Identification of Thyroid Hormone Response Elements in Rodent Pcp-2, a Developmentally Regulated Gene of Cerebellar Purkinje Cells*

(Received for publication, February 3, 1994, and in revised form, March 2, 1994)

Lanling Zou, Steven G. Hagen, Kevin A. Strait, and Jack H. Oppenheimer

From the Thyroid Research Unit, Section of Diabetes, Endocrinology, and Metabolism, the Department of Medicine and the Department of Cell Biology and Neuroanatomy, University of Minnesota, Minneapolis, Minnesota 55455

In a previous study, we have shown that in vivo expression of the cerebellar Purkinje cell-specific gene Pcp-2 is regulated by thyroid hormone (T3) during neonatal development. In addition, transient cotransfection studies using thyroid hormone receptors (TRs) and a Pcp-2-lacZ construct pointed to direct regulation of Pcp-2 gene expression by T3. Therefore, we have initiated the following series of studies to define more precisely the location of the thyroid hormone regulatory elements in the Pcp-2 gene. By transfection and in vitro receptor binding analyses, we have identified two thyroid hormone response elements, A1 (-295/-268) and B1 (+207/+227). A1 contains a central half-site flanked by two similar half-sites, while A1 contains two pairs of alternate half-sites. When these elements were ligated to the modified mouse mammary tumor virus promoter (AMMTV), both induced a 8-14-fold expression of the reporter gene, but only in the presence of T3. Gel mobility assays demonstrated that both A1 and B1 bind TRs in the presence of thyroid hormone receptor auxiliary proteins or the retinoid X receptor. Mutations of the G residues to T within the individual half-site sequences of A1 caused a variable decrease in the transactivation of the AMMTV-CAT construct and a corresponding reduction in TR binding in vitro. Thus, mutational analysis of A1 pointed to the interaction of the flanking half-site motifs with the central AGGTCA half-site. Increasingly, lengthening of the A1 sequence at its 3'-end caused a progressive dampening of the T3 response. The results suggest that the neighboring sequence may function as a silencer of the A1 element. Since thyroid hormone regulation of Pcp-2 is manifest only during the first 2 weeks after birth, we hypothesize that A1 and B1 act as T3-dependent response elements operative only during early neonatal Purkinje cell development and that their function is suppressed by a neighboring silencer element operative when expression of Pcp-2 becomes hormone-independent.

Thyroid hormone (T3) plays an essential role in the normal development of the mammalian brain (1). In the rat, this effect is apparent only during the first 2 weeks of neonatal life. Absence of the hormone during this period leads to irreversible structural damage (2). Previous studies from our laboratory have attempted to analyze the molecular basis of this action. We have documented the effect of thyroid hormone on the expression of four brain genes during the first 2 weeks of life (3). Three of these genes are expressed in cerebellar Purkinje cells, a well recognized cellular target of thyroid hormone during brain development (4). The fourth gene, myelin basic protein, is expressed in oligodendrocytes and contains a previously defined thyroid hormone response element (5). The rise of these mRNAs occurs in the neonate, immediately preceded by a 40-fold surge in the mRNA for the TR3 isoform and the accompanying rise in the level of brain triiodothyronine (6). These findings have prompted us to speculate that the T3-TR3 complex plays a pivotal role in mediating the effects of thyroid hormone in brain development. An examination of the early time course of the three Purkinje cell mRNAs and the myelin basic protein mRNA in euthyroid pups and in pups rendered functionally athyroidal revealed the presence of both T3-dependent and T3-independent components in the increased gene expression observed during neonatal development. In the absence of T3, the levels of all four mRNAs rise slowly, but eventually plateau at the same level as that reached in euthyroid pups, approximately between postnatal days 45 and 60. In contrast, euthyroid animals reach maximal gene expression between postnatal days 15 and 20, coincident with the period of Purkinje cell differentiation and myelination. Given the carefully synchronized program of cellular interactions characteristic of central nervous system development, we have hypothesized that a delay in achieving optimal gene expression could explain the irreversible central nervous system damage observed in the hypothyroid neonate.

