Characterization of Myelin Basic Protein Thyroid Hormone Response Element and Its Function in the Context of Native and Heterologous Promoter*

(Received for publication, January 16, 1992)

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In this report we have characterized further the myelin basic protein (MBP) gene thyroid hormone response element (TRE) by functional and binding analysis. Mutation and deletion experiments revealed that this TRE, confined to the sequences −184 to −167 of the MBP promoter, is able to function as a classical regulatory element in the context of the native and a heterologous promoter. It is comprised of two regions, containing a motif that is highly conserved among other TREs: AGGACA, arranged as an inverted palindrome. Any mutation within the footprinted region impaired receptor binding and function. Moreover, the deletion of sequences outside of the receptor footprinted region (MBP-TRE-18) resulted in a higher triiodothyronine responsiveness and a concomitant increase in receptor-dependent, hormone-independent repression. Results of transfection assays showed that both receptors α and β elicit indistinguishable triiodothyronine responses when the MBP-TRE functions as a regulator of a heterologous promoter activity. However, a preferential β receptor transactivation was observed when the MBP-TRE was placed in the context of its native promoter.

Hormone response elements (HREs) are specific DNA sequences that are recognized by members of the nuclear hormone receptor superfamily. When receptors interact with HREs they can either activate or inhibit expression of target genes (1–5). Growing evidence suggests that a key element in determining the receptor specificity of these HREs is the presence of highly conserved regions as well as the orientation and spacing of two core binding motifs (6, 7). Mutations that alter spacing between binding sites, resulting in interconversion of receptor specificity, have been reported recently (6–8). In addition, it has been shown that consensus sequences arranged as a direct repeat or as a direct or an inverted palindrome dictate a transcriptional response to retinoic acid, estrogen, and T₃ receptors, respectively (7), implying a critical role for the relative orientation of consensus sequences in the selective transcriptional response.

Among the thyroid hormone receptor superfamily, biochemical and functional studies with the rat growth hormone (rGH) gene, wild type or synthetic thyroid hormone responsive elements (TREs) (9–12), the rat myosin heavy chain (rMHC) α gene-TRE (13), or the rat malic enzyme gene (rME)-TRE (14–16) strongly suggest that the T₃ receptor binds to at least two motifs, each consisting of a consensus hexamer, arranged either as a direct or inverted repeat, containing the sequence AGGTAC or its derivatives. Furthermore, two pairs of G residues centered 10 bases apart seem to be required for the specific interaction with the T₃ receptor (10, 16).

Evaluation of the functional properties of HREs such as thyroid hormone response elements, when placed upstream of thymidine kinase or simian virus promoters, directing expression of the luciferase or chloramphenicol acetyltransferase reporter gene, has provided valuable information about the function of hormone receptors. However, they do not allow evaluation of the modulatory effects exerted by the natural chromatin environment. Thus the identification of natural hormone-responsive elements and their functions in the context of native promoters might expand our knowledge about how nuclear receptors work in vivo.

Several studies testing the ability of steroid receptor proteins to interact with various response elements have demonstrated that the presence of the hormone is absolutely required to enhance transcription (17, 18), although the estrogen receptor, with the hormone binding domain deleted, has been shown to bind to an estrogen response element (17). On the other hand, thyroid hormone receptor, in the absence of the ligand, not only binds to DNA (19–22) but, moreover, displays a unique function. It can repress transcription (19, 20, 22, 23), suggesting different mechanisms in the control of gene expression between steroid and thyroid hormone receptors. In addition, it has been shown that the unliganded receptor, when bound to any TRE, functions as a stronger repressor of thymidine kinase promoter activity as compared with the native promoter and its TRE (16), suggesting a role for flanking sequences in T₃ regulation of target genes.

