the DBD and the repertoire of TREs to which it can bind (184, 232).

V. THYROID HORMONE RESPONSE ELEMENTS

Steroid hormone receptors bind as homodimers to conserved palindromic hormone response elements that mediate hormone regulation of target genes (160). In contrast, TRs can bind to TREs as monomers, homodimers, and heterodimers in vitro. In general, most of these TREs are located upstream from the minimal promoter, but in certain cases, also can be located in 3'-flanking sequences downstream from the coding region (40, 600). Mutational analyses of the rat growth hormone gene TRE, and sequence comparison among known TREs from other T3-responsive genes, have suggested a putative consensus hexamer half-site sequence of (G/A)GGT(C/G)A (84, 555). However, there can be considerable variation found in primary nucleotide sequences of TREs as well as the number, spacing, and orientation of their half-sites (555). In particular, TRs can bind to TREs in which half-sites are arranged as palindromes (TRE_pal), direct repeats (DRs), and inverted palindromes (IPs). The optimal spacing for these half-site arrangements are zero, four, and six nucleotides, respectively (TRE_pal0, DR4, and IP6) (Fig. 8). Almost all positively regulated target genes contain two or more half-sites; however, TRs can activate transcription via an artificial single octamer half-site, perhaps even as monomers (240).

Approximately 30 natural TREs have been described so far with DRs, followed by IPs, as the most common motifs (555). Several groups also have observed that TR homo- and heterodimers bind to TREs arranged as IPs and DRs better than palindromes (17, 268, 333, 588). In the case of DRs, it has been shown that VDRs preferentially
transactivate via reporter vectors containing DRs with a three-nucleotide gap (DR3), TRs via a four-nucleotide gap (DR4), and RARs via a gap of five nucleotides (DR5), according to a “3–4–5” rule (160, 532). Heterodimerization with RXR and specific DNA contact points in the DBD may play important roles in determining heterodimer spacing on direct repeats (153, 160, 413). However, the DNA binding and transcriptional activation via these elements do not appear to be absolutely receptor specific because TRs can bind and transactivate weakly on DR5 and DR6, and RARs can bind and transactivate on DR4 (557, 588). VDRs can bind to DR4 and DR5 (587) but cannot transactivate via these HREs. However, VDR has dominant negative activity on T3 and RA-mediated transcription via these elements. Additionally, the primary sequence of the half-site may be important in maintaining receptor-specific binding to DRs as mutation of the third nucleotide of the half-site hexamer from a G to a T enabled VDR binding and ligand-mediated transactivation via a DR4 (418). It also has been shown that flanking and spacing sequences in TREs can affect DNA-binding and transcriptional activation, either by making contacts with TR or local DNA bending (216, 240, 242, 244).

TRs can form heterodimers with RXRs, which also are members of the nuclear hormone receptor superfamily (see below). This enables TR complexes to bind to TREs in a specific orientation relative to the minimal promoter. Palindromic and inverted palindromic TRE half-site sequences are symmetric and thus do not dictate a particular heterodimer orientation on the TRE. On the other hand, TRE half-sites in DR4 have a 5′ to 3′ polarity so the direct repeat motif could specify the heterodimer orientation on the TRE. Several approaches have been used to study this issue. Mutant TRs or RXRs containing amino acid substitutions in the P box of the first zinc finger of the DNA-binding domain that allow preferential binding to a glucocorticoid hormone response element (GRE) half-site have been used to study TR/RXR heterodimer binding to hybrid response elements containing TRE and GRE half-sites (268, 391, 586). These results showed that TR binds to the downstream half-site and RXR to the upstream half-site when TR/RXR heterodimer binds to DR4. Methylation interference studies also showed that TRα-1 and TRα-1/RXR preferentially bind to the downstream half-sites of DR4 and the IP F2 (216). The apparent polarity of TR/RXR complexes on F2 may be due to degeneracy of one of the half-site sequences or possibly contributions by the flanking and spacing sequences. Cotransfection studies in which the orientation of these TREs were reversed also decreased T3-mediated transcriptional activity (216). Taken together, these findings suggest that TR/RXR heterodimers bind with a specific polarity which, in turn, can modulate transcriptional activity. This “shape” of the TR complex may be important in protein-protein interactions with coactivators and corepressors that link the liganded TR/RXR heterodimer with the transcriptional machinery. In this connection, Kurokawa et al. (267) demonstrated that RAR/RXR heterodimers bound to DR1 and DR5 with different polarities. In the former case, they remained bound to a corepressor and mediated constitutive basal repression, and in the latter case, they dissociated from corepressor in the presence of all-trans-retinoic acid and mediated transcriptional activation. Similarly, it has been shown that the TRE sequence can affect corepressor release from TR in the presence of ligand (368).

VI. THYROID HORMONE RECEPTOR COMPLEXES

Early studies of TR binding to specific DNA sequences utilized methods such as the avidin-biotin complex/DNA (ABCD) assay that did not allow direct visualization of TR complexes bound to DNA (63, 161, 585). However, successful employment of electrophoretic mobility shift assays (EMSAs) demonstrated that TRs could bind to synthetic and natural TREs as monomers, homodimers, and heterodimers in vitro (144, 288, 557, 584). TRβ-1 may have a greater tendency than TRα-1 to form homodimers on several different TREs, suggesting that these two TR isoforms may have different dimerization potentials (109, 607). Domain swap experiments have suggested that the amino-terminal region of TRα-1 may inhibit homodimer formation via an allosteric mechanism (208).

Initially, TRs were thought to mediate their effects on transcription as homodimers, similar to steroid hormone receptors. However, two groups observed that TRs surprisingly heterodimerized with proteins from pituitary and liver nuclear extracts (63, 341). These proteins were called TR auxiliary proteins (TRAPs) and enhanced TR binding to TREs (63, 341). Because these proteins were expressed in nuclear extracts from many different tissues and species (63, 287, 341, 494), TR/TRAP heterodimers potentially could be formed in all cells that contain TRs. Because these heterodimers appeared to bind better to TREs than TR homodimers, it was speculated that they played a role in T3-regulated transcription.

Several groups showed that TRs heterodimerize with RXRs, members of the nuclear hormone receptor superfamily which have high homology with RARs (250, 300, 321, 589, 594, 602). RXRs bind their cognate ligand, 9-cis-retinoic acid, with high affinity (202, 304). They can form homodimers as well as heterodimers with RARs, VDRs, and peroxisome proliferator activated receptors (PPARs) (160). Several lines of evidence suggest that RXRs are the major TRAPs and thus play a critical role in T3-mediated transcription. First, they were observed to enhance TR binding to TREs. Second, studies using anti-RXR antibo-
ies showed that the major endogenous TRAPs are RXRs or related proteins (287, 420, 494). Third, TR/TRAP and TR/RXR heterodimer complexes both remained bound to TREs in the presence of T₃ (17, 333, 422, 584, 589) (see below). Fourth, heterodimer-selective, but not homodimer-selective, mutants were able to mediate transcriptional activation of TRE-containing reporters (21, 140, 344, 592). Mutant TRs containing deletions or amino acid substitutions of amino acids at positions 290–310 or in the ninth heptad region of the LBD concomitantly decreased heterodimerization and transactivation (21, 140, 344, 360, 592). Fifth, RXR enhanced T₃ mediated transcription in yeast cells that do not contain endogenous TRAPs (65, 187). And last, RXR enhanced T₃-mediated transcription in a reconstitutable in vitro transcription system (294).

TR/RXR heterodimer formation increases the repertoire of target genes that can be regulated by T₃, as heterodimers bind to TREs with variable sequence and orientation of half-sites (160). Moreover, there are at least three members of the RXR subfamily, so it is possible that different RXR isoforms may form TR/RXR heterodimers that have different TRE-binding specificities and/or abilities to transactivate target genes. Additionally, it is possible that an endogenous ligand like 9-cis-retinoic acid can bind and activate the heterodimer partner of the TR complex. Of note, addition of both 9-cis-retinoic acid and T₃ synergistically activated transcription on two different TRE-containing reporters (227, 429). However, in other cases, TR can block 9-cis-binding to RXR and thereby abrogate retinoid stimulation of target genes (91, 147, 266). Finally, TRs can heterodimerize with other members of the nuclear receptor family including RAR, PPAR, chicken ovalbumin upstream promoter transcription factor (COP-1TF), and VDRs (45, 99, 314, 448, 519, 589). The functional significance of these heterodimers, most of which have been demonstrated in vitro by EMSA, is not known. However, they do not appear to be major endogenous TRAPs and may have restricted roles in particular target genes or tissues (287, 494). If they do have physiological roles, though, these heterodimers increase further the diversity of TR complexes and the potential of receptor cross-talk on target genes.

Although TRs can form monomers, homodimers, and heterodimers on TREs in EMSA (284, 583), the role(s) of TR monomers and homodimers on transcription is not well understood. In contrast to steroid hormone receptors in which ligand enhances receptor binding to HREs, T₃ decreases TRα-1 and TRβ-1 homodimer as well as TRα-1/TRβ-1 dimer binding to several TREs arranged as DRs and IPs (17, 333, 422, 584). Unliganded TR homodimers also interact better with corepressors than unliganded TR/RXR heterodimers in vitro (207, 311). Thus it is possible that unliganded homodimers may form a complex with corepressors that are involved in basal repression of target genes in the absence of T₃, which then dissociates from the TRE in the presence of T₃.

Cross-linking and coimmunoprecipitation studies suggest that TR monomers and TR/RXR heterodimers exist in solution, whereas TR homodimers form weakly in solution (268, 288, 581). T₃ and 9-cis-retinoic acid promote dimerization in solution, and thus may preform a transcriptionally active complex before DNA binding (95, 234). It appears that dimerization promotes DNA binding (392), and DNA binding enhances dimerization (56, 366, 581).

VII. PHOSPHORYLATION OF THYROID HORMONE RECEPTORS

Recently several groups also have observed that increasing the phosphorylation state of cells can enhance T₃-mediated transcriptional activation of target genes (229, 308, 496). The mechanisms for this enhanced transcriptional activation are not known but may involve phosphorylation of TR, RXR, or coactivators. In support of the potential role of TR phosphorylation in transcriptional activation, it recently has been demonstrated that TR can be phosphorylated in vitro and in vivo (165, 167, 308, 492). Chick TRα-1 has at least two serine phosphorylation sites in the amino-terminal A/B domain, but the functional role(s) is not known (167). Additionally, these sites do not appear to be conserved across species. The human TRβ-1 can be phosphorylated in vivo and in vitro (308), although the phosphorylation sites have not been determined. Two groups have used HeLa cytosol extract to in vitro phosphorylate Escherichia coli-expressed TRα-1 (39, 492). Sugawara et al. (492) examined the binding of phosphorylated TRα-1 to several TREs and found that phosphorylation selectively enhanced TR homodimer, but not TR/RXR heterodimer, binding to several different TREs. Bhat et al. (39) showed that phosphorylation enhanced DNA binding by both TR complexes. Interestingly, phosphorylation by protein kinase A can decrease v-erbA and chick TRα-1 monomer binding to TREs (530). These results suggest that phosphorylation may be another mechanism, in addition to T₃ binding, that can modulate TR complex binding to TREs. Additionally, T₃ itself can modulate the phosphorylation state of TR (518).

