We reported previously that deletion of the 50-amino acid NH2-terminal A/B domain of the chicken (c) or rat thyroid hormone (T3) receptor-α (T3Rα) decreased the T3-dependent stimulation of genes regulated by native thyroid hormone response elements (TREs). This requirement of the NH2-terminal A/B domain for transcriptional activation was mapped to amino acids 21–30 of cT3Rα. Expression of transcription factor IIB (TFIIB) in cells was shown to enhance T3-dependent transcriptional activation by cT3Rα, and this enhancement by TFIIB was dependent on the same 10-amino acid sequence. In vitro binding studies indicated that cT3Rα interacts efficiently with TFIIB, and this interaction requires amino acids 23KKRRK27 in the A/B domain. In this study we document the functional importance of these five basic residues in transcriptional activation by cT3Rα, further supporting the biological significance of these residues and their interaction with TFIIB. Interestingly, we also find that the same amino acids also affect DNA binding and dimerization of cT3Rα and Ga1 mobility shift assays reveal that a cT3Rα mutant that has all five basic amino acids changed from 23KKRRK27 to 23TTIT27 binds to a palindromic TRE predominantly as a homodimer, whereas cT3Rα with the wild-type 23KKRRK27 sequence binds predominantly as a monomer. These results from both a marked decrease in the ability of the cT3Rα mutant to bind as a monomer and from an enhanced ability to dimerize as reflected by an increase in DNA-bound T3R-retinoic X receptor heterodimers. These effects of 23KKRRK27 on DNA binding, dimerization, transcriptional activation, and the association of T3Rα with TFIIB support the notion that this basic amino acid motif may influence the overall structure and function of T3Rα and, thus, play a role in determining the distinct context-dependent transactivation poten-vials of the individual T3R isoforms.

Steroid, retinoid, and thyroid hormone nuclear receptors are ligand-dependent transcription factors that couple extracellular signal directly to transcriptional responses. These receptors activate or repress transcription of target genes by binding to specific DNA sequences referred to as hormone response elements (HREs)1 (1). The nuclear receptor superfamily can be divided into the steroid hormone receptor family and the thyroid hormone/retinoid receptor family (1, 2), which includes receptors that mediate the effects of thyroid hormone (l-triiodothyronine (T3) (the T3Rs), all-trans-retinoic acid (the RARs), 9-cis-retinoic acid (the RARs and RXRs), and 1,25-di-hydroxyvitamin D3 as well as several orphan receptors (e.g. COUP-TF, c-erB2a2) whose ligand(s), if any, are unknown (3–5).

The T3Rs are encoded by two distinct but closely related genes (α and β) which, in humans (h), map to chromosomes 17 and 3, respectively (6). Each gene expresses several alternatively spliced isoforms. The T3Rα gene in the rat (r) and man expresses the T3-binding isoform rT3Rα1 along with c-erB2aα2, which does not bind T3 because of alternative splicing at the COOH terminus (3, 7, 8). The closely related chicken (c) α gene expresses only cT3Rα, which is more than 90% similar to the amino acid level to rT3Rα1 and hT3Rα1 (6, 9, 10). The T3Rβ gene expresses T3Rβ1 and T3Rβ2 that differ only in their NH2-terminal A/B regions, which are distinct from the A/B region of T3Rα1 (3, 11). Except for the A/B domains, the T3Rα and T3Rβ receptors are more than 90% similar at the amino acid level. Thus, three T3Rs are expressed which differ primarily in the A/B domain, suggesting that this region may play a role in mediating different effects of these receptors.

One of the central issues in understanding the actions of the T3Rs and other nuclear receptors is elucidation of the details by which target genes are recognized. The T3Rs and certain other members of thyroid hormone/retinoid receptor family bind to their HREs as monomers, homodimers (12–16), or as heterodimers with the RXRs (17–24). In particular, the T3Rs bind to and activate transcription from a wide variety of response elements organized as direct repeats (DR), inverted repeats (IR), or everted repeats (ER) of the optimized AGGTCA hexanucleotide half-site (25–27) and from native half-site motifs that diverge from the AGGTCA core binding sequence (28).

Recognition of specific base pairs within the half-site core binding motif is mediated by the highly conserved DNA binding

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1 The abbreviations used are: HRE(s), hormone response element(s); T3, triiodothyronine; T3R, triiodothyronine receptor; RAR, retinoic acid receptor; RXR, retinoid X receptor; h, human; r, rat; c, chicken; DR, direct repeat; IR, inverted repeat; ER, everted repeat; DBD, DNA binding domain; TFIIB, transcription factor IIB; MVM, mammatory tumor virus; TRE-p, palindromic thyroid hormone response element; CAT, chloramphenicol acetyltransferase; GH, growth hormone; LTR, long terminal repeat; Mal, malic enzyme; TRE-p, TRE-p with single G to C change in one of the half-sites; pEX, pEXPRESS; RSV, Rous sarcoma virus; GST, glutathione S-transferase; 9-cis-RA, 9-cis-retinoic acid; ROR, retinoid orphan receptor.