In our previous report (24) we demonstrated that the expression of a brain-specific gene, the myelin basic protein (MBP) gene, is controlled by thyroid hormone, at least in part, at the level of transcription and that the molecular mechanism of this control is mediated by a specific thyroid hormone receptor interaction with a cis-regulatory element within the MBP 5′-flanking region. Using the DNase I foot-
printing technique and Spodoptera frugiperda 9 (Sf9) nuclear extract infected with recombinant baculovirus expressing thyroid hormone receptor α (TRα), we identified the T3 receptor binding site in the MBP promoter region at position −186/−163. In the present study, we have investigated the structural and functional properties of the MBP-TRE in the context of both the native and heterologous promoters. Results of mutation and deletion analyses have assigned a crucial role to nucleotides within the binding sites, in the region between half-sites, and those flanking a minimal TRE as well.

Our results also show that TRβ is a more efficient T3-dependent activator of the MBP promoter than TRα, whereas no difference between these two T3 receptors was observed when MBP-TRE functioned as an enhancer of thymidine kinase promoter activity.

MATERIALS AND METHODS

Plasmid Construction—The construction of the MBP (−256/+1)-chloramphenicol acetyltransferase (MBP-camCAT) chimeric gene has been described (24). TRE-thymidine kinase CAT (MBP-TRE-TK-CAT) constructs were made according to Desvergne et al. (16). Briefly, synthetic double-stranded oligonucleotides encompassing 5'-flanking MBP sequences (−194/−161) were fused in the linker region of pBLCAT2 reporter plasmid (25) upstream of the −105-base pair tk promoter. Oligonucleotides used in mutation and deletion analyses were as follows (coding strand):

\[
M1 = 5' - \text{CAGAACAATGGGACCTCGGCTGATTACACGGCG-3'}
\]

\[
M2 = 5' - \text{CAGAACAATGGGACCTCGGCTGATTACACGGCG-3'}
\]

\[
M3 = 5' - \text{CAGAACAATGGGACCTCGGCTGAGGACACGGCG-3'}
\]

\[
M4 = 5' - \text{CAGAACAATGGGACCTCGGCTGAGGACACGGCG-3'}
\]

\[
M5 = 5' - \text{CAGAACAATGGGACCTCGGCTGAGGACACGGCG-3'}
\]

\[
M6 = 5' - \text{CAGAACAATGGGACCTCGGCTGAGGACACGGCG-3'}
\]

MBP-TRE 18 = 5' - \text{ACCTCGGCTAGGACACGGCG-3'}

All constructs were sequenced using the dideoxyxynucleotide method (26). The plasmids pRSV-rTRα and pCMV-β-Gal were described previously (15, 16). The rTRβ DNA (pre-erbA-β (−37)) (27), was subcloned into the HpaII/HindIII site of pRSV-CAT (28) to produce pRSV-rTRβ as an expression vector in transfection experiments.

Cell Culture and Transfections—NIH3T3 cells (15–50 passages) were maintained in Dulbecco's modified Eagle's medium containing 10% fetal calf serum. Transfections, CAT assays, and quantitation were performed as described by Desvergne et al. (16). After subculture of the background, the CAT expression (in cpm) was normalized to the units of β-galactosidase activity. Results represent the average of at least three independent experiments.

Gel Mobility Assay—The probe for gel mobility shift assay was a double-stranded oligonucleotide containing sequences of the MBP promoter from −196/−164 end labeled with T4 polynucleotide kinase. Purification by polyacrylamide gel electrophoresis. Binding reactions were carried out by adding 1 μl of nuclear extract from Sf9 cells infected with either the wild type or the recombinant baculovirus overexpressing rTRα (24) to 9 μl of binding buffer containing 100 mM Hepes, 5 mM dithiothreitol, 10 mM MgCl₂, 50% glycerol, 0.5 mg/ml bovine serum albumin (Pharmacia LKB Biotechnology Inc.), and 7 μg of poly(dAdT)-poly(dAdT) as a competitor of nonspecific binding. After 10 min, 1 μl of labeled probe (2 × 10⁶ cpm) was added to the mixing mixture, and incubation proceeded for 50 min. For competition assays, double-stranded oligonucleotides containing mutated and deleted MBP-TRE were added to the binding reactions at the concentrations indicated in the legend to Fig. 2. Autoradiograms were quantitated by densitometric analysis.