In an effort to explore the mechanisms underlying the regulation of the Pcp-2 gene during early brain development, we undertook a series of transient transfection experiments. The results of these studies originally led us to infer that TR3 regulates the Pcp-2 gene in an isoform-specific fashion and that such regulation, although augmented by T3, could occur in the absence of ligand (3). Recent experiments in our laboratory, however, have modified our interpretation of these results. Our inference of isoform specificity was based on the mistaken assumption that both TRα1 and TRβ1 had been inserted into the CDM8 expression vector. In reality, the vector CDM was used for TRβ1 and CDM8 for TRα1. CDM and CDM8 (7) in our system exhibit differential response characteristics. The apparent isoform-specific and ligand-independent effects of TRβ1 could be attributed to the fortuitous circumstance that TRβ1

MMTV, mouse mammary tumor virus; CAT, chloramphenicol acetyltransferase.
had been inserted into CDM and TRal into CDM8. In essence, our newer findings point to the importance of vector-related factors in determining what were interpreted as isoform-specific and ligand-independent phenomena. Despite these reinterpretations of our earlier data, we continue to find that T3 augments expression of Pcp-2-lacZ in transfected CHO cells when sufficient TR is present.²

In this report, we have attempted to analyze these regulatory mechanisms in greater detail. An examination of the Pcp-2 nucleotide sequence revealed one region in the 5'-upstream region and one in intron 1 compatible with thyroid hormone response elements (TREs). We have attempted to determine whether these sequences confer thyroid hormone responsiveness to a heterologous promoter and whether they bind TRs in a specific fashion. Our studies have also generated evidence that a nucleotide sequence adjacent to the TRE in the upstream region may act as a silencer of T3-mediated regulation, a finding that may help to explain the mechanism responsible for restricting T3 influence on brain development to a well defined interval in the neonatal rat.

MATERIALS AND METHODS

DNAs and Transient Transfection Assays—Oligonucleotides were synthesized on a 391 DNA synthesizer PCR-MATE (Applied Biosystems, Inc.). All oligonucleotides contained a HindIII site on both ends to allow for ligase. Paired oligonucleotides were annealed and ligated into the HindIII site of plMMTV-CAT (8). The sequence and orientation were confirmed by restriction mapping and DNA sequencing. A1 extension fragments were prepared by PCR using a Coy Laboratory Products Temp Cycler II (Model 1105S). Primers were synthesized to generate the desired fragment, and PCR was carried out for 35 cycles with the following steps: 1) denaturation for 60 s at 94 °C, 2) annealing for 60 s at 50 °C, and 3) extension for 60 s at 72 °C. Standard reaction conditions were as follows: 10 nm Tris (pH 8.3), 50 nm KCl, 2.5 nm MgCl₂, 0.5 nm dNTPs, 0.5 nm primers, 1 ng of template DNA, and 2.5 units of Taq polymerase in a total volume of 100 μl. PCP-2 polymer products were analyzed by gel electrophoresis. Transfections were performed as described by Strait et al. (3).

Preparation of in Vitro Translated Receptors and Hormone Binding Assay—The α2 cDNA from rat brain was from C. C. Thompson and R. M. Evans (Gene Expression Laboratory, The Salk Institute). The β1 cDNA was a gift from Howard C. Towle (Department of Biochemistry, University of Minnesota Medical School). The mouse RYR β2 cDNA was from Dr. Keiko Ozato (National Institute of Child Health and Human Development, National Institutes of Health, Bethesda, MD) and was modified and subcloned by Howard C. Towle. All three cDNAs were subcloned into pET28R containing the T7 promoter and translational enhancer from alfalfa mosaic virus (9, 10). The cDNAs were transcribed, and the resulting mRNA was translated as previously described by Schueler et al. (11). The in vitro translated products were purified by 125I[methionine incorporation and SDS-polyacrylamide gel electrophoresis and quantitated by the T3 binding capacity determined by saturation analysis (11).

Nuclear Extract Isolation—Nuclear extracts from rat liver, cerebellum, and cerebral cortex were obtained by the method of Oppenheimer et al. (12). The final desalting step was performed by dialyzing overnight in buffer (20 mm Hepes, 20% glycerol, 0.1 mm KCI, 0.2 mm EDTA, 2-5 mm phenylmethylsulfonyl fluoride, and 1 mm dithiothreitol). Nuclear extracts from CHO cells were obtained by the method described by Andrews and Feller (13). Protein concentrations were determined by the assay of Lowry et al. (14) or Bradford (Bio-Rad) (26). Nuclear extracts were aliquoted and stored at -80 °C.