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domain (DBD), which defines the nuclear receptor superfamily. This highly conserved DBD contains 66–68 amino acids that are organized into two zinc finger structures that include 9 perfectly conserved cysteines followed by a carboxyl-terminal extension (29, 30). A helix in the carboxyl-terminal extension, with its extensive minor groove contacts, effectively extends the contact surface of the DBD beyond the consensus 6-base pair half-site (31). The ability of nuclear hormone receptors to distinguish among specific HREs is conferred by 3 amino acids at the base of the first zinc finger in the DBD (the P box) (32). This region is organized into an α-helix that penetrates the major groove and recognizes the specific nucleotide sequence of the HRE.

Amino acids at the base of the second zinc finger (the D box) are thought to provide a dimerization interface for protein–protein interactions on certain HREs (32). Structural studies indicate that the DBDs of certain thyroid hormone/retinoid family members form a cooperative dimerization interface, when bound to DRs but not to IREs and ERs (31). For IREs and ERs, binding of homodimers or heterodimers is thought to result from a dimerization interface located within the ligand binding domain. In addition to homodimer and heterodimer binding, the T3R also bind IREs, DRs, and other DNA configurations as monomers (14). In the absence of RXR, T3R binds more efficiently as monomers to these elements, and T3Rβ isoforms bind more efficiently as homodimers (33).

We reported previously that a 10-amino acid sequence within the A/B domain of cT3R or rT3Rα1 was essential for ligand-dependent activation of native HREs and for interaction of T3R with TFIIIB (34). Interestingly, deletion of the 50-amino acid A/B domain of cT3R markedly reduced homomer binding and increased homodimer binding of the receptor, suggesting that the A/B domain of T3R imposes preferential monomer binding of the receptor (34). In this study we show that the same 5 basic amino acids 23KKRRK27 which are necessary for efficient binding to TFIIIB are required for transcriptional activity of cT3R. These same amino acids are also responsible for imposing preferential monomer binding and influencing the efficiency of heterodimer formation with RXR. To our knowledge this is the first identification of specific NH2-terminal residues involved in the differential binding of T3R isoforms to DNA.

**EXPERIMENTAL PROCEDURES**

Plasmids—ΔMTV-TREp-CAT (14, 25), ΔMTV-TRE-GH-CAT (25), and ΔMTV-TRE-Mal-CAT (35) have been described previously. These CAT reporter genes contain a single copy of each TRE cloned into the HindIII site at −88 of ΔMTV-CAT, a mouse mammary tumor viral LTR-CAT reporter that lacks glucocorticoid response elements (25, 32). The TREp (also known as the TRE-IR) is an inverted repeat of optimized AGGTCA half-sites (AGGTCA TGACCT). TRE-GH and TRE-Mal are from the rat growth hormone (13) and rat malic enzyme genes (36), respectively, and contain direct and inverted repeats. The TREp is the same as the TREp except that it contains a single G to C change in one of the half-sites (AGGTCA to AGGTCC).

Full-length cT3Rα cDNA, corresponding to amino acids 1–408, was cloned into a pEXPRESS vector (pEX-cT3Rα) (37). pEX vectors contain the Bous sarcoma viral (BSV) LTR linked to a phage T7 RNA polymerase promoter from pET8c (38), an Asp718(PcoI)-containing Rous sarcoma viral (RSV) LTR linked to a phage T7 RNA polymerase promoter (37). pEX vectors contain the Bous sarcoma viral (BSV) LTR linked to a phage T7 RNA polymerase promoter from pET8c (38), an Asp718(PcoI)-containing Rous sarcoma viral (RSV) LTR linked to a phage T7 RNA polymerase promoter (37). pRSV-T7-cT3Rα was made by inserting an oligonucleotide containing a polylinker (5′-GACGTCGAC-3′) into the digested vector. The polylinker was designed so that the 5′-HindIII site is inactivated, but the 3′-HindIII site is regenerated upon insertion, allowing for future cloning at this site. pRSV-T7-cT3Rα (21–408) was formed by digesting pRSV-T7-cT3Rα (31–408) with HindIII and PfMI and inserting an oligonucleotide corresponding to amino acids 21–30. pRSV-T7-cT3Rα (31–408), which lacks the first 30 amino acids of cT3Rα, was constructed by removing the HindIII-PfMI fragment corresponding to amino acids 1–30 of cT3Rα from pRSV-T7-cT3Rα and inserting an oligonucleotide containing a polylinker (5′-HindIII-PstI-3′) into the digested vector. The PstI site in this and other pRSV-T7-cT3Rα mutants contains the ATG initiation codon. pEX-cT3Rα (51–408), lacking the entire A/B region, was constructed using PCR and cloned into pET8c (38).

