Capacity for Cooperative Binding of Thyroid Hormone (T3) Receptor Dimers Defines Wild Type T3 Response Elements

G. A. Brent*, G. R. Williams†, J. W. Harney, B. M. Forman‡, H. H. Samuels, D. D. Moore, and P. R. Larsen

Thyroid Division (G.A.B., G.R.W., J.W.H., P.R.L.) and Howard Hughes Medical Institute (G.R.W., J.W.H., P.R.L.)
Brigham and Women's Hospital
Boston, Massachusetts 02115

Department of Molecular Biology (D.D.M.)
Massachusetts General Hospital
Boston, Massachusetts 02114

Department of Medicine (G.A.B., G.R.W., P.R.L.)
Department of Genetics (D.D.M.)
Harvard Medical School
Boston, Massachusetts 02115

Division of Molecular Endocrinology
Department of Pharmacology (B.M.F., H.H.S.) and Department of Medicine (H.H.S.)
New York University Medical Center
New York, New York 10016

Downloaded from https://academic.oup.com/mend/article/6/4/502/2714532 by guest on 16 August 2022

Thyroid hormone response elements (T3REs) have been identified in a variety of promoters including those directing expression of rat GH (rGH), α-myosin heavy chain (rMHC), and malic enzyme (rME). A detailed biochemical and genetic analysis of the rGH element has shown that it consists of three hexamers related to the consensus [(A/G)GGT(C/A)A]. We have extended this analysis to the rMHC and rME elements. Binding of highly purified thyroid hormone receptor (T3R) to T3REs was determined using the gel shift assay, and thyroid hormone (T3) induction was measured in transient transfections. We show that the wild type version of each of the three elements binds T3R dimers cooperatively. Mutational analysis of the rMHC and rME elements identified domains important for binding T3R dimers and allowed a direct determination of the relationship between T3R binding and function. In each element two hexamers are required for dimer binding, and mutations that interfere with dimer formation significantly reduce T3 induction. Similar to the rGH element, the rMHC T3RE contains three hexameric domains arranged as a direct repeat followed by an inverted copy, although the third domain is weaker than in rGH. All three are required for full function and T3R binding. The rME T3RE is a two-hexamer direct repeat T3RE, which also binds T3R monomer and dimer. Across a series of mutant elements, there was a strong correlation between dimer binding *in vitro* and function *in vivo* for rMHC ($r = 0.99$, $P < 0.01$) and rME ($r = 0.67$, $P < 0.05$) T3REs. Our results demonstrate a similar pattern of T3R dimer binding to a diverse array of hexameric sequences and arrangements in three wild type T3REs. Addition of nuclear protein enhanced T3R binding but did not alter the specificity of binding to wild type or mutant elements. Binding of purified T3R to T3REs was highly correlated with function, both with and without the addition of nuclear protein. T3R dimer formation is the common feature which defines the capacity of these elements to confer T3 induction. (Molecular Endocrinology 6: 502–514, 1992)

0888-8809/92/0502–0514$03.00/0
Molecular Endocrinology
Copyright © 1992 by The Endocrine Society

INTRODUCTION

Thyroid hormone (T3) regulates a wide range of physiological processes as a consequence, at least in part, of the direct effect of the T3 receptor (T3R) on gene expression (1). Investigation of promoter elements which bind T3R and confer T3 responsiveness to target genes has yielded a diverse family of sequences (2).

These wild type T3 response elements (T3REs) generally consist of multiple copies of a hexameric sequence related to a consensus (A/G)GGT(C/A)A arranged as direct and inverted repeats. A synthetic element consisting of two hexamers in a palindromic arrangement, similar to those described for estrogen, progesterone, and glucocorticoids (3), also functions as a T3RE and has been called TREpal (4).

We originally described the rat GH (rGH) T3RE as a direct repeat of a hexameric sequence with a 4 base pair (bp) gap based on footprint analysis (5). The direct repeats were termed the A and B domains (6). Subsequent functional analyses demonstrated that the rGH T3RE consists of three domains which match the hexameric consensus, with a palindrome added to the direct repeat (7). All three of these hexamers, termed the A, B, and C domains, are required for full T3 response (7).

The availability of highly purified T3R has allowed detailed analyses of T3R binding to the rGH T3RE. T3R can bind cooperatively as a dimer to either the direct repeats A and B or the palindromic B and C domains of the wild type rGH T3RE (8). Increases and decreases in binding are highly correlated with T3 response for both up and down mutations. Mutations which increase T3 induction or conditions of high T3R concentration result in binding of T3R to all three hexamers.

Based on the sequence and arrangements of the rGH hexamers, we proposed tentative models for the arrangements of T3 response elements from a number of T3-regulated promoters (2, 7). The α-myosin heavy chain (αMHC) promoter region has been cloned in rat (9) and human (10), and the region which confers T3 responsiveness has been identified (11, 12). The sequence contains three hexamers with a similar orientation to the rGH T3RE (7). Our assignment of the direct repeat hexamers of the MHC T3RE was recently confirmed by limited mutational analysis (13), although the third inverted domain was not included in that study. A strong T3 response element has been identified in the rat malic enzyme gene 5′-flanking region (14, 15) with several hexamers matching those in the rGH T3RE. T3REs have been identified in a variety of other genes including chicken lysozyme (16), the third intron of rGH (17), and the murine leukemia virus long terminal repeat (18). These elements all contain hexamers which closely match the consensus sequence, but which are variable in number and arrangement.

Analysis of T3REs has been limited by the availability of a highly purified, active T3R preparation to allow binding analysis independent of accessory proteins. The variation in hexamer number, orientation, and spacing in wild type T3REs has made it difficult to identify a unifying property of elements which bind T3R and confer T3 responsiveness. T3R binding to the rGH T3RE demonstrates surprising flexibility in interactions with various hexamer arrangements which may generalize to other T3REs. In the current study, we have analyzed wild type T3REs based on the rGH hexamer domain pattern, to determine common features which are responsible for conferring T3 responsiveness. Binding of highly purified T3R, overexpressed in *Escherichia coli*, to T3REs was determined by gel mobility shift assay, and T3 response was measured in transient transfections. Our results demonstrate that, despite a diversity of T3RE hexameric sequence and arrangement, there is a similar pattern of T3R dimer binding. The capacity for a T3RE to bind T3R dimers was highly correlated with function, whether or not nuclear proteins were present, although addition of nuclear protein enhanced T3R binding. Two hexamers are required for dimer formation, and the capacity to form dimers represents the common feature which defines the capacity of these elements to confer T3 induction.

RESULTS

Rat αMHC T3RE Contains Three Hexameric Binding Domains

The sequence of elements studied are shown in comparison to the rGH T3RE hexamer pattern in Fig. 1. The T3REs contain two or three hexamers arranged as a direct repeat with a 4-bp gap, a palindrome with a 1- or 2-bp gap, or a combination of arrangements. Purified T3R, overexpressed in *E. coli*, was incubated with the wild type rMHC T3RE (−130 to −159; Fig. 2A), and binding was analyzed in a gel shift assay. Two specifically retarded bands were observed. The faster migrating band represents binding of monomer and the more slowly migrating upper band represents dimer binding (8, 19). Mutations were designed based on the similarity of the rMHC sequence to those described for the rGH T3RE and the domains labeled in a similar fashion.

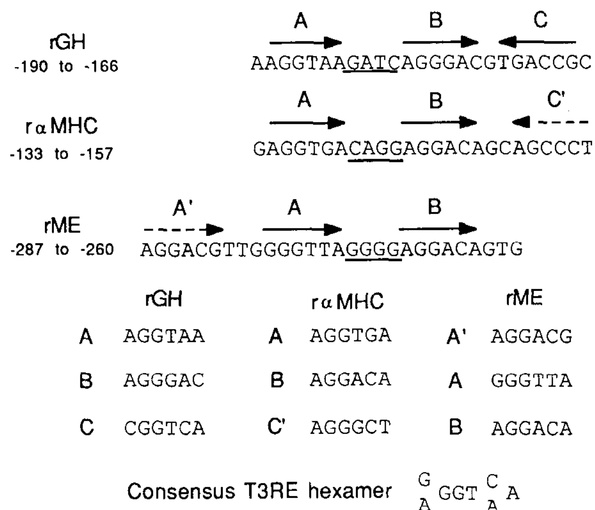

Fig. 1. Sequence of T3REs Studied

Sequences of T3REs from rGH, rαMHC (antisense strand shown), and rME promoters. Hexamer assignments are based on four of six bases matching the consensus T3RE hexamer as shown.

