In vivo regulation of β-MHC gene in rodent heart: role of T₃ and evidence for an upstream enhancer

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Wright, Carola E., F. Haddad, A. X. Qin, P. W. Bodell, and K. M. Baldwin. In vivo regulation of β-MHC gene in rodent heart: role of T₃ and evidence for an upstream enhancer. Am. J. Physiol. 276 (Cell Physiol. 45): C883–C891, 1999.—Cardiac β-myosin heavy chain (β-MHC) gene expression is mainly regulated through transcriptional processes. Although these results are based primarily on in vitro cell culture models, relatively little information is available concerning the interaction of key regulatory factors thought to modulate MHC expression in the intact rodent heart. Using a direct gene transfer approach, we studied the in vivo transcriptional activity of different-length β-MHC promoter fragments in normal control and in altered thyroid states. The test β-MHC promoter was fused to a firefly luciferase reporter gene, whereas the control α-MHC promoter was fused to the Renilla luciferase reporter gene and was used to account for variations in transfection efficiency. Absolute reporter gene activities showed that β- and α-MHC genes were individually and reciprocally regulated by thyroid hormone. The β-to-α ratios of reporter gene expression demonstrated an almost threefold larger β-MHC gene expression in the longest than in the shorter promoter fragments in normal control animals, implying the existence of an upstream enhancer. A mutation in the putative thyroid response element of the −408bp β-MHC promoter construct caused transcriptional activity to drop to null. When studied in the −3,500bp β-MHC promoter, construct activity was reduced (~100-fold) while thyroid hormone responsiveness was retained. These findings suggest that, even though the bulk of the thyroid hormone responsiveness of the gene is contained within the first 215bp of the β-MHC promoter sequence, the exact mechanism of triiodothyronine (T₃) action remains to be elucidated.

transcription; dual luciferase; in vivo gene transfer; thyroid response element; β-myosin heavy chain

ADULT MAMMALIAN CARDIAC muscle expresses two genes encoding myosin heavy chains (MHCs), which have been designated α- and β-MHC (20, 27, 28). The α-MHC gene encodes the α-MHC protein product, homodimers of which form a native myosin designated the high-ATPase, V1 isoform. In contrast, the β-MHC gene encodes the β-MHC protein product, homodimers of which form the low-ATPase, V3 isoform. Posttranslational assembly of the α and β products also gives rise to a protein heterodimer, designated the moderate-ATPase, V2 isoform. Each of these native myosin isozymes contains the same complement of myosin light chains. The α- and β-MHC genes are members of a multigene family in which each of the genes is expressed in a muscle type- and developmental stage-specific fashion (25, 27). Whereas α-MHC is expressed only in the heart, β-MHC is expressed in the heart and is also the major myosin isoform in slow-twitch skeletal muscle (27). The α- and β-MHC genes are arranged in tandem in the genome, separated by only ~4 kb of intergenic sequence (26). Expression of the two genes is closely linked and tightly regulated in a reciprocal fashion (25, 27, 28).

The relative expression of these MHCs is highly plastic in cardiac cells of different mammalian species, spanning a wide variety of pathophysiological states (1, 17, 25, 27, 28, 32, 39). Moreover, the differential expression of the MHCs impacts significantly the intrinsic functional properties of the heart. During embryonic and fetal development, β-MHC is the predominant isoform expressed in the heart. In rats, shortly after birth, most of the β-MHC is quickly replaced by α-MHC. In the adult rodent the α-MHC is the predominant isoform expressed in the euthyroid animal, accounting for ~85–90% of the total MHC protein pool, whereas the β-MHC accounts for the remaining 10–15%, a pattern that is consistent with the steady-state level of the MHC mRNA that is expressed (17). However, this profile can be altered by a variety of experimental interventions, such that the β-MHC relative expression is significantly upregulated to various levels depending on the particular intervention.