Recently, Davis et al. (113) have shown that TRβ-1 associates with mitogen-activated protein (MAP) kinase in coimmunoprecipitation studies and that ligand binding may stimulate TR phosphorylation by MAP kinase. Interestingly, previous studies have shown that MAP kinase can modulate transcriptional activity of ER and PPARγ by phosphorylation of the receptor (238, 406). Moreover, MAP kinase phosphorylation of the steroidogenic factor-1 (SF-1) and ER AF-1 regions leads to enhanced coactivator
recruitment and transcriptional activation (188, 528). Stimulation of the protein kinase A pathway also potentiated T3-mediated transcription in a cell-specific manner (301), suggesting multiple kinase pathways may modulate transcriptional activity of TR. Power et al. (405) have demonstrated that some nuclear hormone receptors can be activated by dopamine stimulation in the absence of ligand, mostly likely via receptor phosphorylation. It is possible that some ligand-independent effects by TR may be due to receptor phosphorylation by cell-specific kinases or phosphatases, or due to cell-specific expression of certain membrane-signaling receptors that can activate transcription by unliganded TR. In this connection, cell-type specific phosphorylation may stabilize TRβ-1 protein (517). Additionally, it has been shown that DNA binding by the alternative splice variant of TRα-1, c-erbAα-2, is regulated by casein kinase II phosphorylation of serines in its carboxy terminus (239). This phosphorylation is critical for determining the dominant negative activity (ability to block wild-type TR action) by c-erbAα-2. These findings suggest that phosphorylation potentially may regulate diverse and important TR functions, although the precise location of phosphorylation sites, their regulation, and their functional roles remain to be elucidated.

VIII. MOLECULAR MECHANISMS OF THYROID HORMONE RECEPTOR ACTION

A. Corepressors/Basal Repression

In contrast to steroid hormone receptors that are transcriptionally inactive in the absence of ligand, unliganded TRs bind to TREs and may modulate transcription of target genes (Fig. 9). Several laboratories showed that unliganded TRs can repress basal transcription of positively regulated TREs in cotransfection studies (26, 53, 603). It was not known initially whether these observations were physiologically relevant or peculiar to cotransfection systems. Early observations showing that T3 decreased TR homodimer binding to TREs led to the hypothesis that unliganded TR homo- and/or heterodimers might mediate basal repression (584). This notion was further supported by the demonstration that TR binding to TREs was important for mediating basal repression, as mutations in TRβ-1 DBD or the TRE primary sequence abrogated basal repression (26, 592). Unliganded TRs have been shown to interact directly with TFIIB, a key component of the basal transcription machinery (25, 183, 394, 520, 523), and potentially can interfere with the assembly of a functional preinitiation complex at the promoter. Studies of TR action in vitro transcription systems (142) suggested that direct interaction between TRs and the basal transcriptional machinery could help mediate ligand-dependent basal repression. On the other hand, several studies also suggested that soluble corepressors may be critical for mediating basal repression (407, 522).

The cloning and functional characterization of several corepressors have greatly enhanced our understanding of basal repression and shed light on these previous issues (Fig. 10). Several laboratories used the yeast two-hybrid system or biochemical purification to clone proteins that exhibited decreased interaction with TR and RAR in the presence of their cognate ligands (83, 211, 295, 442). One of them was a 270-kDa protein called nuclear
receptor corepressor (NCoR), which also was isolated as an RXR-interacting protein, RIP 13 (211, 459). It contains three transferable repression domains and two carboxy-terminal α-helical interaction domains. NCoR was able to mediate basal repression by TR and RAR, as well as orphan members of the nuclear hormone receptor family such as rev-erbA and COUP-TF. It had little or no interaction with steroid hormone receptors and did not mediate basal repression by these receptors. NCoR also has been shown to interact with TFIIB, TAFII32, and TAFII70, so part of its ability to repress transcription may be due to its ability to interact with the basal transcriptional machinery. Recently, a truncated version of NCoR, NCoRi, which is missing the repressor region, has been identified, which may represent an alternative-splice variant of NCoR (207). This protein blocks basal repression by NCoR and potentially may serve as a natural antagonist for NCoR if it is expressed in significant amounts in tissues. Another corepressor, SMRT, has been identified and has homology with NCoR (83, 295, 442). The original sequence for SMRT was derived from a partial clone, but it is now appreciated that full-length SMRT is similar in size and has similar repression and nuclear receptor interaction domains as NCoR. SMRT also is able to mediate basal repression of TR and RAR in cotransfection studies. Another protein, small ubiquitious nuclear corepressor (SUN-CoR), was isolated and enhanced basal repression (596). This 16-kDa protein may form part of a corepressor complex as it interacts with NCoR.

Studies of TR and v-erbA have defined the importance of the hinge region for interactions with NCoR and SMRT, because mutations in this region abrogate basal repression without affecting transcriptional activation (83, 211, 324). Interestingly, rev-erbA contains two amino-terminal regions which interact with NCoR and are required for basal repression, suggesting that nuclear hormone receptors may have different interaction sites with corepressors (597). Within the interaction domains of NCoR and SMRT are consensus LXXI/HIXXXI/L sequences that resemble the LXXLL sequences that enable coactivators to interact with nuclear hormone receptors (214, 348, 390). Interestingly, these motifs allow both corepressors and coactivators to interact with similar amino acid residues on helices 3, 5, and 6, which are part of the ligand pocket of TR. Differences in the length and specific sequences of the corepressor and coactivator interaction sites coupled with the conformational changes in the AF-2 region upon ligand binding may determine whether corepressor or coactivator binds to TR (390). Additionally, corepressors can bind to the TR heterodimer partner RXR. It appears that helix 12 of RXR masks a corepressor binding site in RXR, which is unmasked upon heterodimerization with TR (599).

Recently, several groups have shown that corepressors can complex with other repressors, Sin3 and histone deacetylase 1 (HDAC1), that are mammalian homologs of well-characterized yeast transcriptional repressors RPD1 and RPD3 (13, 200, 271, 347, 352). Thus local histone deacetylation may play a critical role in basal repression by nuclear hormone receptors. Moreover, this mechanism of basal repression may be employed by other transcription factors such as Mad/Max and Myc/Mxi heterodimers (13, 200, 271). Anti-NCoR antibodies have been shown to coimmunoprecipitate HDAC activity (200). Additionally, microinjection of specific antibodies generated against mSin3 and RPD3 were able to block basal repression by NCoR (200). Recent studies by Lazar (286) and Wong (J. M. Wong, personal communication) suggest that HDAC4 may interact directly with NCoR at a different repressor site than Sin3 and HDAC1. Recent coimmuno-precipitation studies also suggest that HDAC3 may be the major HDAC associated with SMRT (286). Another component of the corepressor may be the protooncogene c-Ski which has been shown to be involved in transcriptional repression by Mad and TR (354). It is likely that histone deacetylation by unliganded TR/corepressor complex may help maintain local chromatin structure in a state that shuts down basal transcription. In this connection, studies examining TRB3 promoter in a Xenopus oocyte system showed that simultaneous chromatin assembly and TR/RXR binding were required for basal repression of transcription (562, 563). This repression was relieved by addition of T₃ and was accompanied by chromatin remodeling. These data demonstrate that histone acetylation and deacetylation, and the consequent changes in chromatin structure and nucleosome positioning, may be important determinants of gene transcription (Fig. 11). Additionally, DNA methylation may play a role.
in basal repression as methyl-CpG-binding proteins can associate with a corepressor complex containing Sin3 and HDACs (351, 543). 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.

TR also can activate transcription in the absence of ligand. Samuels and co-workers (201) showed that unliganded TR surprisingly could transactivate via rGH and PRL TREs in pituitary cell lines (89). Additionally, several groups have reported ligand-independent gene transcription in neuroblastoma and Xenopus cells (346, 385). It is not known whether cell-specific activators or inhibitors or other mechanisms account for these observations. Last, TRs can activate transcription of negatively regulated genes such as RSV LTR, glycoprotein hormone α-subunit, TSHβ, and TRH in the absence of ligand (88, 206, 305, 342, 433). Brent et al. (57) showed that a negative TRE (nTRE) from the glycoprotein hormone α-subunit gene could be placed in different positions relative to the rat growth hormone minimal promoter (even downstream), and still mediate negative regulation. They showed that the nTRE sequence may play a more important role than its position relative to the minimal promoter. Additionally, several groups have found nTREs located in the 3'-untranslated region of target genes (40, 600). Recent studies have suggested that ligand-independent activation may be mediated by TR recruitment of corepressors, and this may be mediated by protein-protein interactions of DNA (500, 501). Interestingly, these effects could be blocked by pharmacological inhibition of HDAC activity. These findings suggest that histone deacetylation may promote ligand-independent activation of a negatively regulated target gene. Another study showed that T3-mediated negative regulation may also require HDAC activity (445). On the other hand, NCoRi (which does not contain the repression domains) enhances ligand-independent regulation in a reporter containing the TRH promoter (207). NCoRi activation function is stronger than NCoR, so the ligand-independent activation function may map to the carboxy terminus of
NCoR (93). These studies suggest a role for corepressors in ligand-independent activation of some negatively regulated target genes, but the precise mechanism needs to be further defined.

The physiological role of corepressors is only partially understood. Corepressors have been implicated in leukemias that have RAR fusions and acute myelogenous leukemia/eight-twenty-one (ETO) fusions from chromosomal translocations (158, 176). Additionally, the TRα/ TRβ double-knockout mice have a milder phenotype than congenitally hypothyroid mice, suggesting that basal repression by unliganded receptor may have more deleterious effects on transcription in target tissues. Lazar (286) recently found that transducin B-like protein comimunoprecipitated with SMRT. This histone-binding protein was deleted in patients with congenital sensory-neural deafness (33). Interestingly, TRβ knockout mice and affected patients from a family with resistance to TH due to absence of TRβ also have sensory neural deafness (149, 414). It is interesting to speculate that absence of TRβ or an associated repressor complex protein can cause a similar phenotype. Last, Feng et al. (135) have targeted a dominant negative NCoRi to the liver in transgenic mice. They observed that basal transcription of spot 14 and BcE3 (a target gene identified by cDNA microarray) was higher in hypothyroid transgenic mice than in littermate controls. When mice were treated with T₃, both genes were induced to similar levels. These findings suggest that NCoR-mediated basal repression in vivo could be reversed by overexpression of NCoRi without effects on transcriptional activation. Interestingly, transcription of several other target genes was unchanged in hypothyroid transgenic mice, suggesting that there may be gene-specific repression by different corepressors.

B. Coactivators/Transcriptional Activation

Recent studies using Far-Western and immunoprecipitation approaches showed that liganded TR may interact with multiple nuclear proteins that potentially can form a transcriptionally active complex (141, 394). Work by several groups previously showed that TRs can interact with general transcription factors (25, 142, 183, 520). Moreover, these interactions seemed to be ligand dependent, suggesting that direct contact between TR and general transcription factors could play a role in derepression and transcriptional activation (27, 142). In support of this possibility, ER was shown to interact with TBP, TAFII 30, and TFIIB (217, 221, 434), and PR could interact with TAFII 110. Petty et al. (396) also showed that liganded TRβ interacts with several Drosophila TAFs, particularly TAFII 60 and TAFII 110, and the latter could augment T₃-dependent transcription (396).

Previous studies using cotransfection and in vitro transcription systems highlighted the importance of minimal promoters in mediating T₃-regulated transcription (129, 491, 580). These studies raised the possibility that adapter proteins, or coactivators, might bridge the liganded TR complex with components of the basal transcriptional machinery. Previously, several viral transcription factors were shown to interact with coactivators to enhance transcription (62, 170, 310). Similarly, several potential coactivators for hormone-regulated transcription were reported, including CREB binding protein (CBP) which interacts with CREB (269). Also, several groups identified proteins that interacted with ER in a ligand-dependent manner and thus could potentially participate in estrogen-dependent transcriptional activation (72, 186, 221). These putative coactivators interacted with the AF-2 region located in helix 12 of the LBD. This region has high homology among many members of the nuclear hormone receptor family and was shown to be important for ligand-dependent transcription for several receptors (27, 31, 105, 278, 601) (Fig. 6). This region contains a critical ϕϕXXϕϕ sequence in which ϕ represents a hydrophobic amino acid and X represents any amino acid.