RESULTS

Potential Thyroid Hormone Regulatory Regions of Pcp-2—Previous transfection experiments in our laboratory used a construct containing ~800 bp of the Pcp-2 gene: 408 bp of upstream promoter, the first exon, the first intron, and part of the second exon constituting the untranslated region of the Pcp-2 mRNA fused to lacZ as a reporter (15). When this construct was cotransfected into CHO cells with TRβ1, we observed a statistically significant (~50%) increase in lacZ expression in response to T3 (3). A subsequent search of these regions of the Pcp-2 gene for potential TREs revealed three sequences of interest (Fig. 1). Two of these sequences reside in the 5'-upstream region of the gene and are designated A1 (~295/-268) and A2 (~227/~206). The other is situated in the first intron and is designated B1 (+207/~227). These sequences were selected according to currently accepted criteria suggesting that TREs consist of two hexamers containing homology to the sequence AGGTCA and separated from each other by 4 base pairs (16).

As shown in Fig. 1, A1 contains three potential half-sites: an "ideal" AGGTCA half-site flanked by a second upstream half-site with 4-nucleotide spacing as well as a downstream half-site with 6-nucleotide spacing. A1 also has two potential half-sites located on the opposite strand. A2 shows a loose homology to the palindromic TRE sequence (AGGTCACTGACCT) of Glass et al. (17). Finally, B1 contains two potential halves of half-sites: an

² The experimental basis for our reinterpretation of our earlier data is as follows. Use of the CDM vector for the expression of TRal resulted in an increase in Pcp-2-lacZ expression comparable to that observed when TRβ1 was expressed by the same vector. This was the case in both the absence and presence of T3. Moreover, even without expression of TR, insertionless CDM by itself augmented the expression of Pcp-2-lacZ. The mechanism underlying such regulation remains unclear, but could involve stimulation of Pcp-2 gene expression by the binding of a negatively regulating transacting factor. However, in the absence of expressed receptors, T3 had no effect on the regulation of Pcp-2. These observations suggest that CDM vector rather than TR is important in determining the ligand-independent and apparently TR isoform-specific phenomena observed.

Fig. 1. Schematic representation of the Pcp-2 gene. Top, the sequence of the Pcp-2 gene from positions ~408 to +433 was examined for regions of similarity to the consensus TRE half-site AGGTCA sequence. Shaded ovals indicate the positions of three potential regions of interest (A1, A2, and B1). A1 and A2 are located in the 5'-upstream region of the gene, while B1 is found in the first intron. EX1, INT1, and EX2 are the first exon, the first intron, and a portion of the second exon that compose the 5'-untranslated region of the Pcp-2 mRNA, respectively. Bottom, the precise positions as well as the sequences of each of the three sites of interest are shown. For A1 and B1, the exact positions and orientations of potential half-sites are indicated (underlined). For A2, homology to the ideal TRE palindrome is indicated with vertical lines. asterisks indicate a mismatch due to the lack of a corresponding nucleotide.
ideal AGGTCA half-site 5’ to a second potential half-site with 4-nucleotide spacing and, on the same strand, a second pair of potential half-sites also with 4-bp spacing. We tested the potential activity of these sequences as TREs by synthesizing corresponding oligonucleotides and inserting them into the modified ΔMMTV promoter construct fused to a CAT reporter. The site of insertion in MMTV was the deleted glucocorticoid response elements (−88/−190) of the MMTV long terminal repeat (8, 18).

Cotransfection of CHO cells with TR81 or TRα1 and ΔMMTV-CAT inserted with A1, B1, or TREpal resulted in a 7.5–14.5-fold increase in CAT activity only when T3 was present (Table I). Thus, both A1 and B1 are capable of acting as TREs in CHO transfection assays. In contrast, when A2 was inserted into ΔMMTV-CAT, the stimulation of CAT activity, either in the presence or absence of T3, did not differ from that observed with the insertionless ΔMMTV-CAT vector. The slight 2-fold induction by T3 of the insertionless ΔMMTV-CAT vector required cotransfection of TRs and was a consistent finding in our studies (data not shown). We surmise that this phenomenon may be the result of a weak thyroid hormone response element located in the MMTV long terminal repeat (−1200 bp) as it was not seen when the basal MMTV promoter (−88/+1) was used. This activation is similar to reports of a TRE in the thymidine kinase promoter (18). Therefore, these findings make it unlikely that A2 contains a conventional TRE.