**Fig. 1. Maximal transcriptional activation of native TREs by cT3Rα requires amino acids 23KKRRK27.** Panel A, HeLa cells were transfected by electroporation with 5 μg of a reporter containing an idealized TREp inverted repeat (AGGTCA TGACCT) inserted at position −88 of ΔMTV-CAT (ΔMTV-TREp-CAT) (14). Cells were cotransfected with 2 μg of either cT3Rα (21–408) or cT3Rα (21–408, 7/8) expression vector and incubated with or without T3 (1 μM) for 48 h. Panel B, same as in panel A except that the reporter was ΔMTV-TRE-GH-CAT, which contains the native TRE from the rat growth hormone gene promoter. Panel C, HeLa cells were transfected with 5 μg of reporter ΔMTV-TRE-Mal-CAT containing the native TRE from the rat malic enzyme gene promoter (35). Cells were cotransfected with 0.75 μg of either cT3Rα (21–408) or cT3Rα (21–408, 7/8) expression vector and incubated with or without T3 (1 μM) for 48 h.
from pEX-cT3R(51–157) and pEX-cT3R(21–408). DNA corresponding to amino acids 119–408 was excised from pEX-cT3R(21–408)53 and subcloned into pEX-cT3R(51–157) after digestion of the vector with MscI, which cleaves at codon 118, and Asp718, which cleaves just after codon 157. pEX-cT3R(51–157) was constructed by polymerase chain reaction of wild-type cT3R using appropriate primers. pRSV-T7-cT3R(21–408, 7/8) was constructed by cleaving pRSV-T7-cT3R(21–408, 7/8) with HindIII and PflI and replacing the DNA corresponding to amino acids 21–30 with an oligonucleotide that changed amino acids 23KRKRK27 to 23TIKIT27. pEX-TPFII was made from human TFIIB in pET11d (39). The TFIIB cDNA was cloned into pGEX2T (40). This plasmid was constructed in the same way using an oligonucleotide that changed amino acids 119–408 into the NcoI and MscI sites of a pEX vector that contained a 50-fold excess of cT3R(21–408, 7/8) vector (lanes 1–3). No binding to the TREp was detected with 0.5, 1.5, or 4.5 μl of control lysate (lanes 1–3, respectively). No homodimer binding was detected with 0.5, 1.5, or 4.5 μl of cT3R(51–408)-expressing lysate (lanes 4–6, respectively). Monomer binding was detected using 1.5 μl of cT3R(21–408, 7/8) and cT3R(51–408)–expressing lysates (lanes 8 and 11, respectively), and both monomer and homodimer binding were detected using 4.5 μl of the same lysates (lanes 9 and 12, respectively). Panel B, no homodimer binding was detected with 1.5, 3.0, or 4.5 μl of reticulocyte lysate expressing cT3R(21–408) (lanes 1–3, respectively). Monomer binding was detected using 1.5 μl of lysate expressing cT3R(21–408, 7/8) (lane 4), and both monomer and homodimer binding were detected using 3.0 and 4.5 μl of the same lysate (lanes 5 and 6, respectively).

FIG. 2. Residues 23KRKRK27 affect DNA binding of cT3R. Wild-type cT3R, cT3R(21–408), cT3R(21–408, 7/8), and cT3R(51–408) were expressed by in vitro translation in reticulocyte lysates and incubated with 5 fmol (30,000 dpm) of the 3P-labeled TREp element. The conditions for gel mobility shift assays are described under “Experimental Procedures” (24, 34). The gels were then dried and autoradiographed. The assignment of receptor monomers (M) and homodimers (D) is based on the mobility of purified cT3R(14). Panel A, no binding to the TREp was detected using 0.5, 1.5, or 4.5 μl of control lysate (lanes 1–3, respectively). No homodimer binding was detected with 0.5, 1.5, or 4.5 μl of cT3R-expressing reticulocyte lysate (lanes 4–6, respectively). Monomer binding was detected using 1.5 μl of cT3R(21–408, 7/8) and cT3R(51–408)–expressing lysates (lanes 8 and 11, respectively), and both monomer and homodimer binding were detected using 4.5 μl of the same lysates (lanes 9 and 12, respectively). Panel B, no homodimer binding was detected with 1.5, 3.0, or 4.5 μl of reticulocyte lysate expressing cT3R(21–408) (lanes 1–3, respectively). Monomer binding was detected using 1.5 μl of lysate expressing cT3R(21–408, 7/8) (lane 4), and both monomer and homodimer binding were detected using 3.0 and 4.5 μl of the same lysate (lanes 5 and 6, respectively).
RESULTS

Residues $^{23}$KRRK$^{27}$ of the NH$_2$-terminal A/B Domain of cT$\beta$R Are Necessary for Maximal Transcriptional Activation of Native TRES—Our previous studies aimed at elucidating the functional role of the NH$_2$-terminal A/B region of cT$\beta$R indicated that amino acids 21–30 of cT$\beta$R, which are also conserved in the NH$_2$-terminal A/B domains of hT$\beta$R1 and hT$\beta$R1, are essential for both transcriptional activation and for efficient binding to TFIIB (34). More detailed in vitro binding studies revealed that residues $^{23}$KRRK$^{27}$ centered within amino acids 21–30 are required for efficient binding of cT$\beta$R with TFIIB (34). This suggests that the functional activity that we originally mapped to amino acids 21–30 may depend solely on these 5 basic residues. To test this possibility we compared the functional activities of cT$\beta$R(21–408) and cT$\beta$R(21–408, 7/8) in which KRRK$^{27}$ was changed to TITIT$^{27}$. In transfection experiments with a reporter gene regulated by a single TRE, cT$\beta$R(21–408) binds preferentially as a homodimer $^{(1–408)}$, which are also conserved in the NH$_2$-terminal A/B domain of the cT$\beta$R(21–408, 7/8) showed similar activity (Fig. 1A). In contrast, with $\Delta$MTV-TRE-GH-CAT or $\Delta$MTV-TRE-Mal-CAT, which contain native TREs, cT$\beta$R(21–408) was much more active (Fig. 1C). These results are similar to our previous findings that showed that the functional effect of the NH$_2$ terminus is much more prominent on reporters containing lower affinity native TREs compared with reporters containing the idealized higher affinity TREp (34). Newly, coexpression of TFIIB, although enhancing the activity of both receptors, results in a much higher level of T$_3$-dependent stimulation by cT$\beta$R(21–408) (Fig. 1C). The ability of TFIIB to enhance the activity of cT$\beta$R(21–408, 7/8) partially is not altogether unexpected and is similar to our previous results with cT$\beta$R(51–408). This most likely stems from the low affinity of amino acids 119–154 of the cT$\beta$R D region for TFIIB (34).