Recently, O’Malley and co-workers (369) used a yeast two-hybrid system to clone a putative factor that enhanced transcriptional activation for steroid hormone receptors, which they called steroid receptor coactivator-1 (SRC-1) (Fig. 12). This protein also associates with several other members of the nuclear hormone receptor superfamily, including TRs, and enhances their ligand-dependent transcription. Subsequent work has shown that the original cDNA clone was only partial, and the full-length clone encoded a 160-kDa protein (235, 369, 507). Additionally, there may be alternative splicing of SRC-1 mRNA, leading to multiple SRC-1 isoforms. The functional significance of the SRC-1 isoforms currently is not known; however, one splice variant, SRC-1E, which lacks 56 amino acids of SRC-1 and had unique 14 amino acids at the carboxy terminus, enhanced T₃-mediated transcriptional activation better than SRC-1 (194, 235). Another 160-kDa protein, TIFII/GRIP-1/SRC-2, has been identified that interacts with liganded nuclear hormone receptors, including TRs, and has partial sequence homology with SRC-1. These findings suggest there may be a family of coactivators related to SRC-1 (210, 541). Indeed, another family member, AIB1/pCIP/ACTR/TRAM1/RAC3/SRC-3, has been identified that augments transcription by nuclear hormone receptors (19, 82, 306, 503, 526). As seen in Figure 12, there are several common features among these putative coactivators. First, there are multiple putative nuclear hormone receptor interaction sites that seem to bear a signature LXXLL sequence motif in which X represents any amino acid. This sequence has been shown to be important for coactivator binding to nuclear hormone receptors (199, 325). These coactivators also have a polyglutamine region, similar to androgen recep-
tors. There is polymorphism observed in the length of these trinucleotide repeats of SRC-3, but the functional significance, if any, is not known (198). Additionally, in the amino-terminal region, there is a basic helix-loop-helix (bHLH) motif, suggesting that these coactivators may bind to DNA. Also located in this region is the so-called Per-Arnt-Sim (PAS) domain, which interestingly, also is seen in several transcription factors that regulate the circadian rhythm and in the heterodimer partner of the dioxin receptor (387, 402). Thus the PAS region may serve as a dimerization interface and potentially allow cross-talk among other coactivators or transcription factors. SRC-1 can be phosphorylated by MAP kinase, and its activity may be regulated by membrane-bound receptors such as epidermal growth factor (431). There also are multiple nuclear receptor interaction domains in the SRCs that may confer receptor specificity (122, 316, 505, 540). Among the SRC family members, there may be a certain degree of functional redundancy, as SRC-1 knockout mice were viable and fertile and had only a modest decrease in growth and development in reproductive tissues and a mild disturbance of the hypothalamic/pituitary/thyroid (HPT) axis (550, 570). Interestingly, there was a concomitant increase in TIF2/SRC-2 mRNA in these mice, suggesting that upregulation of TIF2 may compensate for some of the loss of SRC-1 function. Interestingly, the SRC-3 knockout mouse has a much more severe phenotype with decreased growth, delayed puberty, and decreased litter size (569). The effects on the HPT axis and potential contribution to the phenotype are not known at the present time.

Recently, several groups have shown that SRC-1 can interact with the CBP, the putative coactivator for cAMP-stimulated transcription as well as the related protein, p300, which interacts with the viral coactivator E1A (73, 235, 476, 577). CBP/p300 can serve as a coactivator for CREB, p53, AP-1, and NFkB. It is possible that CBP/p300 may serve as an integrator molecule for different signaling inputs (262) (Fig. 10). The biological importance of CBP in humans is exemplified by patients with Rubenstein-Taybi syndrome who have mental retardation, short stature, and craniofacial and other anomalies, as well as mutations in CBP (393). p300 knockout mice die in utero and display defects in cell proliferation as well as neural and heart development (578). CBP knockout mice also die in utero with defects in hematopoiesis and angiogenesis (363). These findings suggest that although CBP and p300 behave similarly in enhancing nuclear hormone receptor action in cotransfection studies, they are not functionally equivalent in vivo.

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 (42, 574). Like GCN5, PCAF has intrinsic histone acetyltransferase (HAT) activity. The HAT activity of PCAF is directed primarily toward H3 and H4 histones. PCAF itself is part of a preformed complex that contains TBP associated factors (TAFs) which have been shown to interact with SRC-1 and SRC-3 (82, 361, 479). CBP also has been shown to be part of a stable complex with RNA polymerase (pol) II (350). Thus PCAF and CBP can serve as adaptors of nuclear receptors to the basal transcriptional machinery and have an enzymatic function (HAT activity). These dual roles likely contribute to nuclear receptor transcriptional activity (Fig. 12). Subsequent studies showed that CBP/p300, as well as SRC-1 and SRC-3, have HAT activity, although their specificity for histone substrates differed (82, 262, 362, 479). Recruitment of different coactivators with different HAT activities may be a mechanism for promoter- or cell-specific regulation of target genes.

Recently, Lanz et al. (277) reported that an RNA transcript, steroid receptor RNA activator (SRA), could enhance AP-1-mediated transcription of the progesterone
receptor and was part of the SRC-1 complex (277). It is possible that SRA may be part of a ribonucleoprotein scaffold that many help recruit SRC-1 to nuclear hormone receptors. Additionally, Stallcup recently showed that a methyltransferase, CARM-1, can be associated with GRIP-1 and methylate the histone H3 (81). These findings suggest that histone methylation as well as acetylation may be employed to alter local chromatin structure.

In vivo foot-printing studies also have suggested conformational changes in the chromatin structure near TREs and retinoic acid response elements after ligand addition (118, 143, 243, 562). Additionally, liganded receptor binding to the hormone response element allowed other enhancer elements in the promoter to be foot-printed (118). In studies on TRβA promoter in Xenopus oocytes, Wolffe et al. (561, 562) have shown that chromatin assembly is critical for basal repression by unliganded TR/RXR heterodimers. Moreover, trichostatin A, a HDAC inhibitor, relieves this basal repression but does not disrupt chromatin structure. In contrast, liganded TR/RXR disrupts chromatin structure, releases the corepressor complex, and recruits coactivators such as CBP and PCAF with HAT activity. Recent studies with green fluorescent proteins have shown that TRs are predominantly in the nucleus and that T3 can mediate intranuclearily reorganization in the presence of ligand (34). Moreover, this change does not require DNA binding. This reorganization may be due to association with chromatin, active transcriptional complexes, and/or nuclear matrix components (34, 213, 482).

Recently, the groups of Freedman (411, 412) and Roeder (595) have isolated a complex of proteins (DRIPs, vitamin D receptor interacting proteins; TRAPs, TR-associated proteins) ranging from 70 to 230 kDa that interacted with liganded VDRs and TRs in GST-pulldown studies. These proteins had similar sizes as a previously reported complex of proteins originally isolated by coinmunoprecipitation with an antibody against epitope-tagged TRα. A critical coactivator in this complex is DRIP205/TRAP220, which contains a LXXLL motif similar to SRC-1 homology with SRC-1 but interacts with liganded TR and contains an LXXLL motif (334). Another protein called TRIP230 (TR and Rb interacting protein of 230 kDa) was identified a putative coactivator, p120, that does not bear homology to SRC-1-LBD. A TRα coactivator is a critical coactivator in this complex is DRIP205/ TRAP220, which contains a LXXLL motif similar to SRC family members and appears to anchor ~15 other proteins. Interestingly, none of these proteins is a member of the SRC family and their associated proteins. Instead, several DRIP/TRAP components are mammalian homologs of the yeast Mediator complex, which associates with RNA Pol II (179, 219). DRIP/TRAP complex is virtually identical to a previously described SRB-MED-containing cofactor complex (SMCC) that can interact with p53 and VP16 to enhance transcription (219). These findings suggest that TR may recruit DRIP/TRAP complex which, in turn, may recruit or stabilize RNA Pol II holoenzyme by virtue of shared subunits. Additionally, DRIP/TRAP complex does not appear to have intrinsic HAT activity (411). Taken together, these findings suggest a model in which there may be at least two distinct coactivator complexes for nuclear hormone receptors; however, the precise functional roles of the two different coactivator complexes are not known. It is possible that the p160/SRC complex may initiate transcriptional activity by recruiting cofactors with HAT activity to ligand-bound nuclear hormone receptors, and DRIP/TRAP complex may then bind to nuclear receptors that can then recruit RNA Pol II holoenzyme to promote transcription of target genes (Fig. 11). Recently, it has been shown that CBP can acetylate ACTR (SRC-3) and promote its dissociation from nuclear hormone receptors, so acetylation of components of the p160/SRC complex from HAT activity may facilitate the exchange of complexes (82). Balasubramanian and Moore (22) recently showed that mammalian homologs of Swi/Snf, BRG-1 and BRM-1, can associate with TR in vitro and activate transcription. These chromatin remodeling proteins have ATPase activity and previously were shown to enhance GR-mediated transcriptional activity in yeast (67). Recently, the sequential recruitment of coactivator complexes and correlation with transcription has been demonstrated for ER and TR (461a, 461b). Understanding the interplay of these coactivator complexes and the mechanisms by which they activate transcription will certainly be the subjects of intense study in the future.

In addition to the p160/SRC and DRIP/TRAP complexes, a number of coactivators have been identified, but their relationship to these complexes, if any, is not known. Moore and co-workers (295, 296) have identified several other proteins that interact with TRβ-1 LBD. These proteins, called TRIPs (TR-interacting proteins), are diverse; one of them is the human homolog of a yeast transcription factor, another a new member of a class of nonhistone chromosomal proteins, and yet another contains a conserved domain associated with ubiquitination of specific target proteins. Monden et al. (334) have identified a putative coactivator, p120, that does not bear homology with SRC-1 but interacts with liganded TR and contains an LXXLL motif (334). Another protein called TRIP230 (TR and Rb interacting protein of 230 kDa) was initially cloned on the basis of its ability to interact with the retinoblastoma protein (74). This protein binds to liganded TR and augments T3-mediated transcriptional activation. Interestingly, cotransfection of Rb blocks the TRIP230 enhancement of transcription, suggesting crosstalk between TR- and Rb-mediated pathways. Another coactivator, TR-binding protein (TRBP, ASC-2, RAP250), interacts with TR via a LXXLL motif and stimulates transcription of several nuclear hormone receptors, AP-1, CREB, and NFκB (66, 252, 298). It also interacts with DRIP 130 and CBP/p300. Yet another protein, TR uncoupling protein (TRUP), binds to TR/RXR heterodimers and prevents their binding to TREs (64). In contrast to coactivators, it blocks T3-mediated transcription, presumably by the foregoing mechanism. Additionally, there are a number of other nuclear proteins that have been identi-
fied by two-hybrid screening that interact with steroid hormone receptors and also may interact with TRs (212, 326). These findings suggest that there may be different classes of coactivators in addition to the SRC and DRIP/TRAP complexes. However, the functional significance of these coactivators on TR-mediated transcription remains to be further defined.

C. Cross-talk With Other Nuclear Hormone Receptors

TRs and other nuclear hormone receptors can modulate transcriptional activities of each other. This cross-talk can occur via several mechanisms: promiscuous binding to HREs, formation of heterodimers, and competition for cofactors (450, 582). The first two mechanisms seem to be the most prevalent among nuclear hormone receptors and can result in either enhanced or decreased transcriptional activity. As mentioned earlier, TR/RXR heterodimers may mediate dual-ligand regulation of transcription in some instances and block ligand-dependent transcription via RXR in others (162, 297, 314, 429, 493). Similar mechanisms also may occur for TR blockade of RAR-mediated transcription (29, 297, 589).

Carlberg and co-workers (448) demonstrated that VDR and TR formed heterodimers on the mouse and rat calbindin HREs which are arranged as DR3 and DR4, respectively, and both vitamin D and T3 could coregulate transcription via these elements. VDR/RXR, but not VDR/TR, bound to several TREs, and VDR exhibited dominant negative activity as it blocked T3-mediated transcriptional activity on DR4 and the chick lysozyme TRE, F2 (587). Aranda and co-workers (156) also have found that VDR blocked TR-mediated transcription in the rGH TRE. Interestingly, cotransfection of SRC-1 or SRC-3 could augment VDR-mediated transcription on TRE- and RARE-containing reporters (DR4 and DR5) (504).