Silencer of T3 Regulation—Since the A1 sequence inserted into ΔMMTV-CAT confers responsiveness to T3, it is tempting to consider the possibility that A1 functions in the induction of the Pcp-2 gene by T3 in early neonates. However, only a minimal T3-dependent effect was observed when the entire 205-bp A fragment (−408/−204), which includes the 28-bp A1 sequence (−295/−268), was inserted into ΔMMTV-CAT (3). These findings suggest that other elements in the larger A fragment (−408/−204) “silence” the T3-dependent TRE properties of the A1 sequence.

To clarify further the significance of the 28-mer A1 sequence within the larger 205-bp A fragment, we applied PCR techniques to extend the length of A1 in the 5’-direction. Fig. 2 shows the nucleotide coordinates of these larger fragments containing A1 and indicates the -fold induction in CAT activity elicited by T3. The results show a gradual decrease in the T3 response as the A1 sequence is extended 3’. These findings suggest that neighboring sequences suppress the response of the A1 element to T3. The ability to inhibit T3 response was sequence-specific since a random 75-bp fragment from pTZ18R ligated to A1 did not suppress the T3 response. Regulation of T3

### Table I

<table>
<thead>
<tr>
<th>ΔMMTV-CAT constructs</th>
<th>T3</th>
<th>TR81</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pep-2 A1</td>
<td>13.6 ± 3.6</td>
<td>13.1 ± 1.1</td>
</tr>
<tr>
<td>Pep-2 B1</td>
<td>7.5 ± 1.2</td>
<td>13.9 ± 4.3</td>
</tr>
<tr>
<td>Pep-2 A2</td>
<td>2.1 ± 0.7</td>
<td>1.9 ± 0.5</td>
</tr>
<tr>
<td>TREpal</td>
<td>13.7 ± 2.8</td>
<td>14.5 ± 3.3</td>
</tr>
</tbody>
</table>

### Fig. 2

A1 activity is silenced by its neighboring sequence. The sizes and positions of the A1 extension fragments at the 3’-end are listed. Fragments of the Pcp-2 gene were prepared by PCR as described under “Materials and Methods.” These fragments were inserted into ΔMMTV-CAT, and the T3 responsiveness was examined by transient transfection of CHO cells. The -fold induction is the ratio of CAT activity when cotransfected with TR81 in the presence and absence of T3. The 75 bp of random DNA was obtained from restriction enzyme digestion of pTZ18R.

### Table II

<table>
<thead>
<tr>
<th>A1</th>
<th>Fold +/− T3</th>
</tr>
</thead>
<tbody>
<tr>
<td>268</td>
<td>11.8 ± 2.3</td>
</tr>
<tr>
<td>-253</td>
<td>5.8 ± 1.7</td>
</tr>
<tr>
<td>-199</td>
<td>2.6 ± 0.4</td>
</tr>
<tr>
<td>75 bp of Random DNA</td>
<td>12.7 ± 2.8</td>
</tr>
</tbody>
</table>

### Fig. 3

Binding of TR81 to Pcp-2 A fragment (−408/−204) in presence of various nuclear extracts. The preparation of in vitro translated TR81 or TR81 and the nuclear extracts from rat tissues is described under “Materials and Methods.” The 204-bp 32P-labeled Pcp-2 probe was incubated with 5 μl of TR81 translation products and 1.5 μg of nuclear protein from rat liver (Li), cerebellum (CB), or cerebrum (CE) or no nuclear extract (−). “Silencer” function would provide a means of altering the responsiveness of the Pcp-2 gene to T3 during brain development.