RESULTS

Mutation and Deletion Analysis of Myelin Basic Protein-Thyroid Hormone-Response Element—We have shown previously that the region −256/+1 of the myelin basic protein gene promoter is sufficient to confer the T3 receptor-dependent transactivation in NIH3T3 and NG108-15 cell lines and that it contains a cis-regulatory element (−186/−163) to which the receptor binds, as defined by DNase I footprinting analysis (24). To examine the ability of this element to function as an independent TRE we used a double-stranded oligonucleotide (−196/−164) encompassing the footprinted region, flanked by SphI/BamHI restriction sites. A single copy of this oligonucleotide (MBP-TRE-33), was cloned in the linker region of pBLCAT2 reporter plasmid, upstream of the thymidine kinase promoter (−105/+51).

The wild type MBP-TRE-33 conferred −100-fold T3 inducibility to the thymidine kinase promoter in the presence of rTRβ when cotransfected into NIH3T3 cells, indicating the regulatory property of this element linked to a heterologous promoter. Identical results were obtained with rTRα (data not shown).

To define precisely the MBP-TRE core motif and the role of single nucleotides in its function, we generated several mutation and deletion constructs and examined their ability to confer T3 responsiveness. This series of oligonucleotides was made as derivatives of MBP-TRE-33 and was inserted in the pBLCAT2 expression plasmid.

We have shown previously that in ME-TRE two pairs of G residues, 10 base pairs apart, are necessary for the specific interaction with the T3 receptor (16). Thus we mutated two G residues to T at positions −173 and −172 in MBP-TRE-33 (mutant M1) and at positions −185 and −184 (mutant M3). In the MBP-TRE-33 these two groups of G residues are centered 13 base pairs apart (Fig. 1). The results showed that the mutation of G to T in M1 virtually eliminated MBP-TRE function whereas M3 in which the G to T mutation is at position −185/−184 had no effect on T3 responsiveness. This indicates that homology of the MBP-TRE sequence with that of ME-TRE is not the critical determinant for TRE function. Since two sets of crucial G residues in the ME-TRE (16) and growth hormone-TRE (10) are separated by eight nucleotides, we mutated 2 cytidine residues (C to A), at positions −182 and −183 (mutant M4) located 10 base pairs from the essential set of two G residues in the mutant M1. The mutation in M4

![Fig. 1. Mutation and deletion analysis of myelin basic protein gene TRE.](image-url)
abolished T₃ responsiveness as did that in M1. Thus, these two sets of nucleotides may represent the core of two binding motifs in MBP-TRE arranged in an inverted palindromic configuration.

To define further the function of these two potential modules in T₃ responsiveness we synthesized TREs containing point mutations across and adjacent to the footprinted region (–186/–163). A mutation of a G to T at –175 (mutant M2) and two point mutations, a C to A at –180 and a G to T at –179 (mutant M5), showed ~70 and 90% decrease in T₃ inducibility, respectively, indicating that other sequences within the footprinted region are important but to a lesser extent than the essential GG and CC residues located 10 base pairs apart.

To explore a potential role of nucleotides in a putative spacing region between half-sites, we mutated four contiguous nucleotides at position –178/–175 (mutant M6). It appears that these nucleotides separating the core motifs play a role in TRE function since we observed ~85% inhibition in T₃ inducibility when compared with MBP-TRE-33. These results indicate that changes in any nucleotide within the footprinted region, except at positions –184/–185 (M3), significantly affected TRE function.

To find a minimal T₃ response element we constructed a deletion of MBP-TRE-33. Deletion of 12 base pairs at the 5’ end and 3 base pairs at the 3’ end gave the mutant MBP-TRE-18 (Fig. 1). Surprisingly, this construct showed ~4-fold higher T₃ responsiveness than MBP-TRE-33. In accord with the footprinting analysis these data indicate that the most efficient functional TRE is confined to the region –184/–167 (MBP-TRE-18) and suggest that sequences flanking the TRE may be responsible for repressing its full activity.