Residues $^{23}$KRRK$^{27}$ Affect DNA Binding of cT$\beta$R as Monomers and Homodimers—The NH$_2$ terminus of T$_3$R isoforms may confer cell type and promoter specificity not just through divergent, isotype-distinct, interactions with other transcription factors (34, 44) but also through conferring distinct DNA binding properties to receptor isotypes (45, 46). For example, the DNA binding properties of v-erbA differ from those of c-erbA (cT$\beta$R) and more closely resemble those of the RARs. The structural basis behind this difference in DNA recognition by v-erbA results from one or more changes within the v-erbA NH$_2$-terminal domain (45, 47).

In a previous study we found that in the absence of RXR, cT$\beta$R binds to the TREp predominantly as a monomer, whereas cT$\beta$R(51–408) binds preferentially as a homodimer (34). Similar results were included in a study by Wong and Privalsky (47) but were not discussed further. This finding suggests that, in addition to the DBD, all or part of the NH$_2$-terminal A/B domain may affect the DNA binding properties of cT$\beta$R. We also found that without RXR, cT$\beta$R(21–408) binds the native rat TRE-GH as a monomer, whereas cT$\beta$R(51–408) binds this element poorly as a monomer (34). To determine whether this different DNA binding of NH$_2$-terminal cT$\beta$R mutants is influenced by the basic amino acid residues KRRK$^{27}$, we compared the binding of cT$\beta$R(1–408), cT$\beta$R(51–408), and cT$\beta$R(21–408, 7/8) to the TREp. As shown in Fig. 2A, both cT$\beta$R(21–408, 7/8) (lanes 7–9) and cT$\beta$R(51–408) (lanes 10–12) bind much more efficiently as homodimers when compared with wild-type cT$\beta$R(1–408) (lanes 4–6). That this difference results from the KRRK$^{27}$ sequence is shown...
by comparing the DNA binding of cT3Rα (21–408) and cT3Rα (21–408, 7/8) (Fig. 2B, lanes 1–3 and 4–6, respectively). Increasing amounts of cT3Rα (21–408) result in an increase in monomer binding, whereas cT3Rα (21–408, 7/8) binds as a homodimer even at very low concentrations. Therefore, the basic amino acid sequence 23KRKRK27 affects not only receptor transactivation potential and its binding to TFIIB but its DNA binding properties as well.

**T3 Inhibits Both Monomer and Homodimer DNA Binding of cT3Rα NH2-terminal Mutants**—The preferential binding of cT3Rα (21–408, 7/8) to the TEP as a homodimer compared with the predominant monomeric binding of cT3Rα (21–408) or wild-type cT3Rα may result from an increased potential to homodimerize and/or a decreased potential to bind DNA as a monomer. This latter instance might result in an increased amount of this mutant receptor available to bind as a homodimer. To assess effects of the 23KRKRK27 sequence in the NH2 terminus on monomer binding, we compared the binding of cT3Rα (21–408) and cT3Rα (21–408, 7/8) with a TRE1/2 in the absence or presence of T3 (Fig. 4). The TRE1/2 is the same as the TEP except that it contains a single G to C change in one of the half-sites (AGGTCA to ACGTCA). Whereas cT3Rα (21–408) binds to the TEP, cT3Rα (21–408, 7/8) and cT3Rα (51–408) bound as homodimers and somewhat less efficiently as monomers (lanes 5 and 7). Surprisingly (lanes 6 and 8), T3 almost completely eliminates monomer and homodimer DNA binding of cT3Rα (21–408, 7/8) and cT3Rα (51–408) in the absence or presence of T3. As shown in Fig. 3, cT3Rα (1–408) and cT3Rα (21–408) in the absence of T3 bind the TEP predominantly as monomers (lanes 1 and 3). As expected, T3 increased the electrophoretic mobility of these complexes without altering their apparent stability (lanes 2 and 4). Thus, deletion of the first 20 amino acids from the A/B domain does not affect receptor DNA binding in either the absence or presence of T3.

**NH2-terminal Mutants of cT3Rα Bind Poorly to a TRE1/2**—The preferential binding of cT3Rα (21–408, 7/8) or cT3Rα (51–408) to the TEP as a homodimer compared with the predominant monomeric binding of cT3Rα (21–408) or wild-type cT3Rα may result from an increased potential to homodimerize and/or a decreased potential to bind DNA as a monomer. This latter instance might result in an increased amount of this mutant receptor available to bind as a homodimer. To assess effects of the 23KRKRK27 sequence in the NH2 terminus on monomer binding, we compared the binding of cT3Rα (21–408) and cT3Rα (21–408, 7/8) with a TRE1/2 in the absence or presence of T3 (Fig. 4). The TRE1/2 is the same as the TEP except that it contains a single G to C change in one of the half-sites (AGGTCA to ACGTCA). Whereas cT3Rα (21–408) binds to the
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The DNA Binding Properties of cT3R, cT3R, and cT3R were not directly related to the Number of Basic Residues Contained within cT3R.