TRs and ERs also exhibit cross-talk. TRs and ERs have identical “P box” sequences and recognize the same consensus half-site sequence, AGGTCA (160). However, ERs bind as homodimers to EREs arranged as palindromes separated by three nucleotides. Although TRs can bind to estrogen response elements (ERE), they are unable to transactivate via these elements (420, 456). Nonetheless, TRs can block estrogen stimulation of vitellogenin gene expression possibly by forming transcriptionally inactive complexes (e.g., TR/RXR heterodimers) on the ERE (161, 456, 609). Zhu and co-workers (397, 608) have shown that TR can bind to the EREs of pro-enkephalin and progesterone receptor promoters in rat hypothalamic cells and decrease estrogen-mediated proenkephalin mRNA synthesis. Because these two estrogen-regulated target genes are important in the hypothalamic regulation of sexual behavior in rats, coexpression of TR with ER may be an important modulator of estrogen response in this neuroendocrine tissue.

ERs also can block T3-regulated transcription by TR (579, 591). The mechanism for transcriptional blockade may depend on the TREs of particular target genes. In one example, ERα blocked the T3-mediated negative regulation of the glycoprotein hormone α-subunit gene (579). ER bound to a hormone response element containing an imperfect palindrome sequence. TR monomer also bound to this composite response element and negatively regulated transcription. Thus ER competition with TR for DNA binding to the response element may block T3-mediated negative regulation of this target gene. Additionally, ER blocked T3-mediated transcriptional activation via DR4 and F2 (591). In contrast to the glycoprotein hormone α-subunit gene, this blockade did not require DNA binding since the transcriptional blockade was observed with ER mutants that did not bind to TREs or EREs. Similar results also were observed using GR and GR truncation mutants. Interestingly, neither ER or GR had any effect on repression of basal transcription by unliganded TR, suggesting that this blockade by the steroid hormone receptors had a selective effect on these two TR functions. It is likely that overexpression of ER and GR titrated limiting amounts of a cofactor(s) critical for transcriptional activation by liganded TR, but not basal repression, by unliganded GR. Similar results were observed for PR- and TR-mediated transcription in an in vitro transcription system (601). There also are several other examples of cross-talk between other nuclear hormone receptors and TRs. PPARs can decrease T3-mediated transcription, possibly via competition for binding to HREs and/or titration of RXRs or coactivators (45, 233, 332). COUP-TF also can interfere with TR-mediated as well asRAR- and VDR-mediated transcriptional activity (99).

Nuclear hormone receptors have been shown to have cross-talk with c-jun and c-fos protooncogene (AP-1) proteins (450). On the proliferin promoter, positive or negative glucocorticoid regulation was regulated by c-jun or c-fos and binding to a composite element containing overlapping an AP-1 binding site and glucocorticoid response element (119). In other cases, c-jun and GR have reciprocal antagonism mediated via protein-protein interactions (451, 576). Similarly, several groups have shown a ligand-dependent repression of AP-1 activity by TRs (312, 388, 604). Interestingly, Lopez et al. (312) also have shown that unliganded TR can positively regulate AP-1 activity on some elements (312). Additionally, c-jun can antagonize T3-mediated transcription via positively and negatively regulated TREs (312, 388, 560, 604). The mechanism for these reciprocal effects seems to be protein-protein interactions between TR and c-jun because several groups have observed formation of TR/c-jun complexes in solution (312, 560, 604). Furthermore, TR and AP-1 com-
plexes can both interact with CBP, providing another potential point for cross-talk. Recently, Yamamoto and co-workers (426) examined TR- and GR-mediated repression of collagenase gene transcription by jun. Interestingly, two jun mutants disrupted TR-mediated repression but not GR-mediated repression. Also, the repression by TR, but not GR, could be blocked by the histone deacetylase inhibitor trichostatin A. These findings suggest that TR- and GR-mediated repression on this gene occur via distinct mechanisms.

D. Nongenomic Effects of TH

There is general agreement that most of the effects of T₃ are mediated by TR regulation of target gene transcription in the nucleus. However, there are a number of reports on nongenomic effects by T₃ and T₄ (116). Evidence for these nongenomic effects include the lack of dependence on nuclear TRs and structure-function relationships of TH analogs that are different from their affinities for TRs. There also can be rapid onset of action (typically seconds to minutes), and utilization of membrane-signaling pathways, typically involving kinases or calmodulin, that have not been implicated in direct TR function. The putative nongenomic effects by TH are diverse. However, the biological significance is not well understood in many cases.

Transport of T₃ across plasma and nuclear membranes have been subjects of interest over the years. T₃ is lipophilic and generally thought to diffuse passively across the plasma and nuclear membranes. However, there is some evidence for facilitated transport across plasma membranes and high-affinity TH binding sites in the plasma membranes of different cells (274, 335, 378, 421, 439). In one study of human erythrocytes, T₃ is concentrated 55-fold inside cells. There also is evidence for a stereo-specific transporter of T₃ into the nucleus as there was a 58-fold higher concentration of l-T₃ and 4-fold higher concentration of d-T₃ in the nucleus than in the cytoplasm using isotope dilution methods, although different affinities for TR may also contribute to this difference (378). However, the identification of the specific proteins that might be involved in T₃ transport has remained elusive. One potential transporter may be the multidrug resistance P-glycoprotein that can modulate TH concentration when overexpressed in cells (421). Another family of transporters may be the organic anion transporter proteins that have been shown to import TH into hepatocytes (1, 154).

In addition to transporters, some other potential targets of T₃ in the plasma membrane include Ca²⁺-ATPase, adenylate cyclase, and glucose transporters (112, 115, 274, 335, 378, 439, 454, 455). In the last case, it has been long appreciated that T₃ can enhance uptake of sugars in a variety of tissues via a mechanism that does not require new protein synthesis, suggesting a direct effect on the plasma membrane transport system (453–455). Additionally, T₃ has been shown to bind to an endoplasmic reticulum-associated protein, prolyl hydroxylase, and also to a subunit of pyruvate kinase when the enzyme is monomeric but not tetrameric (20, 85, 237). It is not known whether T₃ modulates the activities of these enzymes or whether these enzymes may subserve other functions related to T₃ action such as transport or storage. In this connection, it is of interest that thyroxine, T₄, can inhibit deiodinase type II activity by an allosteric mechanism and may promote targeting of a substrate-binding subunit to endosomes (132). Recently, Stensapir et al. (481) also have shown that deiodinase type II can be proteosomally degraded in the presence of T₄ and/or T₃.

Sterling and colleagues (483–485) reported the presence of specific mitochondrial receptors over 20 years ago and thus provided an attractive unifying model for T₃ effects on mitochondrial activity and cellular energy state. There is some evidence that the site of TH action in mitochondria is the adenine nucleotide translocase of the inner mitochondrial membrane (428, 433). However, this work has been difficult to confirm as there are conflicting reports in the literature on the site of TH action in mitochondria (116, 185, 276). Recently, a 43-kDa protein related to TRα-1 LBD has been described in mitochondria that also could bind to TREs and mitochondrial DNA sequences (70, 567). Moreover, transfection of TRα-1 in CV-1 cells resulted in mitochondrial localization and stimulation of mitochondrial activity. These results suggest that there indeed may be specific mitochondrial receptors for T₃ which also may serve as transcription factors in mitochondria.

There also are reports of nongenomic effects on cell structure proteins by THs. Actin depolymerization blocks type II deiodinase activation by T₄ in cAMP-stimulated glial cells, suggesting that an intact actin cytoskeleton is important for this downregulation of deiodinase activity (131, 132). Interestingly, T₄, but not T₃, can promote actin polymerization in astrocytes (468) and thus may influence the downregulation of type II deiodinase activity by a secondary mechanism, perhaps by targeting to lysosomes (131, 132). Moreover, the regulation of actin polymerization also could contribute to the effects of TH on arborization, axonal transport, and cell-cell contacts during brain development. In this connection, Farwell et al. (133) have shown that T₄ was required for integrin clustering and attachment to laminin by integrin in astrocytes.

Finally, Davis and co-workers (307) have observed that the antiviral effects of interferon-γ can be potentiated by T₄ and T₃. These effects were rapid and did not require protein synthesis because they were not blocked by cycloheximide treatment and required protein kinase C and protein kinase A activation. These data suggest that cir-
culturating levels of T₄ may play an important role in modulating cytokine effects in early host defense.

IX. THYROID HORMONE EFFECTS ON TARGET ISSUES

TRs are expressed in virtually all tissues, although the relative expression of TR isoforms may vary among tissues (128, 204, 487). As mentioned previously, TRβ-1 mRNA is highly expressed in liver but also expressed in almost all other tissues, whereas TRβ-2 mRNA is most highly expressed in the anterior pituitary. TRα-1 is expressed in almost all tissues. In addition to this variable expression of TR isoforms in different tissues, the role of TH can vary in different tissues. Indeed, the myriad effects by a single hormone on so many different tissues is surprising and underscores TH’s vital role in cellular function. Thus, in addition to its role on the metabolism of macronutrients and overall energy and oxygen consumption, TH also regulates important functions in specific tissues. Recently, the transcriptional regulation of target genes in some of these tissues has been studied. We highlight some of the effects of TH in its major target tissues, and if known, describe their effects on the transcription of target genes in those tissues.

A. Bone

TH is critical for normal bone growth and development. In children, hypothyroidism can cause short stature and delayed closure of the epiphyses. Biochemical studies have shown that TH can affect the expression of various bone markers in serum, reflecting changes in both bone formation and resorption (10, 337, 430). TH increases alkaline phosphatase and osteocalcin in osteoblasts. Additionally, osteoclast markers such as urinary hydroxyproline, urinary pyridinium, and deoxypyridinium cross-links are increased in hyperthyroid patients. These observations suggest that both osteoblast and osteoclast activities are stimulated by TH. Indeed, there is enhanced calcification and bone formation coupled to increased bone resorption in hyperthyroid patients (328, 337). Additionally, the time interval between formation and subsequent mineralization of osteoid is shortened. The net effect on these bone cells is bone resorption and loss of trabecular bone thickness in hyperthyroidism. There is also marked increase in porosity and decreased cortical thickness in cortical bone in hyperthyroid patients (30, 175, 328, 430). These effects can lead to osteoporosis and increased fractures.

TH may act on bone via TH stimulation of growth hormone and insulin-like growth factor I (IGF-I) or by direct effects on target genes. Mundy et al. (339) demonstrated that T₃ had a direct effect on bone resorption in organ culture, suggesting the latter mechanism could occur. Recent studies have shown that T₃ also can directly stimulate IGF-I production in osteoblasts and enhance T₃ stimulation of [³H]proline incorporation, alkaline phosphatase, and osteocalcin (215). TRs recently have been demonstrated in osteoblast cell lines, osteoclasts derived from an ostoclastoma, as well as in rat and human bone samples (5, 6, 12, 329, 554). Both TRα-1 and TRβ-1 as well as c-erbAα-2 are expressed in most osteoblast cell lines, although there is a predominance of TRα-1 in some cell lines (e.g., ROS25/1 and UMR 106) (554). Williams et al. (554) have speculated that osteoblast phenotype expression may correlate with specific TR isoform expression as the more undifferentiated cell lines expressed predomnantly TRα-1. TRβ-1 and c-erbAα-2 are highly expressed in chondrocytes (5). Moreover, TH inhibits growth and stimulates differentiation of chondrocytes in culture (218). Recent studies have shown that TRβ2 mRNA was unexpectedly expressed in human chondrocytes and osteoclasts in situ (6).