### Localization of TR-binding Site within 205-bp A Region

We performed gel retardation analyses to determine the ability of the Pcp-2 sequences to bind TRs in vitro (Fig. 3). The 205-bp Pcp-2 A fragment (−408/−204) containing both A1 and A2 sequences was isolated and end-labeled with 32P. When in vitro translated TR81 was incubated with the 32P-labeled A fragment and analyzed on a nondenaturing gel, no complexes with retarded electrophoretic migration were detected (lane 1). Addition of the nuclear extracts from various rat tissues resulted in the formation of multiple retarded bands (lanes 2–4). The requirement of nuclear proteins in the binding assay is consistent with reports that auxiliary nuclear binding proteins are essential for receptor binding in vitro (19, 20). The tissue-specific pattern of the DNA-protein complexes suggests that multiple proteins or tissue-specific modification of a single protein is involved in the binding of the DNA by TR81. Although two major complexes were detected with the nuclear extracts from rat liver, cerebellum, and cerebrum, the migration patterns of shifted bands differed slightly in these tissues (lanes 2 and 4). Mixing the probe with the nuclear extracts or the unpro-
of cerebellar brain nuclear protein extract were incubated with an increasing molar ratio of unlabeled TREpal (lanes 5–7), TREpal (lanes 5–7), Pcp-2 A1 (lanes 5–7), Pcp-2 A2 (lanes 8–10), or a random 25-bp sequence (lanes 11 and 12). After 10 min of incubation at room temperature, the 32P-labeled Pcp-2 A fragment (−4081–204) was added to the reaction, and incubations were continued for an additional 30 min. TREpal binding was analyzed on a 4.5% nondenaturing gel. Arrows indicate the thyroid hormone receptor-specific complexes.

Similarly, the finding that the A1 sequence efficiently competed for the protein binding with the A fragment (lanes 5–7) whereas the A2 sequence did not (lanes 8–10) suggests that A1 and not A2 contains a TR-binding site within the larger A fragment. The B1 sequence was also effective at competing with the A fragment (data not shown). These findings are in accord with the conclusion reached from the transfection assays.

Both A1 and B1 Oligonucleotides Bind TRs Efficiently in Presence of RXRβ—Recent studies have demonstrated that RXRβ can form heterodimers with TRs and thus functions as a thyroid hormone auxiliary protein (21–23). To determine whether RXRβ also functions as an auxiliary protein for Pcp-2 TREs and to demonstrate that A1 and B1 can bind TR directly, we substituted in vitro translated RXRβ for the nuclear extract in a gel retardation assay using labeled A1 and B1 sequences (Fig. 5). The A1 and B1 sequences bound both TRα1 and TRβ1 equally well. As expected, the positive control TREpal also bound both receptors when RXRβ was present, whereas the random sequence failed to bind either receptor. These results confirm that A1 and B1 contain functional TREs that resemble TREpal in that they appear to bind TR as heterodimers with auxiliary proteins such as RXRβ. The sequence specificity of the TR–RXRβ heterodimers was demonstrated by displacement with the specific unlabeled oligonucleotides (data not shown).

For the relative binding affinity of these oligonucleotides was determined by a competition assay using labeled TREpal, TRβ1, RXRβ, and increasing amounts of unlabeled TREpal, A1, B1, or a random oligonucleotide (Fig. 6). The results show that the concentration required for complete displacement is much lower for both A1 and B1 than for TREpal. The results suggest that Pcp-2 TREs (A1 and B1) have a TR-binding affinity higher than that of TREpal. The random sequence oligonucleotide did not bind the receptor.

**AGGTCA Is Essential But Not Sufficient for TR Binding and T3 Response**—The Pcp-2 A1 sequence contains three potential half-sites (Fig. 1). To delineate further the importance of these half-sites, the GG residues in each of the three potential half-sites were mutated to TT. The effects of these mutations on the ability of T3 to transactivate were tested using the AMMTV-CAT construct in transfected CHO cells. Fig. 7 defines the mutations and indicates the -fold increase in CAT expression achieved by T3 when each construct was cotransfected with TRβ1. Mutation of the central AGGTCA site (mutation 1) completely abolished the T3 response. Mutations in each of the flanking half-sites (mutations 2 and 3) reduced but did not
abolish T₃ induction of CAT activity. However, when both 5' and 3' half-sites were mutated, the T₃ response was again completely lost. This implies that the central AGGTCA half-site alone is not sufficient to confer T₃ responsiveness. The lack of activity in mutation 4 also indicates that the sequence AGACCA, a potential half-site located 3' to and on the opposite strand of the central AGGTCA sequence, does not function as a TR-binding site. Thus, the A1 element appears to consist of AGGCCT 5'-site is palindromic, and thus, the identical sequence is also present on the opposite strand of DNA. Therefore, dimer binding could occur as either a direct repeat with 4-base spacing or an inverted repeat with 4-base spacing. The ability of the mutated A1 oligonucleotides to bind TRs in vitro was examined by gel retardation assay. The relative affinity of mutants 2-4 was examined by titrating the effect of increasing quantities of TRβ1 on the gel retardation of ³²P-labeled oligonucleotide (Fig. 8). Reaction mixtures contained a fixed amount of RXRβ. The capacity of these oligonucleotides to gel shift correlates with their ability to mediate T₃ regulation in transient transfections. The results show that mutation of the 3'-site (mutant 3) does not dramatically interfere with the binding by TRβ1-RXRβ. However, mutation of the 5' site (mutant 2) caused a marked reduction in binding. Mutation of both 5' and 3'-sites (mutant 4) failed to show any binding at all. In addition, mutation of the central half-site (mutant 1) also failed to bind TRβ1-RXRβ (data not shown). These data suggest that neighboring half-sites are involved in TRβ1-RXRβ binding by forming alternate dimerization sites with the central AGGTCA sequence and that dimers occur at either pair of half-sites. These results also suggest that the middle site and the 5'-half-site containing 4-base spacing are the preferred dimerization binding sites for TRβ1-RXRβ under these in vitro conditions.