Receptor-dependent, Hormone-independent Down-regulation of Thyminde Kinase Promoter Activity by MBP-TRE—Cotransfections of a reporter plasmid enclosing MBP-TRE-33 and the thymidine kinase promoter with the thyroid hormone receptor expression plasmid in the presence of the hormone markedly enhanced MBP-TRE-33-tk-CAT expression. This enhancement was in part the result of an approximately 5-fold decrease in basal CAT activity observed in the presence of the receptor and in the absence of T₃ (Table I). Addition of T₃ to the transfected cell cultures, either NIH3T3 or NG108-15, not only restored the level of CAT expression to that observed without the receptor and hormone but further increased it to about 20-fold above the basal level, with an overall change of ~100 fold. These and similar findings described by others (9, 16) led us to test the MBP-TRE mutants in investigating a role of the nucleotide sequences involved in this unusual receptor function. Mutants M1 and M4, which eliminated the response to T₃, abolished the receptor-mediated repression. The M2, M5, and M6 mutants showed a pattern of inhibition of basal CAT expression, in the presence of receptor, which correlates with the degree of induction when T₃ was added. Similarly the superinduction observed with MBP-TRE-18 was partially due to a 2–3-fold greater repression in the absence of the hormone and presence of cotransfected receptor compared with that observed for MBP-TRE-33. However, the correlation between TRE repression and T₃ induction was not perfect since the mutation in M3, which did not alter T₃-dependent function, repressed the basal CAT activity only about 3-fold. Thus, it appears that the two functions, induction and repression, might be partially independent.

Analysis of T₃ Receptor Binding to MBP-TRE—MBP-TRE mutants were tested in competition binding experiments using gel shift assays to analyze the degree of interaction between MBP-TRE and T₃ receptor (Fig. 2, A and B). A ³²P-labeled oligonucleotide encompassing the MBP-TRE (~196/–164) was incubated with nuclear extracts prepared from Sf9 cells infected with wild type baculovirus. Ten- and 50-fold molar excess of unlabeled oligonucleotide (M1–M6) and MBP-TRE-33 (~194/–161) were used as competitors. An unrelated DNA sequence was used as a nonspecific competitor (N.S.). The mixtures were analyzed by gel mobility assay as described under “Materials and Methods.”

TABLE I

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<td></td>
</tr>
<tr>
<td></td>
<td>–R</td>
<td>+R</td>
</tr>
<tr>
<td>MBP-TRE-33</td>
<td>4,989</td>
<td>896</td>
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<tr>
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Fig. 2. Competition analysis of T₃ receptor binding to MBP-TRE-33. A, double-stranded oligonucleotide MBP-TRE-33, 5’ end labeled with T₃ polynucleotide kinase in the presence of [γ-³²P]ATP, was added to TRα protein obtained as the nuclear extract from Sf9 cells infected with recombinant baculovirus. A control nuclear extract was prepared from Sf9 cells infected with wild type baculovirus. Ten- and 50-fold molar excess of unlabeled oligonucleotide (M1–M6) and MBP-TRE-33 (~194/–161) were used as competitors. An unrelated DNA sequence was used as a nonspecific competitor (N.S.). The mixtures were analyzed by gel mobility assay as described under “Materials and Methods.” B, quantitation of the competition assay. Using a 10-fold molar excess of competitor the amount of receptor bound to the labeled MBP-TRE-33 was quantitated by densitometry scanning of autoradiograms and expressed as a percentage of maximal binding in the absence of competitor.
infected with the recombinant baculovirus expressing rTrα and varying concentrations of unlabeled competitor. As expected, addition of unlabeled MBP-TRE-33 resulted in a marked inhibition of receptor binding to labeled MBP-TRE-33 (see Fig. 2B). On the other hand, mutations M1 and M4 that abolished T3 inducibility were no longer effective competitors of receptor specific binding. Oligonucleotides M2, M5, and M6 gave an intermediate pattern of competition correlating with the functional data obtained in the transfection experiments.