Little is known about direct TH effects on osteoclasts because of the difficulty in obtaining primary cultures or cell lines. Recently, TR protein was detected in a human ostoclastoma and in human bone samples by immunostaining (5, 12), suggesting that TH might have direct effects on osteoclasts. However, two groups have used a bone slice resorption assay to show that functionally isolated osteoclasts were unable to respond directly to T₃ by increasing bone resorption, and could only do so if other bone cells were present (9, 58). These results would suggest that TH may not have a direct effect on bone resorption but may mediate its effects via paracrine factors secreted by osteoclast cells. Indeed, TH stimulated prostaglandins and IGF-I in organ culture studies (247). Additionally, there have been reports of interferon-γ and cyclosporin A inhibiting bone resorption by TH, suggesting cytokines may also serve as mediators of TH effects on osteoclasts (247, 273). Additionally, a clinical study suggests that interleukin-6 may be involved in mediating bone loss in hyperthyroidism (272).

Although TH increases the activities of osteoblasts and osteoclasts in vivo and in culture, little is known about its effects on the transcription of target genes in these cells. There are a number of osteoblast proteins that are stimulated by TH. These include proteins involved in matrix formation such as alkaline phosphatase, osteocalcin, and collagen (10, 247). Additionally, IGF-I and IGF-binding protein-2 mRNA are stimulated by T₃ in rat primary cultures and osteoblastic cell lines (247). However, it is not known whether TH directly regulates transcription of these target genes. Varga and co-workers (163) have used subtractive hybridization of TH-treated mouse osteoblastic cells to isolate a cDNA that corresponded to the insulin growth factor binding protein-4 (IGFBP-4) (163). IGFBP-4 mRNA was stimulated by T₃ and retinoic
acids in mouse osteoblastic cells after 48 h of treatment and remained elevated after 14 days of treatment. The T\(_3\) stimulation of IGF and IGFBPs suggests that TH may participate in osteoblast differentiation and proliferation by regulating growth factor synthesis and action. As mentioned earlier, little is known about the direct role of T\(_3\) on osteoclasts. Thus far, no T\(_3\)-regulated target genes have been described in osteoclasts.

**B. Heart**

TH lowers systemic vascular resistance, increases blood volume, and has inotropic and chronotropic effects on cardiac function (248). The combination of these effects on both the circulation and the heart itself results in increased cardiac output. Hyperthyroid patients have a high output circulation state, whereas hypothyroid patients have low cardiac output, decreased stroke volume, decreased vascular volume, and increased systemic vascular resistance (248). These changes in cardiac function by TH ultimately depend on the regulation of target genes within the heart and indirect effects due to hemodynamic changes by TH.

TH enhances overall total protein synthesis in the heart (120, 121). Additionally, it regulates the transcription of several specific proteins that are critical for cardiac function such as myosin heavy chain (MHC) genes (120). The myosin holoenzyme has a molecular mass of 500,000 kDa and is composed of two MHCs and four light chains. There are two heavy myosin genes (\(\alpha\) and \(\beta\)) whose products dimerize to form three different myosin chain isoenzymes: myosin V1 (\(\alpha/\alpha\)), myosin V2 (\(\alpha/\beta\)), and myosin V3 (\(\beta/\beta\)). The relative expression of the MHC genes in ventricles is species dependent, since \(\alpha\)-MHC gene is highly expressed in rodents and rabbits, whereas \(\beta\)-MHC gene is predominantly expressed in humans. Myosin V1 has higher ATPase activity and increased velocity of fiber shortening than myosin V3, so the relative expression of isoenzymes in the heart can determine cardiac contractility. In hypothyroid rats, myosin V3 predominates so the less active myosin subtype participates in the contractile process resulting in decreased velocity of fiber shortening. In contrast, T\(_3\) treatment stimulates \(\alpha\)-MHC gene expression and decreases \(\beta\)-MHC gene expression, leading to increased myosin V1, and enhanced cardiac contractility. These opposing effects on MHC gene expression have been demonstrated in whole animals as well as neonatal rat myocytes in culture (23, 120, 336).

TH also regulates myosin isoenzyme expression during development (336). In the rat fetus, \(\alpha\)-MHC mRNA expression is high in the atria, whereas \(\beta\)-MHC mRNA expression is high in the ventricles. \(\alpha\)-MHC mRNA then increases shortly after birth in the ventricles and almost completely replaces \(\beta\)-MHC mRNA 7 days after birth. There is no change in \(\alpha\)-MHC mRNA level in the atria. This developmental switch in the rat ventricles likely is triggered by the surge in circulating TH levels that occurs just after birth. It should be noted that in humans and higher mammals, there is a different developmental pattern as myosin V3 (\(\beta/\beta\)) is predominantly expressed in the ventricles from fetal development to adulthood, whereas V1 (\(\alpha/\alpha\)) is expressed in the atria. Additionally, the effects of T\(_3\) on induction of \(\alpha\)-MHC mRNA and protein have been smaller in higher mammals, suggesting that other factors in addition to a switch in MHC isofoms may be playing a role in the inotropic action of TH in these species compared with rodents and rabbits. However, in one clinical study, a man with profound hypothyroidism and severe cardiomyopathy underwent serial ventricular biopsies before and after T\(_4\) replacement (270). The patient’s ventricular sample showed a low \(\alpha\)-MHC mRNA level before treatment that increased 11-fold after T\(_4\) treatment. The patient had significant improvement in several indices of cardiac function, suggesting that induction of \(\alpha\)-MHC by TH likely contributed to his clinical outcome.

The promoter and upstream regions of \(\alpha\)- and \(\beta\)-MHC genes have been analyzed, and putative TREs for both of these genes have been reported (336). In the \(\alpha\)-MHC promoter there are two TREs that are imperfect direct repeats separated by six nucleotides located between −100 and −150. In vitro binding studies and cotransfection studies suggested that the upstream TRE may be more important for T\(_3\)-mediated transcription. However, transgenic studies in which the transgene contained the \(\alpha\)-MHC promoter linked to the chloramphenicol acetyltransferase (CAT) cDNA showed that mutation of the downstream, rather than the upstream TRE, reduced CAT activity in the atria and ventricles of euthyroid mice (490). \(\beta\)-MHC is negatively regulated by T\(_3\) (364). A putative TRE containing a single half-site that is located adjacent to the TATA box may play a role in this negative regulation (138).

The rate of diastolic relaxation of the heart is related to intracellular Ca\(^{2+}\) concentration and sarcoplasmic reticulum Ca\(^{2+}\)-ATPase (SERCA2) activity. The ATPase is an ion pump that removes calcium from the cytosol and stores it in the sarcoplasmic reticulum during diastole. This decrease in the intracellular Ca\(^{2+}\) generated during systole then leads to muscle relaxation. Hypothyroid rats had decreased levels of SERCA2 mRNA that could be markedly stimulated by T\(_3\) administration (427). Similar findings also were observed in fetal chicken cardiac myocytes (598). These findings suggest that induction of this ATPase may account for TH enhancement of cardiac output by relaxing the heart with greater speed (lusitropic effect). Three different TREs that are arranged as DRs and IPs in the promoter region of the SERCA2 gene that confer T\(_3\) responsiveness (191, 471). T\(_3\) also has been shown to regulate expression of several ion channels in...
the heart such as the voltage-gated potassium channel Kv1.5, Na⁺-K⁺-ATPase, and the hyperpolarization-activated cyclic nucleotide-gated channel (365, 379, 572). Additionally, TH can regulate β-adrenergic receptor number in the heart and may thereby enhance sensitivity to catecholamines (558).

Recently, a dominant negative mutant TRβ was targeted to the heart in transgenic mice. α-MHC and SERCA2 mRNA were decreased, but β-MHC mRNA was increased, in the hearts of the transgenic mice (166). Cardiac muscle contraction was prolonged, and the QRS interval was prolonged on electrocardiogram (EKG). TRα−/− knock-out mice showed decreased heart rate and QRS interval prolongation on EKG, whereas TRβ−/− knock-out mice had elevated heart rate that was unresponsive to TH administration (228, 552). These findings suggest that TRα-1 may have a major role in maintaining baseline heart rate, whereas TRβ may mediate TH stimulation of heart rate.

Finally, a novel and potentially exciting therapeutic use of T₃ as an inotropic agent has been in cardiac surgery. Novitsky (356) showed improved cardiac function and hemodynamics when brain-dead organ donors were pretreated with T₃ and cardiac transplant recipients were treated with T₃ postoperatively. A small group of patients that underwent cardiac bypass surgery and were treated postoperatively with T₃ also showed some benefit (357). However, a large randomized study showed that although T₃ increased cardiac output and decreased systemic vascular resistance in patients who underwent coronary-artery bypass surgery, there was no improvement in outcome or changes in postoperative therapy (249).

C. Fat

TH plays important roles in the development and function of brown and white adipose tissue (8). TH can induce white adipose tissue (WAT) differentiation from preadipocytes in young rats as well as in preadipocyte cell lines such as Ob17 and NIH3T3-F442A cells (139, 177, 303, 514). In these studies, T₃ not only induced intracellular lipid accumulation and various adipocyte-specific markers such as malic enzyme and glycerophosphate dehydrogenase, but also stimulated adipocyte cell proliferation and fat cell cluster formation (139, 177).

The mechanism(s) by which T₃ induces WAT differentiation currently is not known but likely involves transcriptional regulation of important target genes by TRs. Both TRα-1 and TRβ-1 are expressed in Ob17 cells, with the TRα-1 as the predominantly expressed TR isoform. Rev-erbAα also is induced during the differentiation of NIH3T3-L1 fibroblasts into adipocytes (80). This induction of rev-erbAα was related to an increase in TRα-1/c-erbBα-2 levels. Additionally, enzymes of the lipogenic pathway, ATP-citrate lyase, malic enzyme, and fatty acid synthase, are induced by T₃ in differentiating adipocytes (43, 159, 245, 389), suggesting T₃ promotes the acquisition of differentiated functions in white adipocyte tissue.

Studies in the adult rat have shown that T₃ plays important roles in regulating basal oxygen consumption, fat stores, lipogenesis, and lipolysis (374, 375). In WAT, T₃ induces key lipogenic enzymes such as acetyl CoA carboxylase, malic enzyme, glucose-6-phosphate dehydrogenase, fatty acid synthase, and spot 14 (43, 159, 245, 338, 389). The expression of these genes is also modulated by other factors such as high-carbohydrate diet, insulin, and cAMP (374, 375). Additionally, T₃ also regulates lipolysis in a coordinate manner with lipogenesis (374, 404). Recently, a member of the nuclear hormone receptor family, PPARγ, has been shown to stimulate differentiation of adipocytes (527). Although prostaglandin J₂ and thiazolidinediones are the major ligands for PPARγ, fatty acids have been shown to stimulate transcriptional activity of PPARs, either by metabolites or by induction of genes that may promote formation of endogenous ligands within the cell (146, 251, 527). Thus TH stimulation of lipolysis may activate other nuclear hormone receptor systems, and thereby promote differentiation.

Recent studies also have shown that both TRα and TRβ-1 are differentially expressed during the development of brown adipose tissue (BAT) (529), a major contributor to facultative thermogenesis in rodents. Facultative thermogenesis occurs in response to cold exposure or overeating and depends on T₃ and adrenergic stimulation of mitochondrial uncoupling protein (UCP) synthesis (164, 203, 375, 432). There are three UCP isoforms, UCP1, UCP2, and UCP3, that are stimulated by T₃ in rat BAT (49, 279, 359, 408). UCP3 mRNA is stimulated by T₃ in mouse BAT and skeletal muscle (168, 226). It is not known whether these effects are directly mediated by T₃ or via downstream signals such as free fatty acids generated by lipolysis. The stimulation of UCP synthesis increases thermogenesis by uncoupling oxidative phosphorylation resulting in energy dissipation as heat. Interestingly, BAT also contains a type II deiodinase whose activity increases in response to cold, thereby enabling BAT to have the important ability to regulate intracellular T₃ concentration in a tissue-specific manner (469, 470). This increase in T₃ concentration likely saturates nuclear TRs and enhances norepinepinephrine stimulation of UCP. The adrenergic stimulation in BAT is predominantly, but not exclusively, mediated by brown fat specific adrenergic β₂-receptors. The dual regulation of UCP by the type II deiodinase and the adrenergic system suggests convergence of nuclear- and membrane-signaling systems in the transcriptional regulation of these important target genes in BAT, but the precise relative contributions and interplay between these regulatory systems need to be further defined. Lowell et al. (313) used the UCP promoter to
target diptheria toxin to BAT in transgenic mice. These animals had decreased amounts of BAT, resulting in decreased cold tolerance and obesity, and suggesting a critical role for BAT in these functions. Recently, UCP3 knockout mice were generated and had normal weight, thermogenesis, and response to T\textsubscript{3}, suggesting that UCP3 is not the main mediator for TH-mediated thermogenesis as originally proposed, or alternatively, compensatory mechanisms can overcome the lack of UCP3 (169, 538).