Expression of the MHC genes in the rodent heart is extremely sensitive to the thyroid status of the animal. Induction of hypothyroidism causes a switch in isofrom expression, so that the β-MHC becomes the major isoform expressed in the myoccardium of these animals (20, 25). On the other hand, administration of additional thyroid hormone has the opposite effect and reduces β-MHC expression to a minimal level while increasing α-MHC expression. This increase/decrease in the β-MHC expression is detected at the protein and mRNA levels, and the intensity of this upregulation/downregulation depends on the potency of the stimulus. Because mRNA signals and protein levels appear to be tightly coupled in a given steady state (16, 17), we interpret these responses to suggest that there are likely transcriptional/translational processes regulating the expression of the β-MHC gene. This notion has been confirmed by us and others using nuclear run-on assays (3, 40).

The molecular signals involved in β-MHC transcriptional control are not well defined. However, recent studies have begun to shed some light on the nuclear factors involved in the in vivo transcriptional regulation of the β-MHC gene. Previous work that focused on functional analyses of the β-MHC gene promoter and used transgenic mice (23, 24, 37), myocytes in culture (10, 11, 21, 22, 38, 42), or direct gene transfer (5, 6,
suggests the interplay of cis and trans factors in the regulation of β-MHC gene expression. Most of these studies have focused on the proximal 400 bp of the promoter sequence. On this 5'-flanking regulatory region, three cis-regulatory elements have been implicated in the positive and an additional two in the negative regulation of β-MHC gene transcription and its tissue-specific expression. These cis elements, when bound by specific nuclear proteins, control the rate of transcription. Transcriptional control of the β-MHC gene involves complex interaction between cis-acting DNA sequences, their cognate trans-acting protein factors, and the basic transcription machinery. One of the negative regulatory elements, a thyroid response element (TRE), has been proposed to be located within the basal promoter, where binding of the transcription machinery is necessary to initiate transcription. The purpose of the present study was to examine the mechanism of transcriptional regulation of the rodent β-MHC gene under normal control and thyroid-manipulated (hypo- or hyperthyroid) conditions with use of an in vivo approach. Here we report that a long 3,500-bp promoter fragment is necessary for optimal transcriptional activity of the β-MHC gene. A putative enhancer element appears to be contained within −2,900 to −3,500 bp of the β-MHC promoter sequence. Our data also suggest that there may be an interaction between this upstream enhancer in combination with its trans-acting protein factor(s) and a downstream element(s) that is located within the first 408 bp of the promoter sequence.

MATERIALS AND METHODS

Animal model and DNA injection procedure. All animal-related procedures described in this study were approved by our institutional animal care and use committee. Young adult female Sprague-Dawley rats (150 g body wt; Taconic Farms, Germantown, NY) were used for all experiments.

For DNA injection into the myocardium the rats were deeply anesthetized with ketamine (40 mg/kg) and acepromazine (1 mg/kg), the abdomen was opened using sterile techniques, and the heart was felt by palpation through the diaphragm. Forty microliters of sterile PBS containing an equimolar (equivalent to 10 µg of −3.5-kb β-MHC pGL3) mixture of two supercoiled DNA plasmids were injected into the myocardium through the diaphragm with use of a 28-gauge needle attached to a 0.5-ml insulin syringe. The diaphragm was slightly pushed against the heart, and a stopper was attached to the needle to facilitate consistent injection into the heart muscle tissue. After the injection, the abdomen was closed with sterile surgical sutures, and the rats were allowed to recover. The mortality rate with this injection technique was <3%.

In experiments testing the thyroid responsiveness of a given β-MHC promoter construct, 30 animals received DNA injections of the same plasmid mixture. After surgery the animals were divided into different experimental groups of 10 animals each. Animals in the hypothyroid group received daily injections of propylthiouracil (PTU, 12 mg/kg body wt ip) to induce hypothyroidism, whereas animals in the hyperthyroid group received daily injections of triiodothyronine (T₃, 150 µg/kg body wt ip) to induce hyperthyroidism. In experiments testing different-length β-MHC promoter constructs in a given thyroid state, subgroups of 10 animals each received injections of a given reporter plasmid mixture. After the injections, all groups in that experiment were left untreated or received daily injections of T₃ or PTU as described above to make them hyper- or hypothyroid. Seven days after the DNA injection, the animals were deeply sedated with a lethal dose of pentobarbital sodium (Nembutal, 100 mg/kg). For each animal the chest was opened to obtain a blood sample via direct cardiac puncture with use of a vacuum container containing EDTA. The plasma was separated by centrifugation and stored at −20°C until subsequent analysis for T₃ and thyroxine (T₄). Next, each heart was rapidly excised; the ventricles were dissected out free of atria and major blood vessels, rinsed in cold saline, blotted dry, weighed, and cut into apex (containing the plasmid-injected area) and base portions (saved for mRNA/protein analysis). The heart portions were then quickly frozen on dry ice and stored at −80°C until processing.