Downloaded from https://academic.oup.com/mend/article/6/4/502/2714532 by guest on 16 August 2022

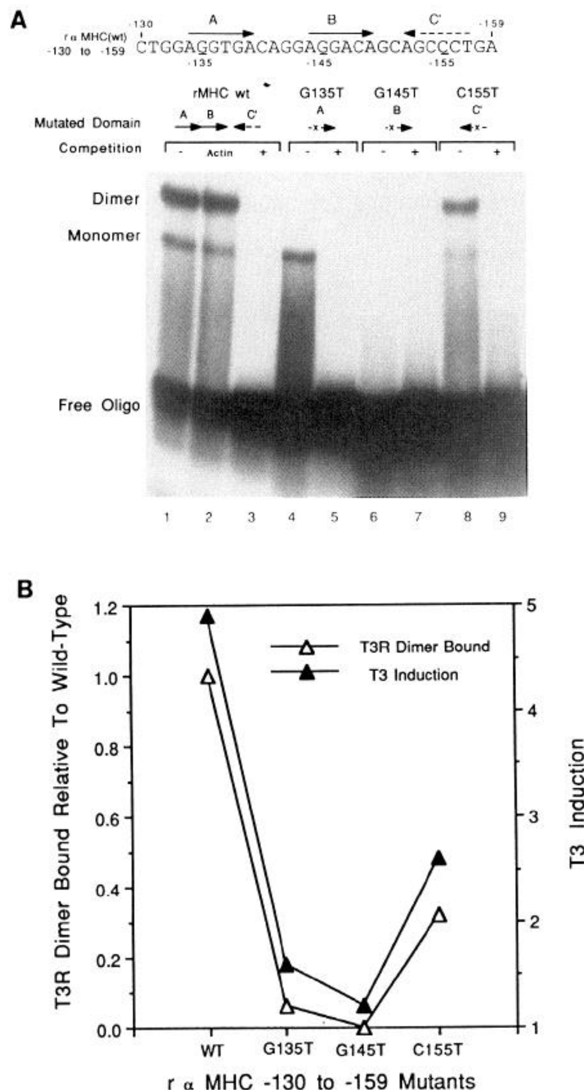

Fig. 2. Binding of T3Rα to rMHC T3RE-Wild Type Element and Mutations

A, T3Rα (44 fmol) overexpressed in *E. coli* was incubated with ^{32}P-labeled DNA probe (7.5 fmol) containing the wild type rMHC T3RE or elements with mutations in the A (G135T), B (G145), or C (C155T) domains. The products were analyzed on a nondenaturing polyacrylamide gel, dried, and autoradiographed at −70 C for 8 h. Two specific retarded T3R/DNA complexes are shown, designated monomer and dimer (see text), along with free oligonucleotide probe. Competition with 200-fold molar excess of cold T3RE (+; lanes 3, 5, 7, and 9) or actin (lane 2) is shown. B, Plot of the total fraction of T3R bound as a dimer relative to wild type (△) and T3 induction upstream of the TK promoter (▲) for the rMHC wild type (WT) and specific mutant elements. The linear correlation coefficient of T3R bound as a dimer *vs.* T3 induction was $r = 0.99$, $P < 0.01$.

Mutating conserved G residues of the putative A, B, or C domains (bases −135, −145, and −155) resulted in reduced total T3R binding to 37%, 3%, and 37% of wild type binding, respectively (Fig. 2A). Dimer formation was retained with mutations in the A or C domains (G135T or C155T), although the A domain mutation formed a weak dimer band seen only at higher T3R concentrations or longer gel exposures. These mutant sequences retain two functional hexamers in either a direct repeat or palindromic arrangement. However, dimer formation was not seen with the B domain mutant (G145T) which contains two functional half-sites (A and C) separated by 12 bp.

The same rMHC wild type and mutant elements were inserted upstream of the herpes simplex thymidine kinase (TK) promoter and tested for T3 response in transient transfections of JEG cells with a cotransfected vector expressing T3Rα. The pUTKAT3 vector alone was stimulated 1.6-fold by T3. T3 induction was reduced from 5.0-fold for the wild type element to 1.6-, 1.1-, and 2.8-fold for the A, B, and C domain mutants, respectively (Fig. 3). Down-mutants resulted in a reduction of both basal and T3-induced expression relative to the rMHC wild type. Similar results were seen with the same oligonucleotides inserted upstream of a truncated rGH promoter (rGH137) and transfected into GH4C1 rat pituitary tumor cells, although the magnitude of T3 induction was higher (14.3-fold for the MHC wild type, reduced to 3.0-, 1.8-, and 9.4-fold for the A, B, and C mutants, respectively).

The ability of a mutant T3RE to confer T3 responsiveness to a heterologous promoter was closely correlated with the capacity of the T3RE to bind T3R dimers in the gel retardation assay (Fig. 2B). A linear plot of T3 induction upstream of the TK promoter *vs.* T3R bound by mutant T3RE relative to the wild type showed a significant positive correlation for total T3R bound ($r = 0.91$, $P < 0.01$) and for T3R bound as a dimer ($r = 0.99$, $P < 0.01$). Similar correlations with T3 induction and binding were found with the elements upstream of the rGH promoter for total T3R bound ($r = 0.80$, $P < 0.05$) and T3R dimer ($r = 0.91$, $P < 0.01$). Monomer binding did not correlate with T3 induction. Mutation of the centrally placed B domain produced the greatest reduction in T3R binding and T3 induction, whereas the C domain mutant resulted in the least reduction. These results closely parallel findings reported for the rGH T3RE, although the C domain is a weaker hexamer than in rGH (8) and is therefore designated as C′ with a *broken line arrow* (Fig. 2A).

A series of point mutations were made in the rMHC T3RE to confirm the hexamer assignment and to assess the potential influences of adjacent sequences. The T3 induction ratios are shown for the various αMHC elements placed upstream of the TK promoter and, in some cases, the rGH137 promoter (Fig. 3). A mutation between putative hexameric domains A and B, G142T, did not reduce T3 induction. Mutation of the last base of the B hexamer (A149T) reduced T3 induction. Mutation of a base between the B and C domains, C151A, resulted in a modest decrease in T3 induction only when placed upstream of the TK promoter, which was further reduced in combination with the B domain mutant (A149T-C151A). Based on the importance of the 1-bp spacing of the palindrome in the rGH T3RE (8), a

Downloaded from https://academic.oup.com/mend/article/6/4/502/2714532 by guest on 16 August 2022

		A → B → ← C	TK Promoter			rGH Promoter
			CAT/hGH			
			-T3	+T3	T3 Ratio	T3 Ratio
Wild-Type:	rMHC wt -130 to -159	CTGGAGGTGACAGGAGGACAGCAGCCCTGA	2.0 (0.2)	9.8 (0.2)	5.0 (0.4)	14.3 (2.0)
Mutants:						
	G135T (A Mut)	-----T------------------------	0.9 (0.4)	1.4 (0.6)	1.6 (0.3)	3.0 (0.3)
	G142T	------------T-----------------	1.3 (0.2)	6.2 (0.4)	4.6 (0.7)	16.8 (1.6)
	G145T (B Mut)	---------------T--------------	1.7 (0.5)	1.9 (0.6)	1.1 (0.1)	1.8 (0.1)
	A149T	-------------------T----------	1.7 (0.3)	3.2 (0.3)	1.9 (0.6)	2.9 (0.4)
	C151A	---------------------A--------	2.4 (0.3)	6.2 (0.3)	2.6 (0.3)	14.8 (2.5)
	A149T, C151A	-------------------T-A--------	2.2 (0.4)	3.7 (0.2)	1.7 (0.3)	2.2 (0.3)
	C155T (C Mut)	-------------------------T----	1.1 (0.3)	3.1 (0.4)	2.8 (0.3)	9.4 (1.3)
	Δ151C	--------------------- --------	1.5 (0.1)	7.1 (0.9)	4.6 (0.6)	------
Up-Mutant Series:						
	A152T	----------------------T-------	1.0 (0.1)	6.0 (0.3)	5.7 (0.8)	------
	C154A	------------------------A-----	1.1 (0.1)	5.7 (0.7)	5.3 (0.6)	------
	A152T, C154A	----------------------T-A-----	0.8 (0.1)	10.9 (0.7)	13.1 (2.0)	------

Fig. 3. Functional Analysis of rMHC T3RE Mutations

The mean T3 induction ratios ± SE are shown for transient transfections in JEG cells (with cotransfected T3Rα) and GH4C1 cells. Constructions (see *Materials and Methods*) are based on elements containing the rMHC wild type sequence rMHC (−130 to −159; antisense strand shown) and various mutants placed upstream of the first 137 bp of the rGH promoter or the herpes simplex TK promoter. Basal and T3-induced CAT/hGH levels (±SE) are shown for elements upstream of the TK promoter.

base deletion (D151C) was made reducing the B/C spacing to 1 base. There was no effect of the deletion on T3 induction.