No Significant Difference of in Vitro Binding between TRα1 and TRβ1—We have also compared in titration experiments the binding of TRα1 and TRβ1 to the Pcp-2 A fragment, which contains both A1 and A2 (data not shown). Increasing amounts of the TRα1 or TRβ1 translation products were incubated with a fixed amount of the probe and RXRβ. We failed to observe any consistent difference in the affinities of TRα1 and TRβ1. Similar results were obtained with the Pcp-2 A1 and B1 probes and with cerebellar or hepatic nuclear protein extracts. These results confirm that the TRβ1 binding sites are the preferred dimerization binding sites for TRβ1-RXRβ under these in vitro conditions.

TRE Comparison:

PCP-2 A1 (295/269) AGGCCCTCTAGGTCAGAGACCAGAGA
PCP-2 B1 (+207/+227) AGGCGAGTCAAGGAAGGAGA
GH (TRE) (-160/+190) AAGGTAAGATCGGGAGCAGTACCCAGGAGA

Fig. 9. Comparison of Pcp-2 A1 and B1 sequences with growth hormone TRE. The Pcp-2 A1 sequence contains three functional half-sites (underlined). Two possible TREs are present in the Pcp-2 B1 sequence. The 4-nucleotide spacing between half-sites is shown with open letters. The sequence of growth hormone (GH) TRE from positions -160 to -190 is also listed for comparison.
sults indicate that, under these in vitro binding conditions, both TRα1 and TRβ1 are capable of binding to Pcp-2 TREs.

**DISCUSSION**

An analysis of Pcp-2 regulation by T₃ is of special interest since this gene may serve as a model of other genes influenced by thyroid hormone during brain development. We have previously suggested that the β1 isoform of the thyroid hormone receptor may play a special role in this process in that the level of TRβ1 rises in the brain concomitant with the increase in brain T₃ and just prior to the well characterized structural and functional changes induced by the hormone (6). The demonstration that the rise in Pcp-2 mRNA is typical of three other mRNAs that are regulated by thyroid hormone further underscores the potential utility of the Pcp-2 model.

Our earlier experiments, however, were disappointing in that transient transfection experiments with the Pcp-2 reporter revealed only a modest 50% increase in reporter gene expression (3). Several explanations deserve consideration. It is possible that the regulation of Pcp-2 expression observed in the neonatal rat is mediated by indirect mechanisms, thus obviating the necessity of TREs. This would be somewhat surprising in view of our data showing the presence of two potentially functional TREs (A1 and B1) situated within the promoter region. The presence of functional TREs suggests that thyroid hormone may serve as a model of other genes influenced by the hormone directly regulates the expression of the myelin basic protein gene in oligodendrocytes. A more likely explanation of the limited increases achieved in transient transfection studies is that CHO cells are not ideal hosts for demonstrating the hormonal regulation of the Pcp-2 promoter.