Plasmid constructs. pG3 basic, a promoterless plasmid containing the firefly luciferase reporter gene, and pRL null plasmid, containing the Renilla luciferase reporter gene, were purchased from Promega. The plasmids (−1,330 to −34) and (−215 to +34) encoding β-MHC chloramphenicol acetyltransferase (CAT) were a kind gift from Dr. P. C. Simpson (University of California, San Francisco) and contained a rat β-MHC genomic fragment fused to the CAT reporter sequence in pUC9. An additional construct, containing a −3,500- to +462-bp [from the transcription start site (TSS)] β-MHC genomic sequence fused to a CAT reporter plasmid, was kindly provided by Dr. Kaie Ojamaa (34).

The −3,500- and −215-bp β-MHC sequences were subcloned into the pGL3 basic vector by use of standard cloning procedures. Both of these constructs and all subsequent constructs were terminated at position +34 from the TSS. The −3,500- to +34-bp β-MHC promoter construct was sequenced in both directions with use of an automated sequence analyzer (Applied Biosystems). The −408- and −914-bp β-MHC pGL3 constructs were generated using unique restriction sites (NheI at position −408 and PstI at position −914) on the β-MHC promoter sequence. The longer −2,256-, −2,521-, and −2,988-bp promoter constructs were generated by PCR using the −3,526-bp construct as a template and Pfu high-fidelity DNA polymerase (Stratagene). The upstream sense primers and the single downstream antisense primer were designed to contain an SacI site for subsequent ligation into the pGL3 multiple cloning site. All these constructs also terminated at +34 from the TSS.

All promoter constructs were analyzed by sequencing to ensure correct orientation and ligation into the vector and were examined for possible unwanted mutations due to the PCR amplification procedure.

Injection of plasmid DNA into heart muscle is associated with variability in DNA uptake by the tissue, resulting in significant variations of reporter gene activity. To account for differences in DNA uptake, a second normalizing reporter plasmid, driven by a promoter different from the promoter of interest, needs to be co-injected with every experiment. Renilla luciferase is the reporter gene of choice for this control plasmid construct, because it has sensitivity similar to the firefly luciferase (Promega) and can be conveniently measured in the same reaction system immediately after the firefly luciferase measurement by using the same tissue extract (Promega). The choice of a suitable promoter to drive Renilla luciferase expression is more difficult and requires careful consideration. We and others have tested the cytomegalovirus (CMV) promoter as a possible choice. This promoter is strong and constitutively active in all types of tissue (muscle...
or nonmuscle). Even though it is a popular choice, we have observed a number of problems with the use of this promoter in gene injection experiments. For example, in our hands, the ratio of firefly to Renilla luciferase expression were highly variable, and varying the amounts of CMV-Renilla plasmid injected did not alleviate the problem (data not shown). Although CMV is used as an indicator of transfection efficiency, we found that, in many cases, CMV-Renilla expression was too low when obviously the tissue had taken up the plasmid solution (as indicated by high levels of β-MHC firefly expression) or too high when β-MHC firefly expression indicated that transfection efficiency overall may not have been high. Another problem associated with the use of the CMV promoter is the possibility of uptake and expression in nonmuscle tissue, which could lead to misinterpretation of the results. Furthermore, CMV activity is highly sensitive to differences in thyroid state, adding even more variability to the system. In our search for an alternative, the α-MHC promoter seemed to be a good choice, because several of the above-mentioned difficulties and variables inherent to the CMV promoter could be eliminated. First, the α-MHC gene is another sarcomeric gene, the expression of which is limited to heart myocytes. Second, α- and β-MHC are endogenously active in the heart. This avoids creating an imbalance of nuclear factors, which easily happens when a very strong promoter, such as CMV, is used. Third, the α-MHC promoter has been well characterized, and even though this gene also is regulated by thyroid hormone, its level of expression is predictably reciprocal to the expression of the β-MHC gene.