To assess whether all or some of the basic residues within cT3R are necessary for preferential monomer binding of wild-type cT3R, we performed gel mobility shift assays with NH2-terminal mutants containing different numbers and combinations of basic and neutral amino acid residues. As shown in Fig. 5A, wild-type cT3R and cT3R bind the TREp predominantly as monomers (lanes 1 and 3), whereas cT3R (51–408) and cT3R (21–408, 7/8) bind predominantly as homodimers (lanes 2 and 4). Interestingly, mutant cT3R (21–408, 9/10), which has only the first 3 basic amino acid residues changed (23KRRK27), binds the TREp very poorly as a monomer and not at all as homodimer (lane 5). In contrast, mutant cT3R (21–408, 11/12, lane 6), which has the last 3 basic amino acid residues changed (23KRTIK27), binds the TREp almost as efficiently as cT3R (21–408, 7/8) or cT3R (51–408) with slightly more monomer than homodimer. Finally, mutant cT3R (21–408, 13/14) (lane 7), which has only the middle basic amino acid preserved (23KTIT27), binds in a way similar to cT3R (21–408, 11/12) but less efficiently. These data indicate that the affinity and mode of binding of these mutants to the TREp are not related directly to the number of the basic amino acid residues within amino acids 21–30. However, all 5 basic amino acid residues 23KRRKR27 are necessary for predominant high affinity monomer binding to TREp.

Fig. 6. Heterodimer formation between RXR and cT3R. cT3R (21–408) and cT3R (21–408) were expressed by in vitro translation in reticulocyte lysates and incubated with 5 fmol (30,000 dpm) of the 32P-labeled TREp element. Weak homodimer (D) and predominant monomer (M) binding of cT3R (21–408) in the absence of T3 (lane 1) was altered to exclusive monomer binding by 1 μM T3 (lane 2). In the presence of baculovirus-expressed mRXRβ (5 ng), cT3R (21–408) formed monomer and heterodimer (HD) complexes (lane 3), which were not altered significantly by either T3 (lane 4) or 9-cis-RA (lane 5) or both (lane 6). Predominant homodimer binding of cT3R (21–408, 7/8) in the absence of T3 (lane 7) was abolished by T3 (lane 8). In the presence of mRXRβ, cT3R (21–408, 7/8) formed almost exclusively heterodimers (lane 9). T3 effected the complete disappearance of the weak homodimer complex without altering the apparent stability of the heterodimer complex (lane 10). 9-cis-RA alone did not affect either heterodimer or weak homodimer complexes (lane 11). Binding in the presence of both T3 and 9-cis-RA was no different from binding in the presence of T3 alone (compare lane 12 with lane 10, respectively).

Amino acids 23KRRK27 affect the Efficiency of T3-RXR Heterodimer Formation—Although amino acids 23KRRK27 influence monomer binding of receptor to DNA, these residues may also affect the ability of the receptor to dimerize. We examined this possibility by assessing the ability of cT3R (21–408) or cT3R (21–408, 7/8) to bind as heterodimers with RXR on the TREp (Fig. 6). Incubations were performed either in the absence or presence of T3 (lanes 1 and 2). As shown previously, cT3R (21–408) alone binds to the TREp predominantly as a monomer, and the electrophoretic mobility of this complex is increased by T3 (Fig. 6, lanes 1 and 2). In contrast, cT3R (21–408, 7/8) binds the TREp predominantly as a homodimer, and this complex is abolished by T3 (lanes 7 and 8). Interestingly, in the absence of T3, cT3R (21–408) forms heterodimeric complexes with RXR less efficiently than cT3R (21–408, 7/8) (lanes 3 and 9), and T3 increases the electrophoretic mobility of both complexes (lanes 4 and 10). 9-cis-RA alone does not affect either complex (lanes 5 and 11), whereas the combination of T3 and 9-cis-RA affects the mobility of both complexes as with T3 alone (lanes 6 and 12). Similar results were also found using a 32P-DR-4 containing AGGTCA half-sites instead of the TREp (data not shown). Importantly, these results indicate that amino acids 23KRRK27 affect both monomer DNA binding and dimerization.
Amino Acids 23KRKRK27 of the A/B Domain Are Required for Maximal Transcriptional Activation of Native TREs by cT3R—A conserved sequence of 10 amino acids from NH2-terminal A/B domain of cT3R, cT3Rα, and hT3Rα1 is essential both for transcriptional activation and for efficient binding to TFIIB (34). Within this sequence, 5 basic amino acid residues, 23KRKRK27, proved absolutely necessary for efficient receptor binding to TFIIB (34). A sequence similar to the

DISCUSSION

Amino Acids 23KRKRK27 of the A/B Domain Are Required for Maximal Transcriptional Activation of Native TREs by cT3Rα—A conserved sequence of 10 amino acids from NH2-terminal A/B domain of cT3Rα, cT3Rα1, and hT3Rα1 is essential both for transcriptional activation and for efficient binding to TFIIB (34). Within this sequence, 5 basic amino acid residues, 23KRKRK27, proved absolutely necessary for efficient receptor binding to TFIIB (34). A sequence similar to the

The results of Fig. 7B imply that the observed difference in the efficiency of heterodimer binding to the TREp results from an intrinsic difference in the formation rate and/or DNA binding of these complexes. To provide evidence for enhanced formation rate and/or DNA binding, we studied the binding of 35S-labeled cT3R to TREp (1,000 × molar excess) was added for different periods of time as indicated. Baculovirus-expressed mRXRβ (5 ng) was included as indicated. Panel B, heterodimeric complexes shown in panel A were quantitated with a Molecular Dynamics PhosphorImager using ImageQuant software and are expressed as arbitrary (relative) units.