The results of this series of experiments provide further support for this explanation. Analysis of the 5'-upstream region and first intron of the Pcp-2 gene identified one sequence in each that could behave as a bona fide TRE when ligated to a heterologous viral promoter. Thus, when CHO cells were cotransfected with TRα1 or TRβ1 and MMTV-CAT containing either the A1 or B1 sequence, addition of T₃ resulted in a 7.5-13.9-fold increase in CAT activity, similar to the increase observed with TREpal. In contrast, another sequence from the upstream region (designated A2) that exhibited a loose homology to TREpal did not confer T₃ responsiveness to MMTV-CAT. Additional evidence for the functional role of the A1 site comes from our demonstration of the importance of the GG doublets in mediating transactivational regulation by T₃ (Fig. 7). Mutation of the GG residues to GT resulted in marked impairment of T₃ regulation in transfection assays as well as diminished TRβ1-ROM binding in gel retardation assays (Fig. 8).

Our gel retardation assays clearly indicate that the proposed response elements bind thyroid hormone receptors. In accordance with previous studies by others (19, 20), our results show that the receptor by itself does not appear to bind strongly to the A1 element. Nuclear extracts from several tissues interact with the thyroid hormone receptor to facilitate receptor binding to TRE and retard the electrophoretic motility of the complex. In accordance with other reports, RXRβ could substitute for nuclear extracts in facilitating TR binding to TRE (21–23). Of particular importance is the demonstration that unlabeled TRE palindrom and A1 successfully compete for the receptor and the auxiliary protein as demonstrated by the inhibition of the gel shift of the labeled A fragment (408-204). To the contrary, A2 does not compete, suggesting that this sequence does not function as a TRE.

In common with the experience of others, there does not appear to be any readily demonstrable isoform specificity in the interaction of TRα1 and TRβ1 with TREs defined for Pcp-2. Thus, neither the transfection assays nor in vitro binding studies have succeeded in elucidating the basis for the putative functional specificity attributed to receptor isoforms. A detailed evaluation of the 25-bp A1 fragment is of special interest. There appear to be three half-sites, either identical to or related to the sequence AGGTCA (Fig. 2) (17). Our data suggest that all three half-sites can play a role in T₃ regulation. The central AGGTCA sequence appears to be critical for TRE function since mutations of this sequence completely disrupt T₃-related regulation. A similar effect is achieved when the GG residues of both the 3'- and 5'-half-sites are simultaneously mutated, leaving only the central half-site intact. The effect of mutating either the 3'- or 5'-sequence alone produces only a partial reduction in transactivation. We hypothesize that minimal transactivation requires at least one set of adjacent half-sites: the central site and the 5'-half-site or the central site and the 3'-site. Full activation involves all three half-sites. The functional effect of mutagenesis was generally paralleled by changes in receptor binding.

Comparison of the Pcp-2 TRE to the TRE of the growth hormone gene (24) revealed several common features (Fig. 9). First, both contain multiple subregions important for T₃ response. Second, only a few of the half-sites are direct repeats with 4 base pairs. Recent studies have emphasized the importance of half-site spacing for receptor activity (16). Third, both growth hormone and Pcp-2 contain a TRE in an intron. In fact, the TRE in the third intron of the growth hormone gene is more efficient than the one in the promoter region (25).

A novel feature of the Pcp-2 gene is the presence of a nucleotide sequence of 75 nucleotides 3' to the A1 site that serves to attenuate the capacity of A1 to mediate thyroid hormone regulation of Pcp-2 gene expression. The specificity of this sequence to silence T₃ regulation is apparent from the finding that the substitution of a random 75-bp sequence fails to modify the regulatory function of the A1 sequence. One can speculate that the silencer sequence may serve to restrict thyroid hormone development effects to the first 2 weeks of life of the newborn rat. If one assumes that the silencer sequence is dependent on a transient transacting factor that appears as the Purkinje cell matures, one could explain the lack of T₃ effect in the adult animal. We are currently attempting to test this hypothesis.

The existence of specific silencer systems that block hormonal regulation may also help to explain the difference in hormonal response of the same gene in different tissues.

**Acknowledgments**—We thank the following people for providing probes: Pcp-2-lucZ, Harry T. Orr (University of Minnesota); TREpal expression construct, Ronald J. Koenig (University of Michigan); TRβ1 expression construct, Howard C. Town; ΔMMTV-CAT, Herb H. Samuels (New York University, New York, NY); and RXRβ expression construct, Keiko Ozato.

**REFERENCES**

TREs in Gene Ppc-2