Based on the weakness of the C′ domain in T3R binding and function, several mutations were designed to create a hexamer more closely matching the consensus sequence (7). T3 induction was increased to 13.1-fold for rMHC A152T-C154A, converting from a four of six to a six of six match for a consensus AGGTCA hexamer. A typical up-mutant pattern was seen with a reduction of basal expression and slight increase in T3-induced expression. Elements containing only one of these mutations (A152T or C154A), however, were only slightly increased over wild type (5.7- and 5.3-fold, respectively). A single mutation, therefore, does not increase T3 induction as seen with the rGH T3RE C domain and confirms that this is a weaker site.

The Influence of Nuclear Proteins on MHC Binding

We have previously shown that addition of nuclear proteins to purified T3R enhances binding as well as promoting higher order complexes (8). We used enhancement by nuclear protein to further investigate the role of the rMHC C domain, as well as the importance of the B domain in T3 monomer binding.

Binding of purified T3 receptor to the rMHC wild type and mutant elements was measured with and without addition of JEG cell nuclear extract (Fig. 4). The JEG extract alone produced a band which migrated above the T3R dimer band. T3R alone, in a small quantity (20 fmol), produced only faint bands on an 8-h, exposure but they were enhanced by the addition of 1 μg JEG nuclear extract. Nuclear protein enhanced both monomer and dimer binding (Fig. 4B), although binding enhancement in the wild type was primarily due to increased dimer binding and in the mutants due to enhanced monomer binding. Despite the enhancement, however, the effect of point mutations in each domain was essentially the same as that seen with T3R alone. The correlation of binding and T3 induction was preserved when nuclear protein was added for T3R dimers ($r = 0.94$, $P < 0.01$) and total T3R binding ($r = 0.87$, $P < 0.05$). As seen for T3R alone, nuclear protein-augmented T3R monomer binding did not correlate with T3 induction. A third oligomer band, as seen with the rGH element (8), was not seen with rMHC. Even with nuclear protein enhancement, however, the C domain mutant bound consistently less dimer and monomer than wild type, confirming a role in T3R binding.

The absence of T3R monomer binding as a result of a mutation in the centrally placed B domain (Fig. 2A) is somewhat surprising given that two intact hexamers remain. It is a consistent finding, however, in both the rGH and rMHC T3REs. The loss of monomer binding is only a relative one since monomers are seen at higher T3R concentrations or when enhanced with nuclear protein. We investigated features of the T3RE which may be responsible for the interaction of T3R monomers with the B domain. Possible explanations for this observation are that the B domain motif has the highest affinity for monomers or alternatively that the central position within the T3RE favors monomer binding. These possibilities were tested by varying domains within the rMHC T3RE. Mutations were made in the rMHC A and B hexamers to create an element with a BAC′ motif arrangement (Fig. 4C).

T3R monomer and dimer binding to the BAC′ ele-

Downloaded from https://academic.oup.com/mend/article/6/4/502/2714532 by guest on 16 August 2022

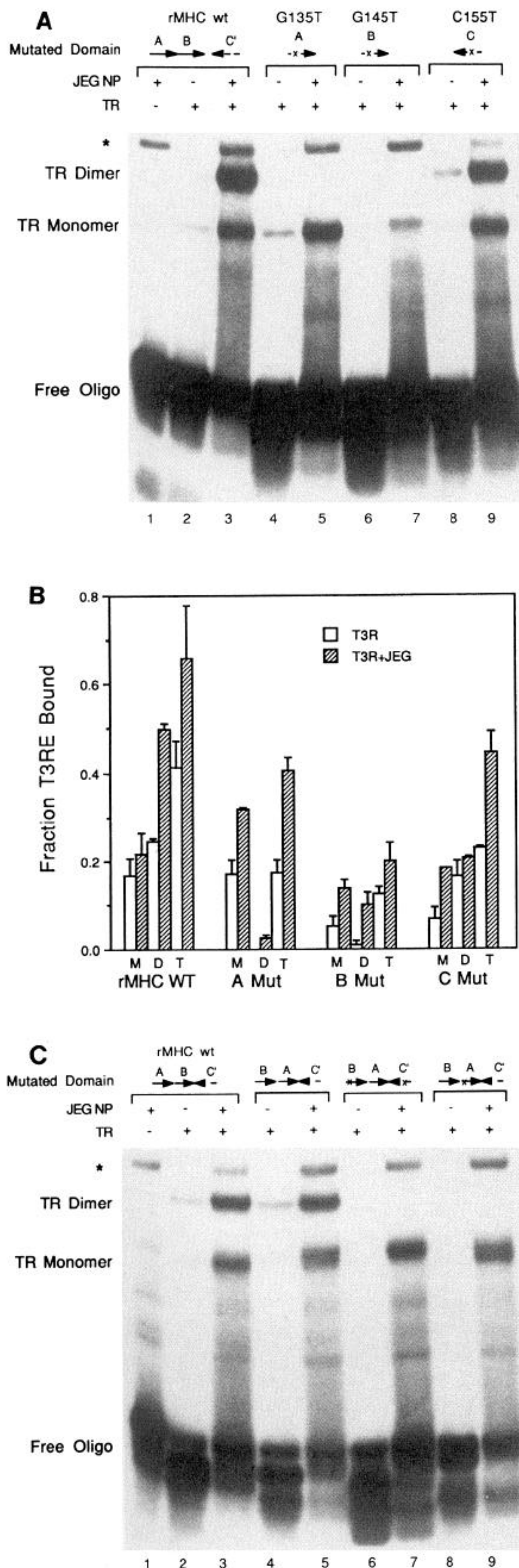

Fig. 4. Binding of T3R to rMHC and Mutants With and Without Addition of Nuclear Proteins

A, T3Rα (20 fmol) overexpressed in *E. Coli* was incubated with ^{32}P-labeled DNA probe (7.5 fmol) containing the wild type rMHC T3RE and mutants of the A, B, or C′ domains. T3R was incubated with (+) or without (−) 1 μg JEG nuclear protein extract (JEG NP). The products were analyzed on a nondenaturing polyacrylamide gel, dried, and autoradiographed at 70 C for 8 h. Lane 1 contains nuclear protein alone. *, JEG-specific retarded band. T3R monomer and dimer bands as well as free oligonucleotide are labeled. B, Fraction of T3RE bound (±SE) is shown for monomer (M), dimer (D), and total bound (T) based on quantification of gels (such as that in A) by laser densitometry. □, T3R alone; ▨, T3R with 1 μg added JEG nuclear protein. C, Gel shift assay as described in A, with rMHC wild type and mutations in the background of an element with the A and B domain motifs inverted (rMHC BAC).

ment, with and without JEG nuclear protein, was essentially unchanged from the rMHC wild type. Mutation of the now centrally placed A domain motif bound only monomer. Mutation of the outer domain positions, B and C′, resulted in a similar level of monomer binding. The B domain, therefore, is not the only hexamer which can bind a monomer, as binding was detected when only an intact A domain was available in the B/C′ mutation. The central position is also not the sole determinant of monomer binding, as binding was detected when the central A domain was mutated. The combination of the central position and an intact B domain motif, therefore, interact to optimize monomer binding. Other factors, including DNA bending and contextual sequences, may further influence this phenomenon.

The Rat Malic Enzyme T3RE Consists of Two Hexamers Arranged As A Direct Repeat

Inspection of the rat malic enzyme (rME) sequence reveals several possible hexamers with a four of six match to the consensus hexamer. We studied the binding of purified T3R to the wild type version of this T3RE and to elements with point mutations affecting putative hexamers. Incubation of purified T3R with the wild type rME T3RE again resulted in a monomer and more slowly migrating dimer band (Fig. 5A), in the identical positions as seen with the rGH and rMHC T3REs. T3R binding, relative to the wild type, was essentially eliminated (2%) by mutation of the putative B domain (G267T) and was strongly reduced (11%) by the A domain mutation, G277T (Fig. 5A). No dimer formation was seen with point mutations in either of these hexamers. These mutations are in hexameric regions closely matching those reported for the direct repeat portion of the rGH T3RE. Mutations in other regions, rME G279T and G271T, resulted in some decrease (84%) and increase (132%) in binding, respectively. Mutations affecting a potential upstream half-site AGGACG (A′; −287 to −281), G285T, and G282T did not significantly alter T3R binding (107% and 100% of wild type, respectively) (Fig. 5, B and C).