The α-MHC promoter (~2,936 – 420) was a kind gift from Dr. Eugene Markin (University of Arizona, Tucson, AZ). This sequence was ligated to the pRL vector, and reporter gene activity from this construct was used to account for variations in in vivo DNA transfer efficiency.

Plasmids were amplified in Escherichia coli cultures according to standard procedures and were purified by anion-exchange chromatography with use of disposable columns (Endofree, Qiagen). Plasmid preparations were examined by ethidium bromide staining after agarose gel electrophoresis.

Site-directed mutagenesis. The MORPH mutagenesis kit and protocol from 5prime – 3prime, Inc. (Boulder, CO) were used for all mutagenesis reactions. A triple base pair mutation was introduced into the basal promoter sequence of the ~408- and the ~3,500-bp β-MHC promoter sequence. The mutation consisted of changing three G bases at position ~54 to ~56 to three T bases. The mutagenic oligonucleotide had the following sequence: 5’-CTGGGTGCAGGTGTTTTGATGGGCCACCC-3’.

Reporter gene assays. Frozen cardiac tissue (~200 mg) from the apex was homogenized in 2 ml of an ice-cold lysis buffer (Promega) supplemented with 5 μg/ml aprotinin, 2.5 μg/ml leupeptin, and 0.2 mM 4-(2-aminoethyl)benzenesulfonyl fluoride (protease inhibitors, Sigma Chemical) with use of a glass homogenizer. The homogenate was centrifuged at 4°C at 10,000 g for 10 min; the supernatant was separated and kept on ice until assayed for luciferase activities. A Promega dual-luciferase detection kit, which measures and distinguishes activities from the two luciferase proteins, was used for luciferase assays. Firefly and Renilla luciferase activities were measured from the same extract in a single tube. Promega’s protocol was used to assay 20 μl of each extract at room temperature. Light output from each specific luciferase activity was measured for 10 s with an analytic luminometer (Monolight 1000-C, Analytical Luminescence Laboratory, Ann Arbor, MI). Background activity levels, based on measurements in noninjected tissue for both luciferases, were established and deducted from the activities measured in the experiments. Activities were expressed as relative light units.

β-MHC mRNA analysis. Total RNA was extracted from frozen tissue (base portion of the injected hearts), as described previously (17). Distribution of α- and β-MHC mRNA was measured by Northern blot analysis of the extracted total RNA (n = 40 for untreated and n = 20 each for hypothyroid and hyperthyroid animals). Oligonucleotides complementary to the 3’-untranslated sequences of the α- and β-MHC isomorphs were used for hybridization, as described previously (17). Band intensities on the autoradiogram were quantitated using a laser scanning densitometer (Molecular Dynamics, Sunnyvale, CA), and each specific absorbance was normalized to its corresponding 18S rRNA signal (17).

Thyroid hormone analysis. Plasma levels of T3 and T4 were measured using a commercially available RIA kit (ICN Pharmaceuticals). Measurements were performed on groups of at least 30 animals for a given thyroid state.

Statistical analysis. Values are means ± SE. Statistical significance was determined by ANOVA followed by the Student-Newman-Keuls test for multiple comparisons. All statistical tests were performed using the GraphPad Prism 2.0 statistical software package. P < 0.05 was taken as the level of statistical significance.