Several human studies have shown that chronic hypothyroidism as well as acute T\textsubscript{3} treatment did not affect serum leptin levels (100, 264, 319, 480). However, one study showed that hypothyroid patients had increased leptin levels, but the increase correlated with adiposity (399). Another study showed that hyperthyroid patients treated with thiamazole increased their leptin levels (610). Studies in the rat have shown that T\textsubscript{3} can decrease leptin levels, but it is not known whether this is due to a direct effect or due to its effects on fat mass (125, 302, 497).

D. Liver

TH has multiple effects on liver function including stimulation of enzymes regulating lipogenesis and lipolysis as well as oxidative processes (375, 376). Some of the lipogenic enzymes that are regulated are malic enzyme, glucose-6-phosphate dehydrogenase, and fatty acid synthase. In the case of malic enzyme, which has been studied extensively, there is a biphasic induction of the malic enzyme mRNA at 4 and 24 h, suggesting that there may be an initial direct stimulation by T\textsubscript{3} and a secondary effect due to stimulation by other gene products that are regulated by T\textsubscript{3} (486). Indeed, it recently has been shown that Spot 14 (S14), a protein that originally was identified by two-dimensional gel electrophoresis of the translational products of total hepatic tissue mRNA from T\textsubscript{3}-treated rats, may regulate a number of lipogenic enzymes, including malic enzyme, in the liver (245, 452). Likewise, at least in the rat, a number of lipogenic enzymes also may be regulated by growth hormone, which is induced by T\textsubscript{3} (375). Interestingly, malic enzyme is very sensitive to T\textsubscript{3} in the liver, but it is unresponsive in the brain, suggesting that tissue-specific factors are important in determining T\textsubscript{3}-mediated stimulation of transcription. Nikodem and co-workers (395) identified a putative TRE in the promoter region of the malic enzyme gene that contains two half-sites arranged as direct repeats separated by four nucleotides.

T\textsubscript{3} regulation of malic enzyme gene transcription also can be regulated by carbohydrate intake, insulin, and cAMP. For instance, T\textsubscript{3} effects on malic enzyme gene transcription are minimal in fasted animals but are most pronounced in animals fed a sucrose-containing fat-free (lipogenic) diet (375, 376). Similar interactions between T\textsubscript{3} and dietary carbohydrate also occur in the gene regulation of other lipogenic enzymes. Another T\textsubscript{3}-regulated gene expressed in liver that has been studied extensively has been the one encoding S14 protein (376). Its mRNA is rapidly induced by T\textsubscript{3} after 20 min in hypothyroid rats and precedes the expression of lipogenic enzymes. Additionally, it is coregulated by carbohydrate similar to lipogenic enzymes. Its tissue distribution is similar to those of lipogenic enzymes as it is expressed in liver, white and brown fat, and lactating mammary tissue. Recently, it has been shown that S14 may be localized in the nuclear matrix and thus may participate in regulating the transcription of lipogenic enzymes (245, 246).

It has been appreciated for many years that hypothyroidism is associated with hypercholesterolemia with elevated serum intermediate and low-density lipoprotein (LDL) cholesterol concentrations (52). The major mechanism for these effects may be lower cholesterol clearance resulting from decreased LDL receptors. Furthermore, the genotype of the LDL receptor gene may influence the elevation of serum LDL cholesterol concentrations in hypothyroid patients and their response to thyroxine treatment (559). An additional mechanism may be decreased hepatic lipase activity in hypothyroidism which decreases conversion of intermediate-density lipoproteins to LDL and high-density lipoprotein metabolism (380, 508). It is not known whether these effects are mediated directly or indirectly by TH. Several putative TREs in the distal promoter region of the hepatic lipase and apolipoprotein A1 genes have been identified (457, 513). TH also has been shown to regulate the expression of several important proteins and enzymes involved in cholesterol metabolism and synthesis such as the LDL receptor, cholesterol ester hydrolase, and cholesterol acyltransferase (423, 447, 461). TH also may regulate post-transcriptional editing of apolipoprotein mRNA (111). TR\textbeta-1 is the predominant isoform expressed in liver, whereas TR\textalpha-1 is the major isoform expressed in heart (128, 204, 487). These differences in TR isoform expression have spawned attempts to develop isoform-specific TH analogs that may have cholesterol-lowering effects but minimal cardiac toxicity (86, 512, 533).

Recently, Hayashi and Refetoff (195) have created a mouse model of TH resistance in the liver by transfecting hepatocytes with adenovirus vectors encoding a mutant TR (195). This mutant receptor had dominant negative activity (see below) on wild-type receptor function. The mice that expressed the mutant TR had elevated serum cholesterol levels compared with control animals in both hypothyroid and hyperthyroid states. Additionally, they had blunted induction of S14 protein and 5’-deiodinase mRNA by T\textsubscript{3}. These findings suggest a direct role by TRs in regulating these genes. This model thus may be a useful
tool for determining those hepatic genes that are directly regulated by TH.

To identify novel hepatic target genes and examine gene profiles regulated by T₃, Feng et al. (136) used a quantitative fluorescent cDNA microarray to identify hepatic genes regulated by TH. Fifty-five genes, 45 of which were not previously known to be TH responsive, were found to be regulated by TH (136). Among them, 14 were positively regulated by TH, and surprisingly, 41 were negatively regulated. TH had broad effects as it regulated gene expression of a diverse range of cellular pathways and functions such as gluconeogenesis, lipogenesis, insulin signaling, adenylate cyclase signaling, cell proliferation, and apoptosis. This application of the microarray technique to study hormonal regulation of gene expression in vivo demonstrates the value of large-scale gene expression analyses for future studies of hormone and drug action.

E. Pituitary

TH regulates the synthesis and secretion of several pituitary hormones. Absence of GH has been observed in the pituitaries of hypothyroid rats (440). Additionally, T₃ can stimulate the transcription of GH mRNA and GH synthesis in rat pituitary tumor cells (322, 440, 458). Brent et al. (54) identified a rGH TRE that contains a direct repeat separated by four nucleotides and a palindromic sequence (101, 281). This HRE can also confer sensitivity to retinoids and vitamin D (156, 493). However, in contrast to rodents, T₃ has limited ability to regulate GH synthesis in humans. For example, hypothyroid children have impaired growth but serum growth hormone levels are normal (424, 537). Cotransfection studies using the human growth hormone promoter have not shown stimulation by TH (55, 71). Studies in cultured human somatotrope adenomas showed that T₃ stimulated GH release but had variable effects on transcription (90).

TH also can negatively regulate thyrotropin (TSH) transcription by direct and indirect mechanisms (464). TH can negatively regulate TRH at the transcriptional level, which in turn decreases transcription of TSH mRNA (465, 573). T₃ also can downregulate prolactin mRNA by a similar mechanism, and also by direct effects on transcription (565). The TRH promoter has been analyzed and contains several nTREs (206, 446). TH hormone also can negatively regulate TSH by decreasing transcription of the glycoprotein hormone α-subunit (common to TSH, luteinizing hormone, follicle-stimulating hormone, and human choriongonadotropic hormone) and the TSHβ subunit genes (44, 51, 77, 108, 318, 464, 466). Several nTREs in the promoters of these genes have been described. In some cases, they contain single half-sites, suggesting that TR monomers may be involved in negative regulation. Additionally, these genes display ligand-independent activation in cotransfection studies (207, 501). It also is possible that corepressors, rather than coactivators, may participate in this activation and negative regulation (207, 445, 501). Additionally, it has been shown that TH decreases the stability of TSHβ mRNA by inducing shortening of its poly(A) tail (263). T₃ can stimulate a cytosolic RNA-binding protein to bind to the 3′-untranslated region of TSHβ mRNA and may thereby regulate mRNA stability at a posttranscriptional level (299).

Recent cotransfection and knockout studies suggest that TRβ-2 isoform may be playing the predominant role in regulating TSH (2, 275). In situ hybridization and immunostaining studies have shown that TRβ-2 is highly expressed in thyrotropes in the pituitary (87, 590). Additionally, RXRγ isoform appears to be selectively expressed in thyrotropes, suggesting that it also may play a functional role in the regulation of TSH via isoform-specific TR/RXR complexes or RXRγ homodimers (193, 495). Recent findings of inappropriate TSH secretion in a RXRγ knockout mice, and the suppression of TSH by RXR-specific agonist in humans are consistent with this model (192, 462).

F. Brain

TH has major effects on the developing brain in utero and during the neonatal period (38, 373). Neonatal hypothyroidism due to genetic causes and iodine deficiency in humans can cause mental retardation and neurological defects. Studies in hypothyroid neonatal rats have shown that absence of TH causes diminished axonal growth and dendritic arborization in the cerebral cortex, visual and auditory cortex, hippocampus, and cerebellum (409, 410). In the cerebellum, absence of TH also delays proliferation and migration of granule cells from the external to the internal granular layer. The critical role of TH is further demonstrated by a recent report in which a dominant negative TR was targeted to the cerebellum in transgenic mice. The Purkinje cells showed decreased dendritic arborization, while the granule cells had retarded migration to the internal granular layer (257). The developmental delays in the rat brain can be reversed if TH is administered within 2 wk after birth (539). These findings support the clinical observations that early T₃ treatment of congenital hypothyroidism prevents intellectual impairment in humans and is the major impetus for neonatal screening for congenital hypothyroidism. In utero, monodeiodination of T₂ to T₃ by type II deiodinase and maternal-fetal transfer of T₃ may help maintain normal T₃ concentrations even when the fetus has congenital hypothyroidism (110, 180). Additionally, maternal transfer of thyroxine may be important, particularly during early fetal development (68, 542). Recent studies have suggested that mater-
nal thyroid status may have significant effects on the neuropsychological outcome of children (38, 182, 403).

The ontology of TR isofoms in the brain suggests that specific TR isofoms may be involved in transcription of target genes and in brain development (373, 487). TRα-1 is expressed throughout the brain from early fetal development and accounts for total T3 binding in the fetal brain. TRβ-1 is absent or minimally expressed, except in a few selected areas such as the cochlea and cerebellum (47, 48, 488, 489). However, there is a dramatic 40-fold increase in TRβ-1 mRNA expression throughout the brain shortly after birth that reaches maximum levels 10 days afterward, and then persists until adulthood (487). In contrast, TRα-1 and c-erbAα-2 mRNA undergo a transient twofold increase that decreases to adult levels 2 wk after birth. This early rise in TRβ-1 expression coincides with the neonatal surge in serum T3 and suggests there may be a coordinated temporal developmental program in which critical target genes are regulated by specific TR isofoms. Similar temporal patterns of expression also have been observed in the chick and amphibian tadpole (127, 137, 473).