The same rME elements examined for T3R binding were placed upstream of the heterologous TK promoter

Downloaded from https://academic.oup.com/mend/article/6/4/502/2714532 by guest on 16 August 2022

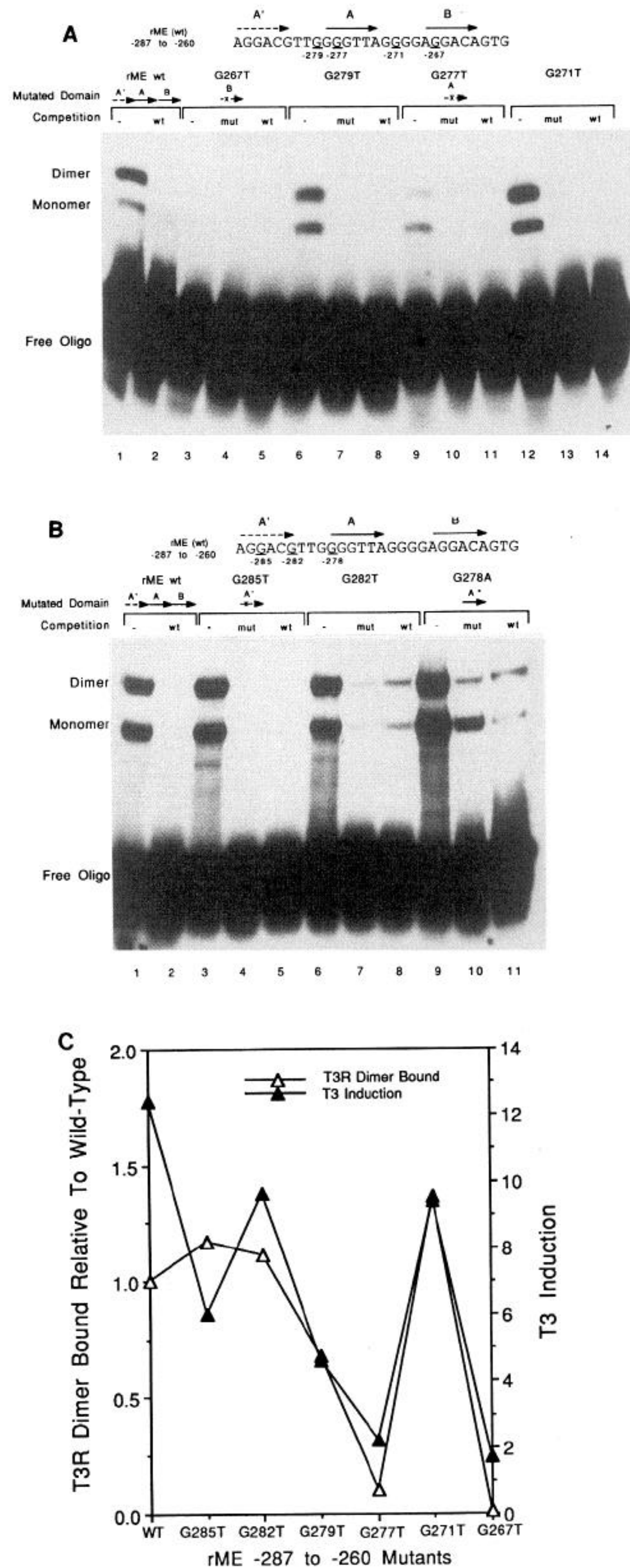

Fig. 5. Binding of T3Rα to rME T3RE Wild Type Element and Mutations

A, T3Rα (44 fmol) overexpressed in *E. coli* was incubated with ^{32}P-labeled DNA probe (7.5 fmol) containing the wild type rME T3RE or with mutations in the A (G277T) or B (G267T) domains. Mutations in flanking areas, G279T and G271T, are also shown. The products were analyzed on a nondenaturing polyacrylamide gel, dried, and autoradiographed at −70 C for 15 h. Two specific retarded T3R/DNA complexes are shown, designated monomer and dimer (see text), along with free oligonucleotide probe. Competition with 100-fold molar excess of cold wild type (wt) rME T3RE (lanes 2, 5, 8, 11, and 14) or mutant (mut) rME T3REs (lanes 4, 7, 10, and 13) are shown. B, rME elements with mutations in the upstream sequences (A′) and an A domain up-mutation (G278A) were incubated with purified T3Rα and analyzed as described above (A). C, Plot of the total fraction of T3R bound as dimer relative to wild type (△) and T3 induction (▲) for the rMHC wild type and specific mutants. The linear correlation coefficient of T3R bound as dimer *vs.* T3 induction was $r = 0.67$, $P < 0.05$.

Downloaded from https://academic.oup.com/mend/article/6/4/502/2714532 by guest on 16 August 2022

		Sequence (A' ----►, A ——►, B ——►)	-T3	+T3	T3 Induction Ratio (SE)
Wild Type:	rME (-287 to -260)	AGGACGTTGGGGTTAGGGGAGGACAGTG	2.1 (0.2)	24.6 (2.9)	11.7 (0.9)
Mutants:	G285T (A' Mut)	--T-------------------------	1.9 (0.1)	11.4 (0.9)	6.0 (0.4)
	G282T	-----T----------------------	1.7 (0.2)	13.3 (0.8)	7.8 (1.0)
	T282ins	---------------------------- (T inserted)	1.7 (0.2)	15.4 (1.0)	9.2 (1.1)
	G279T	--------T-------------------	2.3 (0.6)	10.5 (3.6)	4.6 (0.4)
	G277T (A Mut)	----------T-----------------	1.8 (0.3)	3.6 (0.7)	2.1 (0.1)
	G271T	----------------T-----------	0.4 (0.1)	3.1 (0.4)	8.4 (0.5)
	G267T (B Mut)	--------------------T-------	1.4 (0.3)	2.1 (0.2)	1.5 (0.3)
Up-Mutant:	G278A	---------A------------------	1.3 (0.2)	25.5 (2.4)	19.8 (3.3)
	G278A, T274C	---------A---C--------------	1.1 (0.2)	33.2 (2.8)	30.2 (2.6)

Fig. 6. Functional Analysis of rME T3RE Mutations

The mean basal and T3-induced CAT/hGH ratios (±SE) as well as T3 induction ratios (±SE) are shown for transient transfections in JEG cells with cotransfected T3Rα. Constructions (see *Materials and Methods*) are based on elements containing the rME wild type sequence rME (−287 to −260) and mutants placed upstream of the herpes simplex TK promoter.

and tested in a transient transfection assay (Fig. 6). T3 induction was 11.7-fold for the wild type rME element and was reduced to 2.1- and 1.5-fold with point mutations in the A (G277T) or B (G267T) domains which prevent the binding of T3R dimers. The reduction in T3 induction ratio was primarily the result of a marked reduction in the T3-induced expression. T3 induction was reduced 50% to 6.0-fold with mutations in the upstream A′ region (G285T), significantly more reduced than with mutations in other regions outside the binding hexamers. A plot of T3RE binding and T3 induction (Fig. 5C) shows a close relationship for down-mutants in the A and B domains and a fall in induction with the upstream site mutation but no reduction in binding. A linear plot of T3 induction ratio *vs.* T3R bound by mutant T3RE relative to the wild type showed a significant positive correlation for total T3R bound ($r = 0.59$, $P < 0.05$) and for T3R bound as a dimer ($r = 0.67$, $P < 0.05$), but not for T3R monomers.

We further examined the A domain GGGTTA, which has several possible hexamer assignments based on the grouping of 4 G residues. A mutation which created a hexamer more closely matching the consensus AGGTTA (rME G278A) increased induction to 19.8-fold. Binding to this element by T3R was increased for T3R dimers (160%) and total T3R (170%) (Fig. 5B). An element with two mutations creating a perfect consensus hexamer AGGTCA (G278A, T274C), increased T3 induction further to 30.2-fold (Fig. 6A) and resulted in a reduction in basal and increase in T3-induced expression. These results suggest that the assignment of this hexamer is correct.

Hexamer Sequence and Arrangement Influences T3R Dimer Affinity For Wild Type T3REs

T3R bound to all elements predominantly as a dimer which migrated to the same gel position for each element. The band pattern and position are the same as we have previously reported for the rGH T3RE (8). There was some variation in the amount of T3R bound among the various elements. The fraction of T3RE bound was highest for rMHC (0.39) and rME (0.35) and lowest for rGH (0.26).

Increasing concentrations of T3R were added to a constant amount of labeled T3RE to determine the relative binding affinity of the three wild type elements for T3R (Fig. 7A). The lowest T3R concentration was associated with monomer binding, and dimer binding was favored at higher T3R concentrations. A plot of T3RE bound as dimer as a function of T3R concentration (Fig. 7B) produces a sigmoidal curve. The T3R concentration at which dimer formation is seen varies among the elements. Dimer formation occurs at the lowest concentration of added T3R with rME and MHC; a higher T3R concentration is required for dimer binding to the rGH T3RE.