The relative stability of various DNA-bound cT3Rα complexes was determined by autoradiography to ensure that equal amounts of input radioactivity of the labeled proteins were used.

The NH2-terminal 23KRKRK27 motif influences the formation of cT3Rα/RXR heterodimers in solution. Fig. 8, 25,000 dpm of 35S-labeled T3Rα(21–408) and cT3Rα(21–408, 7/8) were incubated with glutathione-agarose bound GST-RXR or GST in 300 μl of Buffer A (34) for 1 h at 4 °C as described under “Experimental Procedures.” Beads were collected by centrifugation at 4 °C for 5 min at 500 × g and washed three times with 1 ml of Buffer A. The bound proteins were eluted with SDS gel loading buffer and analyzed by SDS-gel electrophoresis followed by autoradiography. 35S-Labeled wild-type or mutant proteins in the binding assays were analyzed by electrophoresis and autoradiography to ensure that equal amounts of input radioactivity of the labeled proteins were used.

Previous studies from our laboratory indicated that the NH2-terminal region of T3Rα1 does not alter its ability to interact with TFIIB or to activate the rat TRE-GH or TRE-Mal in 3MTV-CAT (data not shown). However, all nuclear receptors contain a conserved basic sequence that follows their DBD, which appears to play a role in TFIIB binding (34).

In this study we show that the functional role of the A/B domain in ligand-dependent transcriptional activation by cT3Rα requires the 5 basic amino acid residues 23KRKRK27. This was determined by directly comparing the activities of cT3Rα(21–408) and cT3Rα(21–408, 7/8) in which all 5 basic residues 23KRKRK27 were changed to TIKIT27. Like cT3Rα(51–408), which lacks the entire NH2-terminal A/B domain, cT3Rα(21–408, 7/8) was only slightly less active than cT3Rα(21–408) in transient transfection experiments with a reporter gene containing a single idealized TRE organized as an inverted repeat (3MTV-TREp-CAT, 34, and this study, Fig. 8A). However, in transfection experiments where native TREs from the rat growth hormone or malic enzyme gene promoters were used, cT3Rα(21–408, 7/8) was much less active in the presence or absence of TFIIB (Fig. 1, B and C). Indeed, the requirement for amino acids 21–30 of the A/B domain for optimal transcriptional activation of native TREs appears to depend solely on amino acids 23KRKRK27, cT3Rα(21–408, 13/14), where 23KRKRK27 was changed to TIKIT27, was somewhat more active than cT3Rα(21–408, 7/8) but much less active than wild-type cT3Rα(1–408). The other 23KRKRK27 mutants were closer in activity to cT3Rα(1–408) (not shown) even though their affinity for TFIIB is reduced (34). This probably reflects an integrative effect of the lower affinity of the mutants for TFIIB (34), and as we show in this study, their increased ability to bind to response elements as homodimers and as heterodimers with RXR.

Amino Acids 23KRKRK27 Affect DNA Binding of cT3Rα as Monomers and Homodimers—Previous studies from our laboratory indicated that the NH2-terminal region of cT3Rα might affect DNA binding of receptor to the TREp and the native TRE from the rat growth hormone gene promoter by altering the extent of monomer binding and dimerization (34). Hollenberg et al. (51) also found that the NH2 terminus of hT3Rα1 reduced the ability of the receptor to dimerize. Our current study documents that the basic amino acid sequence 23KRKRK27 plays a key role in both DNA binding and dimerization of cT3Rα.
addition to its role in transcriptional activation and TFIIB binding. First, both cT3R(21–408, 7/8), in which amino acids 23KKRRK27 were changed to 23TTITTT27, and cT3R(51–408) bind to the TREp more efficiently as homodimers than cT3Rα (Fig. 2A). More direct evidence for the involvement of these residues in the DNA binding of receptor is provided by the finding that cT3R(21–408) binds predominantly as a monomer even at high concentrations, whereas cT3Rα(21–408, 7/8) binds efficiently as a homodimer even at low concentrations (Fig. 2A). We obtained results in gel shift studies using a DR+4 containing AAGGCTA half-sites similar to those with the TREp (not shown). Interestingly, although the binding of cT3Rα and cT3Rα(21–408) to the TREp is not affected significantly by T3, the binding of cT3Rα(21–408, 7/8) and cT3Rα(51–408) to this element is inhibited strongly (Fig. 3). Hence, residues 23KKRRK27 may either stabilize a “proper” conformation of the DBD, or they may ensure optimal positioning of the DBD with respect to the TREp half-site. These alternative mechanisms may occur either through a direct interaction between amino acids 23KKRRK27 and the DBD or through interaction of these basic residues with some other region of the receptor such as the ligand binding domain, which may affect the structure of the DBD. Alternatively, residues 23KKRRK27 may affect both the integrity and positioning of the DBD and in addition may contact DNA directly. Irrespective of the actual mechanism by which amino acids 23KKRRK27 affect receptor DNA binding, they seem absolutely necessary in the presence of T3 for the receptor to bind to the TREp (Fig. 3).