Despite the importance of TH in brain development, there are relatively few genes known to be directly regulated by TH, and many have only been partially characterized. Farsetti and co-workers (129, 130) have shown that the gene for myelin basic protein (MBP) is directly regulated by TH and have identified a TRE at position −186 to −169 of the MBP promoter. Recent studies of brain-derived neutrophic factor showed that TH can regulate its expression in a promoter-, developmental-, and region-specific manner (259). TH also regulates several genes that are involved in a wide range of cellular functions: glutamine synthase, protein kinase C, substrate RC3/neurogranin, prostaglandin D2 synthase, hairless (a potential transcription factor), and adhesion molecules such as neural cell adhesion molecule and matrix proteins such as tenascin, and proteins important for neuronal migration (14, 15, 155, 172, 323, 515). Nordquist et al. (355) have identified several genes that are expressed in the cerebellum: calbindin, myo-inositol trisphosphate (OP-3) receptor, and Purkinje cell protein-2 (Pcp2). Study of the mRNA expression of these genes and MBP in the cerebellums of hypothyroid and euthyroid neonatal mice showed that the expression of these genes was delayed in the absence of hormone but eventually reached similar expression levels (489). These data also suggested that there may be three phases in the regulation of these particular genes: a refractory prenatal period, a T3-responsive prenatal period typically from postnatal days 3–20, and a T3-independent period. Identification of a putative silencing element in the promoter of Pcp2 promoter that may be functional during fetal development provides support for this model (16). It is intriguing to speculate there may be developmentally and regionally expressed suppressors that may silence T3-responsive target genes during embryogenesis. One proposed candidate suppressor may be the orphan receptor, Coup-Tf, which binds to the suppressor region in the Pcp2 promoter (16). It remains controversial as to whether these cerebellar genes are specifically regulated by TRβ-1, which is expressed in parallel. Studies in cultured oligodendrocytes have suggested that this may be the case (69, 425); however, no significant developmental delays in Pcp2 and MBP expression were observed in TRβ knockout mice (443). Finally, it is possible that there may be cross-talk between TR and other transcription factors in the regulation of target genes in the brain. The knock- out mouse for the orphan receptor, ROR (staggerer), has cerebellar defects that are almost identical to those observed in hypothyroid mice (258). In this connection, ROR has been shown to modulate transcriptional activity by TH in cotransfection studies (260).

X. RESISTANCE TO THYROID HORMONE

RTH is a syndrome in which patients have hyposensitivity to TH, elevated circulating serum T3 and T4, and elevated or nonsuppressed TSH levels. Refetoff et al. (414) first described this syndrome in two siblings who presented with deaf-mutism, delayed bone age with stippled epiphyses, goiter, and high protein-bound iodine levels. Since this initial report, over 350 subjects have been described who have RTH, with ~80% of the subjects inheriting this disorder (7, 60, 415). The clinical manifestations are variable among families with RTH and also among affected family members. Additionally, patients can have clinical symptoms that have features of hypo- and hyperthyroidism, suggesting variable resistance in different tissues. Some of the clinical features that have been described include goiter, mental retardation, attention-deficit disorder, tachycardia, delayed bone growth and maturation, and hearing abnormalities. There also are examples of pituitary resistance to TH (PRTH), in which patients have resistance predominantly in the pituitary and have signs and symptoms of hyperthyroidism in peripheral tissues (7, 60, 415).

With the exception of the index family, which had autosomal recessive inheritance, RTH displays autosomal dominant inheritance (415). In 1988, Usala et al. (535) demonstrated a tight linkage between the TRβ gene locus and RTH by restriction fragment length polymorphism. Soon afterwards, two groups independently demonstrated mutations in the LBD of TRβ in patients from two families with RTH: glycine-345 replaced by arginine (Mf-1), or proline-453 replaced by histidine (438, 536). Subsequent characterization of other families with RTH have shown TRβ point mutations in the two major “hot spots” that cluster near these original mutations (Fig. 13) (36, 415). Mutations also have been found in a third “hot spot”
in the most amino-terminal portion of the LBD (37, 97, 370, 400, 571). Most patients have mutations due to single amino acid substitutions at a single codon, although single amino acid deletions, frameshift mutations, and truncations due to premature termination of translation from a mutation-generated stop codon also have been identified (7, 60, 415). Almost all mutations cluster around the ligand-binding pocket observed in the TR LBD crystal structure. In vitro transcription and translation of the mutant receptors typically show minimal or reduced T₃-binding affinity (415).

Approximately 60 different mutations in TRβ have been identified in RTH patients from over 100 families (7, 60, 415). In some cases, the same mutations have been described in different families. The clinical phenotype can vary among these families that harbor the same mutation and also vary within a family. This suggests that there may be other genetic modifiers that determine the phenotype. In this connection, the same mutation can cause either GRTH or PRTH in different individuals even within the same family. In general, there does not seem to be a strong correlation between particular mutations and the development of GRTH and PRTH. The R338W mutation, and a ninth heptad mutant, R429Q, may be more prone to a PRTH phenotype; however, patients with these mutations also can exhibit GRTH (7, 140, 415). Interestingly, no germline TRα-1 mutants have been described thus far in humans. It is possible that TRα-1 mutations are lethal in utero, silent, and/or exceedingly rare.

Analyses of the TRβ gene in the index family with RTH showed that the affected members were homozygous for the deletion of the entire coding region of TRβ (502). Thus complete lack of TRβ expression was compatible with life. Interestingly, heterozygous members of the family that contained only one deleted TRβ allele had normal clinical and laboratory findings (Fig. 14). This observation suggested that a single copy of TRβ is sufficient for normal function. Furthermore, it suggests that reduction of TRβ (i.e., gene dosage) did not account for the autosomal dominant inheritance seen in other RTH patients. Instead, it strongly suggested that TRβ point mutations interfere with the normal function(s) of TRs. This interference of wild-type protein function by a mutant protein has been called “dominant negative activity” (223, 582). The amount of dominant negative activity by a mutant TR depends in part on the level of mutant receptor expression. For example, a patient who was homozygous for mutations in both TRβ alleles had severe RTH and mental retardation (371). In contrast, his parents who had mutations in only one TRβ allele had mild RTH. Within any given cell in a RTH patient, mutant TRβ as well as wild-type TRβ and TRα are expressed. The molecular mechanism(s) for the dominant negative activity by the mutant TRβ on wild-type TR function has been the subject of numerous studies. Transient cotransfection studies show that natural TRβ-1 mutations not only fail to mediate normal T₃-regulated transcription, but also block the T₃-regulated transcription by normal TRs (78, 327, 415, 437). The mechanism for this dominant negative activity likely involves binding to TREs by inactive mutant homodimers or TR/RXR heterodimers that cannot bind T₃ and hence cannot activate transcription of target genes. Indeed, DNA binding of mutant TRs and v-erbA is required for their dominant negative activity. Additionally, some natural mutant TRβ-1s, as well as v-erbA, constitutively repress basal transcription even in the presence of T₃ (28, 104, 345, 398, 582, 592). Recent experiments also suggest that dimerization may play an important role in mediating dominant negative activity (94, 196, 344). However, it is not known whether a particular mutant TR complex (i.e., homodimer or heterodimer) mediates this activity in all cases. Heterodimer-specific mutants are able to mediate
dominant negative activity, albeit more weakly than other mutants that can form heterodimers (189, 196). Amino acid substitutions in the ninth heptad of TRβ-1 mutants decreased heterodimerization and dominant negative activity of TRβ-1 mutants (21, 94, 344). These observations have been used to support functional roles for either mutant homo- or heterodimers. However, it may turn out that transcriptionally inactive homo- and heterodimers both can mediate dominant negative activity as long as they compete effectively with wild-type TRs for binding to TREs.

Dominant negative activity in cotransfection studies and severity of clinical phenotype correlate well with impairment of in vitro T3 binding by mutant TRβs (327, 415), although some exceptions have been reported (196, 343). Recent studies on TRβ mutations in the AF-2 region and hinge region have shed some light on this issue. TRβ mutations in the AF-2 region have normal T3 binding, DNA binding, and heterodimerization but are transcriptionally inactive in the presence of ligand due to their inability to interact with coactivators (521). Recently, several groups have described RTH patients who have TRβ mutations in the AF-2 region (96, 498, 549). Furthermore, AF-2 mutants had potent dominant negative activity on wild-type TRs in cotransfection studies (96, 311, 506). Recently, several mutations in the third hot spot also have been characterized. Two mutants (R243Q and R243W) had normal T3-binding affinity but transactivated poorly in the presence of T3 (97, 370, 435, 571). These mutants also exhibited dominant negative activity on wild-type TR. One study showed that mutant homodimers could not be readily dissociated by T3, suggesting that these receptors may have reduced T3-binding affinity after binding to DNA (571). Additionally, some of these mutants may have impaired corepressor release or interaction with coactivators (97, 435).

Yoh et al. (593) have studied SMRT corepressor interaction with a battery of mutant TRs. They observed that mutant TRs had defective dissociation from this corepressor. In general, the release of SMRT correlated with the T3-binding affinity of the mutant receptors. However, two mutants that had only mildly impaired T3 binding affinity were unable to dissociate SMRT even at 1 μM T3. Additionally, two mutants, D432M and D432G, have enhanced association with SMRT. In general, the amount of dominant negative activity correlated with the impairment of SMRT dissociation in the presence of T3. The authors also showed that hinge region mutants that abrogate SMRT interaction also decrease the dominant negative activity. Tagami and Jameson (499) also found similar results when they studied NCoR interaction with a battery of TR mutants. They also observed that a hinge region mutant that abrogated NCoR interaction decreased basal repression of transcription of positively regulated genes and ligand-independent activation of negatively regulated genes. These findings suggest that NCoR could play a role
in mediating dominant negative activity of mutant TRs in positively and negatively regulated target genes. Additionally, mutant TRβs located between the two major hot spots or near the AF-2 region also had impaired corepressor release (92, 436, 498).

Liu et al. (311) studied the interaction of mutant TRs with the corepressor NCoR and coactivator SRC-1. The G345H mutant, which has minimal T3 binding affinity, remained bound to NCoR in the presence of T3 and was unable to bind SRC-1. An AF-2 mutant could release NCoR in the presence of T3 but was unable to bind SRC-1. Both mutant receptors had potent dominant negative activity. A mutant receptor (R320H) with threefold lower T3 binding affinity than wild-type TR was able to release NCoR at higher T3 concentrations and recruit SRC-1 at higher T3 concentrations. Interestingly, this mutant had potent dominant negative activity at low T3 concentrations but weaker dominant negative activity at higher concentrations. Similarly, Chatterjee and co-workers (96, 97) have identified helix 3 and helix 12 mutants from concentrations. Similarly, Chatterjee and co-workers (96, 97) have identified helix 3 and helix 12 mutants from patients with RTH that have impaired recruitment of coactivators. These findings and those showing impaired corepressor release by mutant TRβs (435, 498, 593) reinforce the notion that inability to release corepressors and/or the inability to recruit coactivators (regardless of the mechanism) can result in TR complexes that cannot be activated by ligand and can mediate dominant negative activity.

Refetoff and co-workers (401, 548) recently have reported families with RTH that did not contain TRβ mutations. Additionally, linkage analyses suggested that RTH was not associated with TRα. Nuclear extracts from affected patients showed an additional TR-associating band on electrophoretic mobility shift assay (548). These find-ings suggest the possibility that mutations in cofactors or dysregulation of their expression may be involved in the RTH phenotype of these families. Currently, there is no direct evidence for such "postreceptor" defects. In one example of a coactivator mutation associated with human disease, mutations in CBP were found in patients with Rubinstein-Taybi syndrome (393), an autosomal dominant disorder in which patients have mental retardation, short stature, and craniofacial abnormalities. However, thyroid function tests in these patients were surprisingly normal, suggesting one normal allele is sufficient for TH signaling (367). Ando et al. (18) recently observed an intragenic splice variant of TRβ in a TSH-secreting adenoma that may account for pituitary resistance to TH in that tumor. Thus it is possible that nongenomic mechanisms may account for RTH in some of these families.

There also have been several somatic TR mutations described in tumors. Lin et al. (309) have described somatic mutations in TRα and TRβ from a human hepatoma cell line. It is not known whether these mutant TRs contributed to oncogenesis, although they both exhibited dominant negative activity on wild-type TR. In this connection, a transgenic mouse that overexpressed v-erbA also developed hepatomas (32). A somatic mutation of TRα has been identified in a thyrotropin-secreting tumor and may cause the defective negative regulation observed in these pituitary tumors (18).