Wild Type T3REs Bind T3R Dimers Cooperatively

Inverse plots of the binding results for rMHC and rME were made to determine if binding was cooperative (Fig. 8). The points followed an upwardly curved parabola (all $r > 0.99$), characteristic of positive cooperativity.

Nuclear Protein Enhancement of T3 Receptor Binding

The enhancement of T3R binding by nuclear protein was tested in positive, (rME) and negative (human α-subunit) T3 elements. JEG extract enhanced both monomer and dimer binding to the rME T3RE (Fig. 9A). The human α-subunit binds only a T3R monmer (see Ref.

Downloaded from https://academic.oup.com/mend/article/6/4/502/2714532 by guest on 16 August 2022

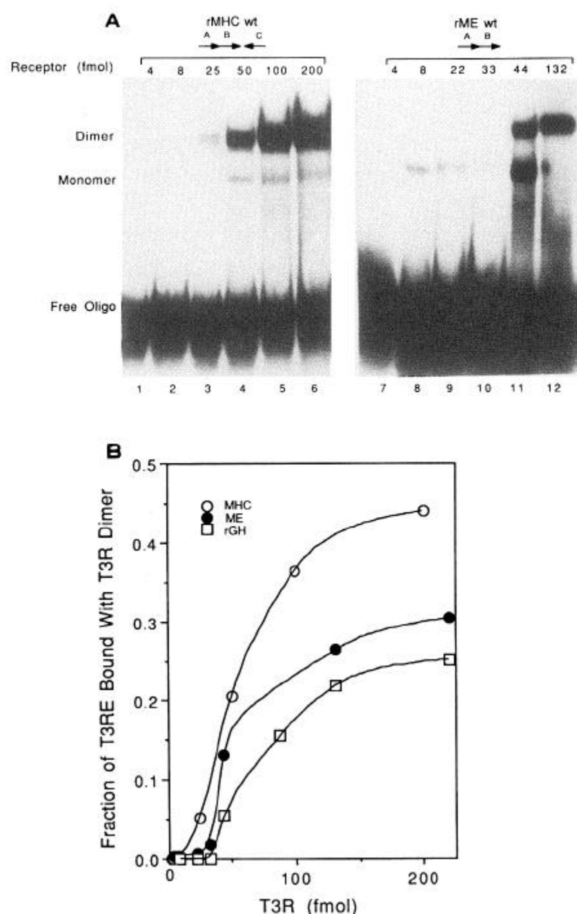

Fig. 7. Binding of Increasing Concentrations of T3R to T3REs

A, Gel retardation assay of increasing concentrations (4–200 fmol) of highly purified T3Rα bound to 15 fmol P^{32}-labeled rMHC and rME T3REs. B, Plot of the fraction of T3RE bound with T3R dimers as a function of increasing T3R concentrations. Curves are calculated from a series of experiments such as those shown in A. The data for rGH are from a previous study (8) and are provided for comparison.

33) which is also enhanced by addition of JEG or COS cell nuclear extract. A dimer band or higher order binding was not seen with up to 5 μg nuclear protein.

Titration of T3R and nuclear protein concentrations binding to the rMHC element defined the lower limits at which enhancement can be seen (Fig. 10). As little as 2 fmol T3 receptor could be detected when 1 μg nuclear protein was added. Enhancement was completely eliminated if the extract was heated for 15 min at 100 C before incubation (not shown). Incubations of T3R with higher or lower poly deoxyinosine deoxycytosine (polydIdC) concentrations (including no polydIdC) retained significant binding enhancement as a result of the addition of nuclear protein (not shown).

DISCUSSION

The functional organization of the rat αMHC T3RE is very similar to the three-hexamer structure that we have previously described for the rGH T3RE (7). As with the rGH T3RE, all three hexamers of the αMHC element must be intact for a full T3 response. In both cases, the C domain appears to make the least contribution toward T3R binding and T3 response in transient transfections. Most striking is the importance and requirement of the central B domain, in both elements, to permit T3R dimer formation. This is consistent with its central position and suggests a model of binding of dimers to either the directly repeated A and B domains or the palindromic B and C domains. The functional correlates of this arrangement are strong, as virtually no T3 induction is observed when this domain is mutated and formation of dimers prevented. The overall T3 induction is stronger for αMHC compared to the rGH T3RE, even with an element containing only the αMHC sequence −133 to −157. Based on the binding results and comparison of the sequences, we conclude that this is because the αMHC A and B hexamers are better matches to the consensus than the rGH sequences (particularly the B domain). The importance of these direct repeats is consistent with a recent report on the αMHC element (13) and with our original description of the importance of direct repeats for T3R binding to the rGH element (5).

Other transcription factors have also been reported to recognize DNA sequences apparently consisting of three monomer binding sites. These include another member of the steroid-thyroid receptor superfamily, vitamin D (20, 21), and the heat shock transcription factor from *Drosophila* (22, 23) and yeast (24). By analogy with the results presented here for T3 receptor and previously described for other members of the receptor superfamily, the vitamin D receptor is likely to bind to its response element as a dimer. However, the heat shock transcription factor interacts with a surprising array of DNA sequences as a trimer, perhaps reflecting flexibility in the ability of individual DNA binding domains to interact with monomer binding sites. The ability of T3R dimers to bind to hexamers arranged as a direct repeat, palindrome, or the inverted repeat lysozyme T3RE (16; our preliminary data) could also be explained by flexibility of DNA binding domains relative to a constant ligand/dimerization domain. Alternatively, the DNA binding domain could remain fixed relative to a ligand/dimerization domain capable of alternative mirror image or tandem interactions.

In contrast to the rGH and rαMHC sites, the wild type rME T3RE appears to consist of only a single dimer binding site corresponding to two hexamers arranged as a direct repeat. Mutations in either the A or B domains prevent dimer formation and abolish T3 response. The results of our biochemical and genetic analyses of the rME T3RE closely match those of Desvergne *et al.* (15), although our mutational studies, based on a larger series of individual point mutations, lead to a different assignment of likely binding sequences. The hexamer assignment defined by the current results is a subset of our prior proposal based on an initial definition of the hexamer consensus (6, 7) and

Downloaded from https://academic.oup.com/mend/article/6/4/502/2714532 by guest on 16 August 2022

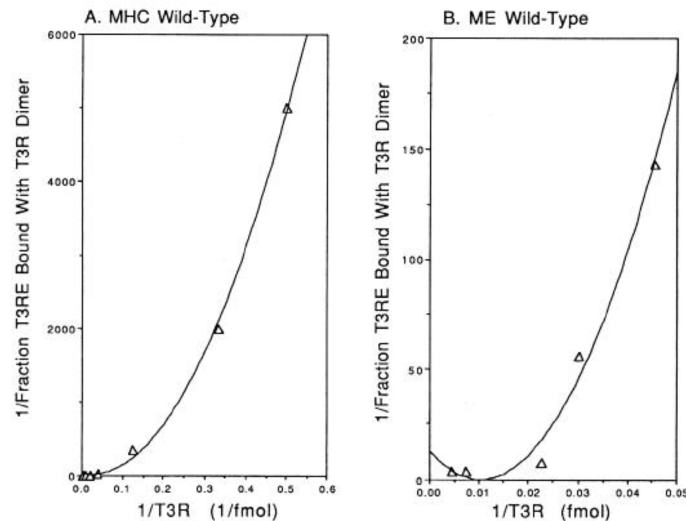

Fig. 8. Double-Reciprocal Plots of Saturation Curves

The data from Fig. 7B for rMHC (A) and rME (B) are graphed as double-reciprocal plots to determine cooperativity. Both lines closely fit an upwardly curved parabola (r ≥ 0.99) characteristic of positive cooperativity.