The predominant DNA binding of cT3Rα(21–408, 7/8) as a homodimer may reflect decreased monomer binding potential, increased homodimerization potential, or a combination of both. DNA binding studies using the TREα to preclude homodimer binding suggest that amino acids 23KKRRK27 affect the ability of receptor to bind DNA as a monomer (Fig. 4). Although cT3Rα(21–408, 7/8) does not bind significantly to the TREα, it does bind to the TREp as a monomer (e.g. compare Fig. 4, lane 3, Fig. 5C, lane 7 with Fig. 2A, lane 8, Fig. 2B, lane 4, Fig. 3, lane 5). One possible explanation for this finding would be that to bind to DNA cT3Rα(21–408, 7/8) has to make an initial contact exclusively as a homodimer. Once this homodimer-DNA contact is established one cT3Rα(21–408, 7/8) molecule could dissociate, leaving a relatively unstable monomer-DNA complex behind.

DNA Binding of cT3Rα as Monomers and Homodimers Is Influenced by Different Basic Amino Acids within the 23KKRRK27 Sequence—The basic residues 23KKRRK27 are not equally important for preferential monomer binding of wild-type α-receptor. For example, cT3Rα(21–408, 9/10), which has amino acids 23TITTRK27, binds to the TREp very poorly and only as a monomer (Fig. 5). In contrast, cT3Rα(21–408, 11/12), which has amino acids 23KRTIT27, binds to the TREp in a way similar to that of cT3Rα(51–408) with slightly more monomer than homodimer. Finally, cT3Rα(21–408, 13/14), which has amino acids 23KTTIK27, binds to the TREp in a way similar to that of cT3Rα(21–408, 11/12) but less efficiently. Hence, the affinity and the mode of binding of these mutants to the TREp are not related directly to the number of the basic amino acid residues within the sequence 23–27, and therefore these residues do not contribute equally to the receptor DNA binding. However, all 5 basic amino acid residues 23KKRRK27 are necessary for predominant high affinity monomer binding to TREp. In contrast, the affinity of cT3Rα for TFIIB does correlate directly with the number of these basic amino acids (data not shown).

The NH2-terminal 23KKRRK27 Sequence Influences the Extent of cT3RαRXR Heterodimer Formation—In addition to the effect of residues 23KKRRK27 on the binding of receptor monomers to DNA, these residues also influence the intrinsic dimerization potential of cT3Rα. Fig. 6 indicates that cT3Rα(21–408, 7/8) binds as a heterodimer with RXR to the TREp element much more efficiently than cT3Rα(21–408). Thus, ~90% of the total TREp-bound cT3Rα(21–408, 7/8) is bound as a heterodimer with RXR, whereas only ~20% of cT3Rα(21–408) participates in such complexes (Fig. 7A, lanes 2 and 7). Both complexes showed similar dissociation rates in the presence of a 1,000-fold excess of unlabeled TREp (Fig. 7A, lanes 2–5 and 7–10, and Fig. 7B), suggesting that the increased amount of heterodimers found with the cT3Rα mutant results from the more efficient formation and/or binding cT3Rα(21–408, 7/8)/RXR heterodimers to DNA. GST binding studies (Fig. 8) suggest that this increase results from the more efficient formation of heterodimers in solution, which then bind to DNA. The increased dimerization potential of the cT3Rα(21–408, 7/8) on the TREp does not result in enhanced activation of the ΔMTV-TREp-CAT reporter compared with cT3Rα(1–408) or cT3Rα(21–408) (Fig. 1A). This discrepancy may reflect the weaker interaction of cT3Rα(21–408, 7/8) with TFIIB, which could result in a number of transcriptionally active cT3Rα(21–408, 7/8)/RXR/TFIIB complexes on the optimized element similar to those with cT3Rα(21–408) or wild-type cT3Rα(1–408).

Influence of the NH2-terminal A/B Domain on DNA Binding of Other Members of the Thyroid Hormone/Retinoid Receptor Subfamily—Several additional reports have provided evidence for involvement of the NH2-termini of related members of the nuclear hormone receptor family in DNA binding. For example, hT3Rα1 amino acids of v-erbA, His12 and Cys32 (which correspond to Arg24 and Tyr44 of cT3Rα) have been shown, in conjunction with amino acid changes in the zinc finger domain, to contribute to a restricted half-site DNA binding specificity (45, 57). RXRα and RXRγ, but not RXRβ, have been suggested to activate transcription by forming tetrameric complexes on DNA elements consisting of four reiterated weak half-sites (53). These isoform-specific DNA binding properties mapped to the NH2-terminal A/B domains. Replacing the RXRβ A/B domain with that of RXRγ resulted in both tetramer binding to DNA and transcriptional activation by the chimeric protein. That the NH2-terminal domain and the zinc finger region of nuclear hormone receptors may functionally cooperate was also shown by a study of the DNA binding properties of the ORs (46, 54). Thus, the differential DNA binding activities of ORα1, ORα2, and ORα3 depend on their distinct NH2 termini, which, when fused to heterologous nuclear hormone receptors (e.g. hT3Rβ1), may impose novel DNA binding specificities. Finally, T3Rα and T3Rβ bind differently to the same DNA element (33). Whereas T3Rα binds predominantly as a monomer to the TREp (14), T3Rβ1 binds predominantly as a homodimer, which is thought to result in part from its increased ability to dimerize (33, 51, 55). Importantly, T3Rβ1 and T3Rα show no homology in their NH2-terminal A/B domains. In particular, T3Rβ1 does not contain a sequence in its A/B domain similar to the T3Rα-specific KRKRK, which may account for the finding that mutant cT3Rα(21–408, 7/8) exhibits certain DNA binding properties more like T3Rβ1 than wild-type cT3Rα.