These results demonstrate a close correlation between receptor-TRE interaction and function as shown previously for other TREs (10, 11, 16).

**DISCUSSION**

The results reported here extend our previous findings concerning identification and characterization of TRE in the myelin basic protein gene promoter. DNase I footprinting and computer-assisted comparison of the nucleotide sequence with other naturally occurring TREs such as ME and myosin heavy chain (MHC) showed a highly conserved motif, AGGACA, which has been identified as crucial for T3 receptor binding and trans-activation. In the MBP-TRE, transversion mutations of either two G residues (M1) or two C residues (M4), each at the center of the palindromic half-sites, abolished trans-activation, indicating that these two mutations play a primary role in MBP-TRE function. Two of these mutations were inefficient competitors of specific receptor binding in gel shift analysis (Fig. 2, A and B). However, each of the eight nucleotides between the center of two motifs is also important for TR binding and function. As proposed previously (16) these results demonstrate clearly that the receptor interaction with TRE is necessary for its function. Furthermore, mutations within the spacer reduced T3 inducibility, although to a lower magnitude and correlated with competitive binding data. Only M3 with the mutated bases outside the core binding motif was equipotent with MBP-TRE-33.

Truncation of MBP-TRE-33 to give MBP-TRE-18 (~184/-167) resulted in a more efficient T3-dependent enhancer of promoter activity, strongly suggesting that sequences flanking the TRE may be responsible for modulation of the MBP-TRE activity.

In accord with this proposition, we observed a difference between TRα and TRβ transactivation of MBP-TRE expression only in the context of its natural promoter. In particular we found a significantly greater increase in TRβ transactivation (~30-fold) as compared with TRα (~4-fold). Interestingly, this difference was not evident when the MBP-TRE was linked to a heterologous promoter. This observation, together with the results obtained with the deletion mutant MBP-TRE-18, suggest that sequences flanking T3 receptor binding sites may be determinants not only of T3-regulated trans-activation but also of receptor subtype specificity. It is noteworthy that MBP gene expression in vivo correlates with the peak of myelination (28-30) and with a striking increase in the level of TRβ expression in the developing neonatal rodent brain (31).

Although the presence of T3 in transfection assays leads to TR-mediated enhancement in promoter activity, the hormone-depleted condition results in TR-mediated repression, which is more notable when the TRE functions as a modulator of a heterologous promoter. This unusual receptor function has thus far only been ascribed to TRs and not to other members of the hormone receptor superfamily. In accord with receptor-related repression is the binding of receptor to TREs.

**Fig. 3.** Differential effect of TR, α and β, on T3-dependent expression of MBP536-CAT and TRE588-TK-CAT and ME115-CAT. NIH3T3 cells were cotransfected with MBP536-CAT (panel A), TRE588-TK-CAT (panel B), or ME115-CAT (panel C) and expression plasmids coding for TRα (pRSV-rTRα) and TRβ (pRSV-rTRβ) and cultured in absence or presence of T3 (10^-7 m). The cell extracts were assayed for the CAT and β-galactosidase activity. The mean T3 induction ratio is the average of eight independent experiments performed in duplicate. Bars show S.E.