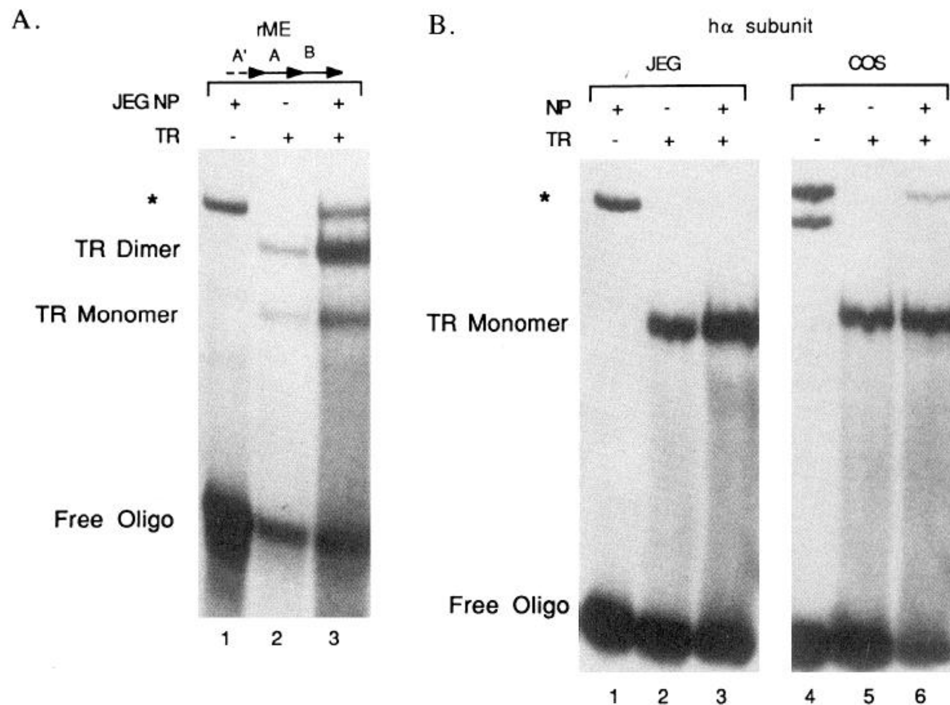

Fig. 9. Enhancement of T3R Binding to A Positive and Negative T3 Element with Addition of Nuclear Protein

A, Purified T3Rα (20 fmol) and ^{32}P-labeled rME T3RE (15 fmol) were incubated with (lane 3) or without (lane 2) 1 μg JEG nuclear protein (JEG NP). Nuclear protein alone is also shown (lane 1). The products were analyzed on a nondenaturing polyacrylamide gel, dried, and autoradiographed at −70 C for 8 h. B, As in A with the human α-subunit element and 1 μg either JEG (lanes 1–3) or COS (lanes 4–6) extract.

is the same as a recent proposal by Umesono *et al.* (13), based on sequence comparison. We assume that the functional potency of the rME T3RE is due, in part, to the good match of the sequences of each hexamer to the consensus, which results in relatively high affinity for receptor. This requirement is essential to permit receptor dimerization which is necessary for full functional potency of a positive TRE. As expected, T3 response was increased further by mutations that bring the A domain even closer to the consensus. In addition, our data and that of Desvergne *et al.* (15) suggest that sequences upstream of the direct repeat motif that

Downloaded from https://academic.oup.com/mend/article/6/4/502/2714532 by guest on 16 August 2022

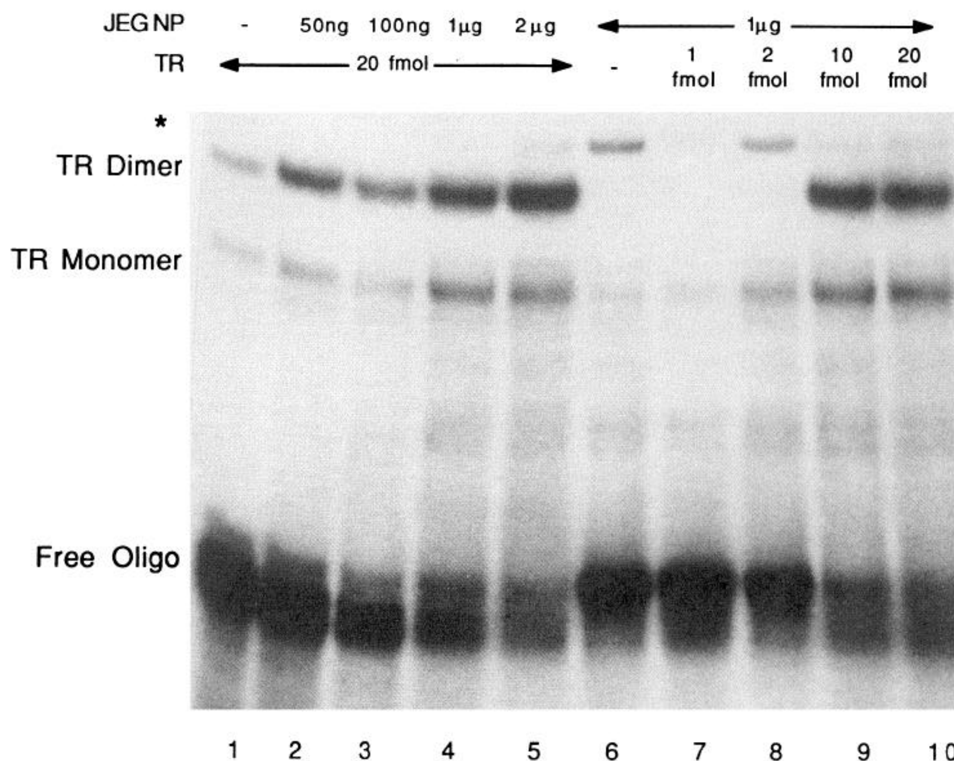

Fig. 10. Titration of T3R and Nuclear Protein Concentration Binding to the rMHC Wild Type T3RE

The rMHC wild type T3RE was ^{32}P-labeled (15 fmol) and incubated with a fixed amount of T3Rα (20 fmol) and increasing concentrations of JEG nuclear protein (0–2 μg; lanes 1–5) or a fixed amount of nuclear protein (1 μg) and increasing concentration of T3Rα (0–20 fmol; lanes 6–10). The products were analyzed on a nondenaturing polyacrylamide gel, dried, and autoradiographed at −70 C for 8 h.

include some matches to the hexamer consensus make some contribution to function *in vivo*. This region may augment T3 induction by interaction with T3 receptor accessory proteins.

The common motif of the elements contained in the elements described here that are positively regulated by T3 is a direct repeat of a conserved sequence with a 4-bp gap, as we originally described for rGH (5). It is clear from our analysis of the rGH and rMHC sites, however, that hexamers outside the direct repeats make significant contributions to both receptor binding *in vitro* and biological function *in vivo*. Further complexity is suggested by the reduction in T3R binding and T3 induction seen with mutations in sequences outside the hexamers in all three of these elements. The lysozyme silencer T3RE appears to consist of two tail-to-tail inverted hexamers separated by a 6-bp gap (16). Our preliminary binding results with this element suggest that this arrangement shows very high affinity for T3R (data not shown). This is supported by Naar *et al.* (25) using synthetic T3REs containing two tail-to-tail inverted consensus hexamers in the ABCD binding assay. However, the relatively modest T3 induction observed with the lysozyme element suggests that its activity *in vivo* may reflect more complex interactions with proteins in addition to T3R. It is apparent that naturally occurring T3 response elements are much more complex than the simple model of direct repeats with a 4-bp gap that has recently been proposed (13).

More significant, and functionally applicable to wild type T3REs, is the ability of a TRE to bind T3R dimers. There is a strong correlation between the capacity of wild type and mutant versions of the rGH, rME, and rMHC elements to bind T3R dimers and the magnitude of their response to T3 in transfections. Addition of nuclear protein enhanced binding but did not change the specificity of T3R binding for the various elements studied or the correlation with induction.

A number of groups, using various T3R preparations and binding assays, have reported an increase in T3R binding to T3REs as a result of the addition of nuclear protein (26–30). A recent study (31) determined binding of *in vitro* translated T3R to wild type T3REs in the ABCD assay. They reported a similar level of nuclear protein-enhanced binding to the rME and rMHC elements (approximately 2-fold) as seen in our assay. Our results are also consistent with a recent analysis of T3R interaction with nuclear proteins in gel shift assay (32). T3R-nuclear protein heterodimers were found to be the most stable complex; however, T3R homodimers were found to predominate in conditions of high T3R concentration relative to nuclear protein. Although we report on T3R homodimer enhancement, we also observed T3R-nuclear protein heterodimer formation in

Downloaded from https://academic.oup.com/mend/article/6/4/502/2714532 by guest on 16 August 2022

our system on specific T3REs when nuclear proteins were in vast excess to T3R (not shown). The relative concentrations of nuclear proteins and T3R that most closely reproduce the *in vivo* situation are not known. However, if accessory proteins bind DNA directly and form heterodimers with the T3R, our results suggest that the specificity of binding of the heterodimer must be very similar to that of a T3R homodimer. We conclude that the capacity for T3R/DNA interactions is the predominant determinant of the potency of an individual T3RE, although nuclear proteins significantly enhance T3R binding and may play a role in dictating specificity under certain circumstances.