RESULTS

Endogenous β- and α-MHC mRNA expression and evidence of altered thyroid states. In rat heart, β- and α-MHC mRNA expression is regulated by T3 in a reciprocal fashion (25, 27). Northern analysis of normal control, hypothyroid (PTU), and hyperthyroid (T3) hearts from young adult rats demonstrates that in the normal control state α-MHC mRNA is 10- to 20-fold more abundant than β-MHC mRNA (Fig. 1). In the hyperthyroid state this pattern is even more exaggerated because of the repression in β-MHC mRNA abundance; thus the α-MHC mRNA is ~100-fold more abundant than β-MHC mRNA. Interestingly, the rat heart in the euthyroid state resembles a heart in a

Fig. 1. Endogenous myosin heavy chain (MHC) mRNA expression in rat heart under 3 different thyroid states. MHC mRNA expression was analyzed by Northern blotting as follows: 5 μg of total RNA were loaded onto gel, transferred onto nylon membrane, and hybridized using α- or β-MHC-specific oligonucleotides. After signal detection, probes were washed off blots, and membranes were rehybridized with an 18S rRNA probe. Signal densities were determined by laser scanning densitometry, and each MHC band was divided by its corresponding 18S rRNA signal. Values are means ± SE. NC, euthyroid (normal control); PTU, hypothyroid (propylthiouracil); T3, hyperthyroid (triiodothyronine). *P < 0.05 vs. NC.
hyperthyroid state with regard to α-MHC mRNA expression, inasmuch as chronic exposure to T3 elevates the α-MHC mRNA pool by only 15% relative to the euthyroid state (unpublished observation). In hypothyroid animals the relationship between α- and β-MHC mRNA completely reverses, and in this state β-MHC mRNA accounts for 99% of the MHC mRNA, with α-MHC mRNA making up the remaining 1% (Fig. 1). These results are typical for young adult animals and are consistent with previous reports (27).

Heart weight-to-body weight ratios and plasma T3 and T4 levels are shown in Table 1. The effects of the PTU or T3 treatment are obvious after only 1 wk of daily drug injection. Heart weight-to-body weight ratios were decreased by ~7% in PTU animals and increased by 44% (P < 0.05) in T3 animals compared with the normal control group. Plasma T3 levels were reduced by 42% in PTU animals and increased by 156% in T3 animals; plasma T4 levels were reduced to 10% (in PTU animals) and ~4% (in T3 animals) of the normal levels. These results collectively suggest that we were successful in altering the thyroid states.

Transcriptional regulation of the α- and β-MHC genes as measured by absolute reporter gene activities. To demonstrate that β- and α-MHC genes are regulated at the transcriptional level, we initially tested the responsiveness of a ~3,500-bp β-MHC firefly luciferase construct and the α-MHC Renilla luciferase construct in the hearts of normal control, hypothyroid (PTU), and hyperthyroid (T3) rats. In Fig. 2 the reporter activity in relative light units per heart per 10 s is shown for both plasmids in the different thyroid states. The β-MHC gene was upregulated about threefold in hypothyroid conditions and downregulated about sevenfold in hyperthyroid conditions compared with the normal control state. The total transcriptional activity of this gene was downregulated ~20-fold in a condition of hyperthyroidism compared with hypothyroidism. In contrast to this pattern, the α-MHC gene showed opposite regulation: this gene was downregulated about threefold in hypothyroidism and upregulated about threefold in hyperthyroidism compared with the expression in normal control (euthyroid) hearts. This suggests that T3 exerts a more potent effect on the transcriptional activity of the α-MHC promoter than on the regulation of the α-MHC mRNA pool (Figs. 1 and 2). The difference in gene expression between hypothyroid and hyperthyroid states was ~10-fold for this gene. These results illustrate that both promoters were highly sensitive to the presence and absence of thyroid hormone and that, despite the lack of correction for the variations in transfection efficiency, the average reporter gene expression resembled the endogenous expression of the two genes. Within a given thyroid state, the coefficient of variation for reporter gene expression was >100%; however, for the β-to-α ratios, the coefficient of variation was decreased to ~20–30% (Table 2). Furthermore, the results obtained with the α-MHC promoter construct were much more consistent than the results obtained in the experiments using the CMV promoter, in which variation was high with or without correction (data not shown). These results indicated that the α-MHC promoter was a satisfactory control expression plasmid for our experiments.