**Fig. 4.** Comparison of the nucleotide sequence of MBP-TRE with other naturally occurring TREs in T3 positively responsive genes. The nucleotide sequences of the T3 receptor binding site of the MBP 5′-flanking region are aligned with those of chicken lysozyme (cLys, 31), rat malic enzyme (rME, 15-16), rat myosin heavy chain a (rMHC, 13), and rat growth hormone (rGH, 9-12) TREs. Numbers, relative to +1, indicate the location of these sequences in the 5′-flanking region of the corresponding gene. Sequence homology is shown in boldface, and motif orientation is indicated by arrows. Shaded boxes designate essential nucleotides for receptor binding and function. Eight-nucleotide spacing is shown by a bracket.
in the absence of hormone. Comparing the results with the MBP-TRE and those with ME-TRE (16), it appears that the degree of repression correlates with the degree of induction when TR is occupied by the hormone. Thus, mutated TREs showing impaired induction of the thymidine kinase promoter function were less efficient repressors of promoter activity as demonstrated by mutations M1, M2, M4, M5, and M6 (Fig. 1). Consequently, in the case of a mutation which causes a greater repression when compared with wild type TRE, addition of the hormone should result in a greater induction of promoter activity. Indeed, deletion of 12 nucleotides upstream of the 5'-end of the DNase I footprint (mutant MBP-TRE-18) results in an approximately 4-fold more efficient TRE in the presence of T3 receptor and hormone than MBP-TRE-33. This increase in T3 responsiveness correlated with an approximately 3-fold greater repression of thymidine kinase promoter activity in the absence of the hormone. On the other hand, the mutant M5 in the presence of the receptor functioned as a repressor of thymidine kinase promoter activity in the absence of T3 but showed no change in T3-dependent transcription activity when compared with MBP-TRE-33. It is likely that some nucleotides may be more important for receptor function in the absence of T3 and others in the presence of the hormone.

Sequence analysis of the T3 receptor footprinted region (~186~/~163) of the 5'-flanking region of the MBP gene encoding the functional TRE showed a high degree of homology to a recently reported T3 response element in the chicken lysozyme gene (32). Sequences comprising MBP and lysozyme TRE motifs are arranged in an inverted palindromic orientation rather than in a direct repeat as found in ME and MHC TREs (Fig. 4). Trans-activation studies with synthetic binding elements containing different arrangements of the idealized GH-TRE, reported recently by Nää et al. (7), showed that the element containing an inverted palindromic arrangement of core motifs separated by three nucleotides displays a very high T3 inducibility in the presence of T3 receptor.

Recently, three independent reports (6,8) attempted to uncover a hormone-dependent receptor specificity within the steroid/thyroid hormone receptor superfamily by investigating the importance of the spacing between two core motifs. Each of these reports introduced its own numbering system and used different HREs, making it difficult to grasp the significance of the spacer size, especially when the core motif could be defined either as a heptamer or a hexamer. Our data with the MBP-TRE together with those of the chicken lysozyme TRE (32), both arranged in an inverted palindromic configuration but separated by either four (this report) or two nucleotides (7), respectively, indicate that the inverted palindromic arrangement of half-sites in naturally occurring TREs with variable spacer gaps confer T3 responsiveness as do the direct repeats with four nucleotide gaps found in the ME and MHC-TRE (6). The precise borders of the core motifs are uncertain, making the spacing somehow arbitrary; however, all known TRE sequences, including ME-TRE and MBP-TRE identified and characterized in our laboratory, when aligned with respect to functionally essential nucleotides (a pair of Gs or GG/CC, (Fig. 4, shaded boxes)), revealed that these pairs are always separated by eight nucleotides. Hence it appears that this spacing is a prerequisite for T3 receptor binding and function with half-sites arranged either as direct repeats (ME, MHC, GH) or inverted palindromes (Lys and MBP). However, a possibility still exists that in addition to two core motifs, appropriately spaced, some other yet unrecognized features required to form thyroid hormone receptor binding need to be uncovered.

In conclusion, we have shown that: 1) Absolutely critical to the TRE of both ME and MBP are two pairs of GG (in ME) and CC and GG pairs (MBP) both separated by 8 bases. 2) All of the intervening bases studied have an appreciable effect on TRE activity. 3) Bases 5' of the footprinted region of the MBP-TRE appear to act as a silencer, implicating the action of an unidentified transcription factor. 4) Mutational analyses also shows that the usual correlation between repression of unliganded receptor does not hold for all mutations. 5) In the homologous MBP-TRE-CAT construct (MBP-TRE-CAT), TRβ is more effective than TRα, and this correlates well with an increase in MBP synthesis and in the concentration of TRβ in fetal and newborn rat brain. However, in a heterologous promoter (MBP→TK-TK-CAT) both receptors are equipotent.

Acknowledgments—We thank Dr. J. E. Rall for manuscript review and also all members of Dr. Nikodem's laboratory for valuable discussion.

REFERENCES