Negative regulation by T3 provides a further level of complexity, and results with the human glycoprotein hormone α-subunit (33) and rGH downstream element (34) suggest that such negative elements may consist of only a single hexamer. Our previous results (33) as well as current data suggest that the human α-subunit element binds only a T3R monomer. Negative regulation by T3, therefore, does not necessarily follow the pattern of cooperative T3R dimer formation that we have described for positive T3REs.

We have demonstrated that wild type T3REs, with a variety of sequences and arrangements of receptor binding hexamers, have the capacity to bind T3R as a dimer. Mutational analysis indicates that T3R dimer formation is required for induction by T3. The strong correlation of binding of purified T3R with function, whether or not nuclear proteins are present, makes it less likely that accessory factors play a dominant role in directing the specificity of T3R-DNA interactions, although they significantly enhance binding. Given the apparent complexity of the interactions of T3R with positive and negative response elements, it seems premature to codify rules for their identification and interpretation.

MATERIALS AND METHODS

Purified Receptor

Chicken T3Rα was overexpressed in *E. coli* strain BL21 DE3 pLYSs using T7 phage polymerase which was inducible by isopropyl-b-D-thiogalactosidase (19). After 1 h induction cells were harvested and lysed by freeze-thawing in GTME-400 (15% glycerol, 25 mM Tris, pH 7.8, 0.05% Triton X-100, 500 μM EDTA, 10 mM β-mercaptoethanol, 1 mM phenylmethylsulfonyl fluoride, and 400 mM KCl). Crude lysates were centrifuged (10 min, 16,000 $\times$ *g*) and the supernatant analyzed for T3R content and quantification by [^{125}I]T3 binding and T3R immunoprecipitation as described (19). The crude lysate was purified by diethylaminoethyl-Sephadex chromatography, ammonium sulfate precipitation, heparin-agarose chromatography, and size exclusion chromatography (Superose 12, Pharmacia, Piscataway, NJ). Purification to apparent homogeneity was confirmed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and silver staining of an overloaded gel, which demonstrated the appropriate sized receptor (46 kilodaltons) and the absence of degradation products (data not shown). Receptor was dispensed into aliquots and stored at −80 C before dilution in GTME-400 for use in gel shift assays.

Nuclear Protein Preparation

JEG and COS cell nuclear extracts were prepared as previously described (8). Protein concentration was measured using the Bio-Rad protein assay method (Bio-Rad Laboratories, Richmond, CA), and aliquots were stored at −70 C.

Binding Reactions and Mobility Shift DNA Assay

Gel shift experiments were performed in two conditions modified from methods described by several groups (19, 26, 35–37). Oligonucleotides containing T3REs and a series of T3RE mutants were radiolabeled with [^{32}P]deoxythymidine triphosphate (New England Nuclear, Boston, MA) by fill in reactions using Klenow large fragment DNA polymerase (38) and purified by nondenaturing polyacrylamide gel electrophoresis. Labeled probe, 15–25,000 cpm (4.5–7.5 fmol), was incubated with purified T3Rα (4–225 fmol) in a 30-μl reaction containing 100 ng polydIdC, 88 mM KCl, 10% glycerol, 25 mM Tris-Cl, 500 μM EDTA, 0.05% Triton X-100, 10 mM β-mercaptoethanol, and 5 μg BSA. These reactions were incubated for 30 min at room temperature and assayed on a 5% nondenaturing polyacrylamide gel in low ionic strength mobility shift buffer (10 mM Tris-Cl, 7.5 mM glacial acetic acid, and 40 μM EDTA, pH 7.8) and electrophoresed in identical buffer with constant circulation, 500 V at 4 C after 30 min prerunning under the same conditions. Competition experiment incubations included 100 ng (1 pmol) unlabeled competitor DNA fragment. Gels were dried under vacuum and autoradiographed at −70 C for 6–15 h.

Quantitative Analysis of Receptor Binding

Quantification of autoradiographs by laser densitometry was performed on a Molecular Dynamics Model 300 Series Computing Densitometer (Molecular Dynamics, Sunnyvale, CA) using Molecular Dynamics ImageQuant software. The density of the retarded (one or more) and free bands was determined at each receptor concentration. The fraction of T3RE bound by receptor in a specific band was calculated by dividing the density of the band by the total density (sum of the densities of retarded and free bands) at a given receptor concentration. Results are presented as the fraction of T3RE bound with T3R dimer *vs.* T3 receptor concentration. Densitometry was performed on shorter exposures than those shown in the figures (5–7 h) in order to determine band density in a linear range of film exposure. The studies presented are representative of at least two separate experiments. Double reciprocal plots of 1/fraction T3RE bound with T3R dimer *vs.* 1/T3R concentration were made for the T3REs to assess cooperativity.

The fraction of T3RE bound with T3R dimers and total T3R bound, relative to the wild type element, were determined in at least two experiments and plotted as a function of T3 induction ratio from transient transfection studies. The best fit lines were calculated (Cricket Graph V1.3, Cricket Software, Malvern, PA) for these plots.

Plasmid Constructs and Oligonucleotides

Standard methods for vector construction were used (38). Double-stranded oligonucleotides were synthesized which contain T3 response elements and mutations. These were ligated into the *Bam*H1 site upstream of rGH137 as described previously (6) or PUTKAT3 (39). The rαMHC promoter sequence (−159 to −130; rMHC) was the basis of subsequent mutations, and oligonucleotide sequences were named based on the location of the mutated base (wild type base on the left, mutated base on the right). rMHC mutations included G135T, G142T, G145T, A149T, C151A, A149T/C151A, C155T, base deletion (Δ151C), A152T, C154A, and A152T/C154A. The rMHC (−159 to −130) sequence was mutated to convert the A domain to a B domain motif and the B domain

Downloaded from https://academic.oup.com/mend/article/6/4/502/2714532 by guest on 16 August 2022

to an A domain motif (T137A, G138C, A147T, and C148G) and was called rMHC BAC. Mutations in the BAC background included mutation of the outer hexamers (G135T, G136T, C155A, and C156A) or the central hexamer (G145T and G146T). The rME promoter sequence (−287 to −260) was the basis of subsequent mutations described as above, G285T, G282T, insertion of a T (T282ins), G279T, G277T, G271T, G267T, and G278A. The oligo, rGHwt, contains the wild type rGH T3RE (−191 to −162; previously rGH34; Ref. 7). All oligonucleotides were inserted in the forward orientation. The nonspecific competitor sequence actin contains sequences (+2077 to +2146) from the third exon of the chicken actin gene. All constructs were sequenced using the dideoxynucleotide method with irreversibly denatured plasmid DNA as template (40).

Transfections

Transfections were carried out as previously described (6) with $CaPO_4$ precipitation in GH4C1 rat pituitary tumor cells and JEG cells for constructs containing the rGH and TK promoter, respectively. The transfections were done in pairs, and each plate contained 15 μg chloramphenicol acetyltransferase (CAT)-expressing plasmid and 5 μg pXGH5 (41), which constitutively expresses hGH, as a control in the GH4C1 cells, or 10 μg CAT-expressing plasmid and 3 μg pTKGH in the JEG cells. JEG cells included cotransfection of 1 μg pCDM13 (which expresses mouse T3Rα; Ref. 42), as previously described (6). CAT activity was determined by a modification of a phase extraction procedure (43). Results are the mean CAT/hGH levels of T3-treated to untreated paired plates, each performed in duplicates of at least two separate transfections which were in close agreement.

Acknowledgments

Yoyi Chen constructed vectors used in these studies. Mark Fleming of the Howard Hughes Medical Institute Biopolymer Facility synthesized oligonucleotides.

Received September 20, 1991. Revision received December 27, 1991. Accepted January 17, 1992.

Address requests for reprints to: Dr. Gregory A. Brent, Thyroid Division, Brigham and Women's Hospital, 75 Francis Street, Boston, Massachusetts 02115.

This work was supported by NIH Grants DK-36256 (to P.R.L.) and DK-16636 (to H.H.S.) and by the Hascoe Foundation.

* Supported by NIH Physician Scientist Award 5-K12-AM01401.

† Supported by a Medical Research Council (UK) Clinician Scientist Fellowship.

‡ Supported by an NIH Medical Scientist Training Grant.