In conclusion, the influence of the NH2-terminus of T3Rα on transcriptional activation, dimerization, and DNA and TFIIB binding supports the idea that this domain may play a role in the selective regulation of specific genes and impart distinct context-dependent transactivation potential to the individual receptor isoforms. The three-dimensional structural studies of the T3Rs performed thus far in either the absence (56) or in the presence of DNA (31) have not included the NH2-terminal A/B.
domain. The marked effect of the KRKKR residues of TαRs on both DNA binding and transactivation suggests important effects of this sequence on receptor structure. Although this interaction may involve the DBD, our finding that mutation of the KRKKR residues alters the effect of ligand on DNA binding also suggests an interaction between the NH2-terminus and the ligand binding domain (Figs. 3 and 5). Thus, structural studies that analyze homo- or heterodimerization or other DNA binding ligand binding domain (Figs. 3 and 5). Thus, structural studies in gene regulation and development.

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REFERENCES

1. Evans, R. M. (1988) Science 240, 889–895
2. Forman, B. M., and Samuels, H. H. (1990) Mol. Endocrinol. 4, 1293–1301
3. Lazar, M. A. (1993) Endocr. Rev. 14, 184–193
4. Giguere, V. (1984) Endocr. Rev. 15, 61–79
5. Glass, C. K. (1994) Endocr. Rev. 15, 391–407
6. Thompson, C. C., Weinberger, C., Lebo, R., and Evans, R. M. (1987) Science 237, 1610–1614
7. Lazar, M. A., Hodin, R. A., Darling, D. S., and Chin, W. W. (1988) Mol. Endocrinol. 2, 893–901
8. Lazar, M. A., Hodin, R. A., and Chin, W. W. (1989) Proc. Natl. Acad. Sci. U.S.A. 86, 7771–7774
9. Sap, J., Munoz, A., Damm, K., Goldberg, Y., Ghysdael, J., Leutz, A., Beug, H., and Vennstrom, B. (1988) Nature 334, 635–640
10. Weinberger, C., Thompson, C. C., Ong, E. S., Lebo, R., Gruij, D. J., and Evans, R. M. (1986) Nature 324, 641–646
11. Hodin, R., Lazar, M. A., Wintman, B. I., Darling, D. S., Koenig, R. J., Larsen, P. R., Moore, D. D., and Chin, W. W. (1989) Science 244, 76–79
12. Selmi, S., and Samuels, H. H. (1991) J. Biol. Chem. 266, 11589–11598
13. Brent, G. A., Williams, G. R., Harney, J. W., Forman, B. M., Samuels, H. H., Moore, D. D., and Larsen, P. R. (1992) Mol. Endocrinol. 6, 502–514
14. Forman, B. M., Casanova, J., Raaka, B. M., Ghysdael, J., and Samuels, H. H. (1992) Mol. Endocrinol. 6, 429–442
15. Ribeiro, R. C., Kushner, P. J., Apriletti, J. W., West, B. L., and Baxter, J. D. (1992) Mol. Endocrinol. 6, 1142–1152
16. Wahlestrom, G. M., Sjoberg, M., Andersson, M., Nordstrom, K., and Vennstrom, B. (1992) Mol. Endocrinol. 6, 1013–1022
17. Yu, V. C., Delseth, C., Andersen, B., Holloway, J. M., Devary, O. V., Naar, A. M., Kim, S. Y., Boutin, J.-M., Glass, C. K., and Rosenfeld, M. G. (1991) Cell 67, 1251–1260
18. Berrodis, T. J., Marks, M. S., Ozato, K., Linney, E., and Lazar, M. A. (1992) Mol. Endocrinol. 6, 1468–1478
19. Buggs, T. H., Pohl, J., Lonny, O., and Stunnenberg, H. G. (1992) EMBO J. 11, 1409–1418
20. Kliever, S. A., Umesono, K., Mangelsdorf, D. J., and Evans, R. M. (1992) Nature 355, 446–449
21. Leid, M., Kastner, P., Lyons, R., Nakahori, H., Saunders, M., Zacharewski, T., Chen, J.-Y., Taub, A., Garnier, J.-M., Mader, S., and Chambon, P. (1992) Cell 68, 377–385
22. Marks, M. S., Hallenbeck, P. L., Nagata, T., Segars, J. H., Appella, E., Nikodem, V. M., and Ozato, K. (1992) EMBO J. 11, 1419–1435