There have been two reported cases of hypersensitivity to TH (222, 320). One patient was euthyroid on the basis of thyroid function tests but had a marked tachycardia. Analyses of T3 binding in lymphocytes showed normal binding affinity but increased binding capacity. Family history showed several family members with paroxysmal tachycardia and elevated T3 binding in lymphocytes. Sequencing of exons 9 and 10 of TRβ showed no mutations. Another patient had serum thyroid function tests suggestive of hypothyroidism but exhibited no clinical symptoms suggestive of hypothyroidism. This patient had a V444A substitution in TRβ resulting in increased affinity for T3. A constitutively active estrogen receptor with a mutation in the AF-2 domain has been described (546), so it is theoretically possible that mutations that enhance interaction with coactivators could generate a TR with constitutive activity or increased ligand-mediated transcription.

XI. GENETICALLY ENGINEERED MOUSE MODELS OF THYROID HORMONE ACTION

Transgenic expression of dominant-negative mutant TRs, and the targeted gene inactivation or knockout of TR isoforms, have been used to study TH action in mice. These mouse models have provided new information on the developmental and physiological effects of TH.

Two groups have examined the effects of ubiquitous expression of v-erbA and a natural mutant TRβ-1 in transgenic mice (32, 564). These transgene products have dominant negative activity on the actions of wild-type TR. The mice that expressed v-erbA had multiple abnormalities including hypothyroidism (due to follicular disorganization in the thyroid), inappropriate TSH response, enlarged seminal vesicles, hepatomas, decreased fertility, and reduced adipose tissue. Because v-erbA has dominant negative activity on retinoic acid-mediated transcription, it is possible that some of the observed effects may not be due solely to blockade of T3-mediated transcription. The transgenic mice that expressed mutant TRβ-1 had elevated T3, inappropriate normal TSH, behavioral abnormalities, decreased fertility, and decreased weight. These findings resembled the clinical phenotype observed in RTH patients harboring this mutation.

Pituitary-specific targeting has been undertaken using natural dominant negative mutants (TRβ G345R, Δ337T) under the regulation of the TSHβ or glycoprotein hormone α-subunit promoters (4, 197). These transgenic
mice had slightly elevated $T_4$, elevated TSH, inability to suppress TSH after $T_3$ administration, and decreased cholesterol. These findings are consistent with TH resistance in the pituitary and TH sensitivity in the liver. Interestingly, one line ($\Delta 337T$) had a blunted rise in TSH compared with littermate controls, suggesting the mutant TR may block the ligand-independent activation of TSH (4). In contrast to these two studies, another transgenic line in which a frameshift mutant TR (448 frameshift) was targeted to the pituitary with the glycoprotein hormone $\alpha$-subunit did not show any abnormalities in the HPT axis (606). It is possible that differences in the expression levels of the mutant TRs may account for this difference in phenotype.

Cardiac-specific targeting of $\text{TR}\beta \Delta 337T$ has resulted in transgenic mice with abnormal papillary muscle contraction and a prolonged QRS on EKG (166). $\alpha$-Myosin heavy chain and sarcoplasmic reticulum Ca$^{2+}$-ATPase mRNA were decreased, whereas $\beta$-myosin heavy chain mRNA was increased in transgenic mice. These findings are similar to those observed in the hypothyroid heart; however, they contrast with the situation in patients with RTH who often have resting sinus tachycardia. The heart normally expresses mostly $\text{TR}\alpha$ and little $\text{TR}\beta$, so the finding in RTH patients may be due to the relatively low amount of dominant negative $\text{TR}\beta$ in the context of elevated circulating TH levels (415). Another study in which the same mutant $\text{TR}\beta$ was targeted to the heart showed decreased contractility in isolated heart preparations (386).

Hayashi et al. (195) have used an adenovirus-based expression system encoding a dominant negative $\text{TR}\beta$-1 mutant, G345R, to create a liver-specific model of RTH (195). They utilized the selective clearing and uptake of injected adenovirus vector by the liver and enabled them to efficiently transfect hepatocytes with either $\text{TR}\beta$ or G345R. They demonstrated resistance to $T_3$ in G345R-transfected mice by measuring $T_3$ regulation of several liver enzymes. Interestingly, serum cholesterol increased more in the $\text{TR}\beta$ and G345R transfected mice compared with controls. These findings suggest that unliganded wild-type $\text{TR}\beta$ and G345R both may have effects on target genes in the cholesterol synthesis pathway.

Two different groups generated $\text{TR}\alpha$ knockout mice that have different phenotypes (152, 552). The structure of the $\text{TR}\alpha$ gene is complex because it encodes $\text{TR}\alpha$-1, c-erbA$\alpha$-2 (which cannot bind $T_3$), and rev-erbA (generated from the opposite strand encoding TR$\alpha$) so the locus of homologous recombination will determine which isoforms will be knocked out (293). Transgenic mice in which both $\text{TR}\alpha$-1 and c-erbA$\alpha$-2 have been deleted ($\text{TR}\alpha$ –/–) have a more severe phenotype with hypothyroidism, intestinal malformation, growth retardation, and early death shortly after weaning (152). The early death can be partially rescued by $T_3$ injection of pups. Transgenic mice that lack only $\text{TR}\alpha$-1 ($\text{TR}\alpha$-1 –/–) have a milder phenotype with decreased body temperature and heart and prolonged Q-T interval on EKG (552). These findings suggest a major role for $\text{TR}\alpha$-1 in regulating cardiac function. The differences between the two phenotypes could be due to specific functions of c-erbA$\alpha$-2, although specific knockout of c-erbA$\alpha$-2 did not affect survival of the pups (151). Samarut and co-workers (75) have reported generation of short TR isoforms from internal translation start sites that can block the actions of TR. It is likely that these TR isoforms may be responsible for the more severe phenotype of the $\text{TR}\alpha$ –/– knockout mice. Indeed, a $\text{TR}\alpha$ knockout that lacks both $\text{TR}\alpha$-1 and c-erbA$\alpha$-2 and does not express these isoforms ($\text{TR}\alpha$ o/o) has a milder phenotype than $\text{TR}\alpha$ –/– and increased $T_3$ sensitivity in tissues expressing TR$\beta$ (316a).

Ablation of $\text{TR}\beta$ by homologous recombination in mice ($\text{TR}\beta$ –/–) produced modest changes in phenotype with elevated TSH and $T_3$, thyroid hyperplasia, as well as hearing defects as the major findings (149, 150). These findings involving the HPT axis resemble those seen in patients with RTH. deafness was observed in the index cases of RTH which had deletion of $\text{TR}\beta$ (414). Taken together, these findings suggest a critical role for $\text{TR}\beta$ in the development of the auditory system. Interestingly, ligand-independent elevation of TSH is normal in hypothyroid $\text{TR}\beta$ –/– mice, but the suppression of TSH by TH is impaired (547). Recently, $\text{TR}\beta$-2 has been selectively knocked out (2). These mice had elevated levels of TH and TSH, implicating $\text{TR}\beta$-2 as the major regulator of TSH. Interestingly, these mice did not have any hearing defects. These findings suggest that $\text{TR}\beta$-2 may not be critical for auditory development, or its function may be compensated by $\text{TR}\beta$-1. Finally, recent studies in $\text{TR}\beta$ –/– mice suggest $\text{TR}\beta$-2 may be involved in retinal development (355a).

Given the relatively mild phenotypes of the $\text{TR}\alpha$-1 and $\text{TR}\beta$ knockout mice, it is likely that the two isoforms may have redundant transcriptional activity and can compensate for each other in most target genes. To examine the effects of abolishing all TR isoforms, $\text{TR}\alpha$-1 –/– $\text{TR}\beta$ –/– knockouts have been generated (171). These mice lack $\text{TR}\alpha$-1 and $\text{TR}\beta$, but still express c-erbA$\alpha$-2. These mice have markedly elevated $T_4$, $T_3$, and TSH with large goiters. These mice also have growth retardation and decreased fertility, as well as impaired bone development and reduced bone mineral content. These mice also have reduced heart rate and impaired control of body temperature, similar to the $\text{TR}\alpha$-1 –/– mice. Although these mice have many symptoms of hypothyroidism, these mice did not have any reduction in activity level. There was no measurable $T_3$ binding in liver and brain nuclear extracts, confirming the absence of any TR in these mice.

$\text{TR}\alpha$ –/–/$\text{TR}\beta$ –/– knockout mice which lack $\text{TR}\alpha$-1, c-erbA$\alpha$-2, and $\text{TR}\beta$, but express short $\text{TR}\alpha$ iso-
forms have been generated (157). They have a similar phenotype as the TRα //−/− mice except for markedly elevated T4, T3, and TSH, and more pronounced malformation in the ileum. TRα o/o/ TRβ //−/−, which lack the TRα-1, c-erbAα-2, and TRβ-1 as well as the shorter TRα isoforms, survive until 6 mo of age and have a similar phenotype similar to the TRα-1 //−/−/ TRβ //−/− mice (171). However, the females are completely sterile, and the males have decreased fertility. They also have markedly elevated T4, T3, and TSH. Comparison of the the latter two double-knockout strains in addition to the c-erbAα-2 knockout mice (151) should provide information on the specific role of c-erbAα-2 on development and thyroid function.

The preceding double-knockout mouse studies demonstrate that absence of TRs is not incompatible with life and that mice without TRs can survive surprisingly well. Many of the effects on peripheral tissues were milder in the double-knockout mouse than those seen in congenital hypothyroidism. The reason(s) for these observations is not known. Given the lack of T3 binding in nuclear extracts of the TRα //−/−/ TRβ //−/− mice, it is unlikely there is an additional TR isoform unless it is expressed during embryogenesis or in very limited subset of cells. It is possible that nongenomic effects of TH may be active in the double-knockout mice but not in congenital hypothyroidism. It also is possible that lack of TRs is less deleterious than having TRs present during hypothryoidism. Given the role of unliganded TRs and corepressors in repressing basal transcription, it is possible that critical target genes may be shut down in the hypothyroid mouse, whereas a certain degree of “leaky” basal transcription occurs in the double-knockout mice.

Recently, Cheng and co-workers (236) have generated a “knock-in” mouse model in which a mutant TRW was introduced into the endogenous TRβ gene locus. Similar to patients with RTH, the heterozygous mice had elevated serum T4 and TSH, mild goiter, hypercholesterolemia, impaired weight gain, and abnormal bone development. Homozygous mice had markedly elevated serum T4 and TSH and a much more severe phenotype. It thus appears that the mutant TR has dominant negative activity on TR function in this mouse model of RTH. It also will be interesting to compare the expression of target genes in these mice with the TRα o/o/ TRβ //−/− knockout mice in the absence of TH, as the latter mice do not have TRs to mediate basal repression. Recently, Wondisford and colleagues (191a) have generated "knock-in" mice expressing mutant TRβ, and these mice have abnormal cerebellar development and function, as well as learning deficits.

Finally, Weiss et al. (550) have shown that loss of a coactivator can also lead to mild RTH. Using SRC-1 knockout mice that previously had been shown to have some resistance to steroid hormones in reproductive tissues, they found that the knockout mice had mildly elevated T4, T3, and moderately elevated TSH levels. Interestingly, suppression of TSH by T3 was impaired in knockout mice, suggesting that SRC-1 may be needed for the negative regulation of TSH production. These findings have clinical implications because several families with RTH have been described that do not have mutations in TRα or TRβ genes. It is possible that mutations in cofactors may account for their RTH phenotype. Additionally, as knockout mice for various coactivators are generated, they will be useful tools in studying TH action on both positively and negatively regulated genes and the effects of coactivators on the function of the HPT axis.

XII. CONCLUSION

Just as our early knowledge of TH action was interwoven with many of the key developments in biomedical science, so it has been the case for the recent study of TH action. Even more than before, our knowledge has benefited from the contributions of outstanding investigators from many different fields and countries. With the recent sequencing of the human genome and the development of new technologies such as microarrays, proteomics, and genetically engineered mouse models, we will have powerful new tools to study the complexities of TH action in the future. It is with eagerness that we look forward to an even deeper understanding of TH action.

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