REFERENCES

1. Samuels HH, Forman BM, Horowitz ZD, Ye Z-S 1988 Regulation of gene expression by thyroid hormone. J Clin Invest 81:957–967
2. Brent GA, Moore DD, Larsen PR 1991 Thyroid hormone regulation of gene expression. Annu Rev Physiol 53:17–35
3. Evans RM 1988 The steroid and thyroid hormone receptor superfamily. Science 240:889–895
4. Glass CK, Franco R, Weinberger C, Albert VR, Evans RM, Rosenfeld MG 1987 A c-erb-A binding site in rat growth hormone mediates trans-activation by thyroid hormone. Nature 329:738–741
5. Koenig RJ, Brent GA, Warne RL, Larsen PR, Moore DD 1987 Thyroid hormone receptor binds to a site in the rat growth hormone promoter required for induction by thyroid hormone. Proc Natl Acad Sci USA 84:5670–5674
6. Brent GA, Larsen PR, Harney JW, Koenig RJ, Moore DD 1989 Functional characterization of the rat growth hormone promoter elements required for induction by thyroid hormone with and without a co-transfected β type thyroid hormone receptor. J Biol Chem 264:178–182
7. Brent GA, Harney JW, Chen Y, Warne RL, Moore DD, Larsen PR 1989 Mutations of the rat growth hormone promoter which increase and decrease response to thyroid hormone define a consensus thyroid hormone response element. Mol Endocrinol 3:1996–2004
8. Williams GR, Harney JW, Forman BM, Samuels HH, Brent GA 1991 Oligomeric binding of T3 receptor is required for maximal T3 response. J Biol Chem 266:19636–19644
9. Mahdavi V, Chambers AP, Nadal-Ginard B 1984 Cardiac α- and β-myosin heavy chain genes are organized in tandem. Proc Natl Acad Sci USA 81:2626–2630
10. Markham BE, Bahl JJ, Gustafson TA, Morkin E 1987 Interaction of a protein factor within a thyroid hormone-sensitive region of rat α-myosin heavy chain gene. J Biol Chem 262:12856–12862
11. Izumo S, Mahdavi V 1988 Thyroid hormone receptor isoforms generated by alternative splicing differentially activate myosin HC gene transcription. Nature 334:539–542
12. Flink IL, Morkin E 1990 Interaction of thyroid hormone receptors with strong and weak *cis*-acting elements in the human α-myosin heavy chain gene promoter. J Biol Chem 265:11233–11237
13. Umesono K, Murakami KK, Thompson CC, Evans RM 1991 Direct repeats as selective response elements for the thyroid hormone, retinoic acid, and vitamin D_3 receptors. Cell 65:1255–1266
14. Petty KJ, Desvergne B, Mitsuhashi T, Nikodem VM 1990 Identification of a thyroid hormone response element in the malic enzyme gene. J Biol Chem 165:7395–7400
15. Desvergne B, Petty KJ, Nikodem VM 1991 Functional characterization and receptor binding studies of the malic enzyme thyroid hormone response element. J Biol Chem 266:1008–1013
16. Baniahmad A, Steiner C, Kohne AC, Renakawitz R 1990 Modular structure of a chicken lysozyme silencer: involvement of an unusual thyroid hormone receptor binding site. Cell 61:505–514
17. Sap J, de Magitris L, Stunnenberg H, Vennstrom B 1990 A major thyroid hormone response element in the third intron of the rat growth hormone gene EMBO J 9:887–896
18. Sap J, Munoz A, Schmitt J, Stunnenberg H, Vennstrom B 1989 Repression of transcription mediated at a thyroid hormone response element by the v-erb-A oncogene product. Nature 340:242–245
19. Forman BM, Samuels HH 1991 pEXPRESS: a family of novel expression vectors containing a single transcription unit that is active *in vitro*, in prokaryotes and in eukaryotes. Gene 105:9–15
20. Demay MB, Roth DA, Kronenberg HM 1989 Regions of the rat osteocalcin gene which mediate the effect of 1,25-dihydroxyvitamin D_3 on gene transcription. J Biol Chem 264:2279–2282
21. Demay MB, Gerardi JM, DeLuca HF, Kronenberg HM 1990 DNA sequences in the rat osteocalcin gene that bind the 1,25-dihydroxyvitamin D_3 receptor and confer responsiveness to 1,25-dihydroxyvitamin D_3. Proc Natl Acad Sci USA 87:369–373
22. Perisic O, Xiao H, Lis JT 1989 Stable binding of *drosophila* heat shock factor to head-to-head and tail-to-tail repeats of a conserved 5 bp recognition unit. Cell 59:797–806
23. Xiao H, Perisic O, Lis JT 1991 Cooperative binding of *drosophila* heat shock factor to arrays of a conserved 5 bp unit. Cell 64:585–593

Downloaded from https://academic.oup.com/mend/article/6/4/502/2714532 by guest on 16 August 2022

24. Sorger PK, Nelson CM 1989 Trimerization of a yeast transcriptional activator via a coiled-coil motif. Cell 59:807–813
25. Naar AM, Boutin J-M, Lipkin SM, Yu VC, Holloway JM, Glass CK, Rosenfeld MG 1991 The orientation and spacing of core DNA-binding motifs dictate selective transcriptional responses to three nuclear receptors. Cell 66:1267–1279
26. Murray MB, Towle HC 1989 Identification of nuclear factors that enhance binding of the thyroid hormone receptor to a thyroid hormone response element. Mol Endocrinol 3:1434–1442
27. Lazar MA, Berrodin TJ 1990 Thyroid hormone receptors form distinct nuclear protein-dependent and independent complexes with a thyroid hormone response element. Mol Endocrinol 4:1627–1635
28. O'Donnell AL, Rosen ED, Darling DS, Koenig RJ 1991 Thyroid hormone receptor mutations that interfere with transcriptional activation also interfere with receptor interaction with a nuclear protein. Mol Endocrinol 5:94–99
29. Darling DS, Beebe JS, Burnside J, Winslow ER, Chin WW 1991 3,5,3′-Triiodothyronine (T_3) receptor-auxiliary protein (TRAP) binds DNA and forms heterodimers with the T_3 receptor. Mol Endocrinol 5:73–84
30. Beebe JS, Darling DS, Chin WW 1991 3,5,3′-Triiodothyronine receptor auxiliary protein (TRAP) enhances receptor binding by interactions within the thyroid hormone response element. Mol Endocrinol 5:85–93
31. Yen PM, Darling DS, Chin WW 1991 Basal and thyroid hormone receptor auxiliary protein-enhanced binding of thyroid hormone receptor isoforms to native thyroid hormone response elements. Endocrinology 129:3331–3336
32. Lazar MA, Berrodin TJ, Harding HP 1991 Differential DNA binding by monomeric, homodimeric, and potentially heteromeric forms of the thyroid hormone receptor. Mol Cell Biol 11:5005–5015
33. Brent GA, Williams GR, Harney JW, Forman BM, Samuels HH, Moore DD, Larsen PR 1991 Effects of varying the position of thyroid hormone response elements within the rat growth hormone promoter: implications for positive and negative regulation by 3,5,3′-triiodothyronine. Mol Endocrinol 5:542–548
34. Crone DE, Kim H-S, Spindler SR 1990 α and β Thyroid hormone receptors bind immediately adjacent to the rat growth hormone gene TATA box in a negatively hormone-responsive promoter region. J Biol Chem 265:1–6
35. Lavin TN, Baxter JD, Horita S 1988 The thyroid hormone receptor binds to multiple domains of the rat growth hormone 5′-flanking sequence. J Biol Chem 263:9418–9426
36. Chatterjee VKK, Lee J-K, Rentoumis A, Jameson JL 1989 Negative regulation of the thyroid-stimulating hormone a gene by thyroid hormone: receptor interaction adjacent to the TATA box. Proc Natl Acad Sci USA 86:9114–9118
37. Fawell SE, Lees JA, White R, Parker MG 1990 Characterization and colocalization of steroid binding and dimerization activities in the mouse estrogen receptor. Cell 60:953–962
38. Aususbel FM, Brent R, Kingston RE, Moore DD, Seidman JG, Smith JA, Struhl K (eds) 1987 Current Protocols in Molecular Biology. John Wiley and Sons Inc., New York
39. Prost E, Moore DD 1986 CAT vectors for analysis of eukaryotic promoters and enhancers. Gene 45:107–111
40. Chen EY, Seeburg PB 1985 Supercoil sequencing: a fast and simple method for sequencing plasmid DNA. DNA 4:165–170
41. Selden RF, Bowie KB, Rowe ME, Goodman HM, Moore DD 1986 Human growth hormone as a reporter gene in regulation studies employing transient gene expression. Mol Cell Biol 6:3173–3179
42. Prost E, Koenig RJ, Moore DD, Larsen PR, Whalen RG 1988 Multiple sequences encoding potential thyroid hormone receptors isolated from mouse skeletal muscle cDNA libraries. Nucleic Acids Res 16:6248
43. Seed B, Sheen J-Y 1988 A simple phase-extraction assay for chloramphenicol acetyltransferase activity. Gene 67:271–277

Downloaded from https://academic.oup.com/mend/article/6/4/502/2714532 by guest on 16 August 2022