Deletion analysis of the β-MHC promoter in normal control hearts corrected for α-MHC activity. In normal control hearts, expression of the α-MHC is relatively constant. Thus, when different-length β-MHC promoter constructs are coelectrotransfected with the same α-MHC pRL construct, the β-to-α ratio is a direct function of the β promoter activity. Seven different-length β-MHC promoter fragments were tested in normal control hearts: −3,500, −2,900, −2,500, −2,000, −1,914, −408, and −215 bp, all extending to +34 bp relative to the TSS. The β-to-α ratios of reporter gene expression show that all five of the intermediate promoter segments drive β-MHC expression at about the same level, in contrast to the long −3,500-bp promoter fragment, which shows two- to threefold higher β-MHC expression levels (Fig. 3). β-MHC expression with use of the short −215-bp promoter construct was reduced by ~30% relative to the intermediate-length promoter constructs. We attribute this latter response to the lack of some critical positive regulatory elements (e.g., βe2

Table 1. Heart weight-to-body weight ratios and plasma T3 and T4 levels in normal control, hypothyroid, and hyperthyroid animals

<table>
<thead>
<tr>
<th></th>
<th>NC</th>
<th>PTU</th>
<th>T3</th>
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<tbody>
<tr>
<td>Heart wt/body</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>wt, mg/g</td>
<td>3.03 ± 0.036</td>
<td>2.84 ± 0.042</td>
<td>4.35 ± 0.052*</td>
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<tr>
<td>T3, ng/dl</td>
<td>88.98 ± 1.89</td>
<td>52.04 ± 2.45*</td>
<td>227.40 ± 20.56*</td>
</tr>
<tr>
<td>T4, μg/dl</td>
<td>4.49 ± 0.15</td>
<td>0.43 ± 0.03*</td>
<td>0.20 ± 0.02*</td>
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</table>

Values are means ± SE. Heart weight-to-body weight ratios in hypothyroid [prop thiouracil (PTU)-treated] and hyperthyroid [triiodothyronine (T3)-treated] animals reflect effect of 1 wk of treatment with respective drugs. Weights were analyzed from 30 animals in each group. Plasma levels of T3 and thyroxine (T4) were measured using a commercially available RIA kit. *P < 0.05 vs. normal control (NC) by one-way ANOVA.

Fig. 2. Total in vivo reporter gene activity in NC, PTU, and T3 hearts. Total β-MHC firefly luciferase (F-luc) activities [expressed as relative light units (RLU)] for long −3,500-bp β-MHC promoter fragment and total α-MHC Renilla luciferase (R-luc) activities (expressed as RLU) when hearts were coelectrotransfected with β-MHC in different thyroid states are shown. Both genes are highly sensitive to thyroid state and show an inverse regulation in response to altered thyroid hormone levels. Values are means ± SE; n = 10/group. *P < 0.05 vs. NC. See Fig. 1 legend for definition of abbreviations.
Genes are regulated by thyroid hormone. All tested luciferase activities were measured using a dual-luciferase assay. Despite variability in DNA uptake by tissue, data show that activity is expressed as equivalent to 10 µg of α-MHC pRL construct and an equal molar amount of either of the β-MHC pGL3 constructs. At 7 days after injection, hearts were removed and extracted, and the 2 luciferase activities were measured using a dual-luciferase assay. Despite variability in DNA uptake by tissue, data show that β- and α-MHC genes are regulated by thyroid hormone. All tested β-MHC pGL3 constructs are highly expressed in hypothyroid state and are dramatically downregulated in hyperthyroid hearts. α-MHC pRL construct shows opposite response in all experiments.

Table 2. β-MHC firefly luciferase and α-MHC Renilla luciferase activities in hyper- and hypothyroid rat hearts

<table>
<thead>
<tr>
<th>Injected Plasmid Constructs</th>
<th>Hypothyroid</th>
<th>Hyperthyroid</th>
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<tr>
<td>Firefly (β)</td>
<td>Renilla (α)</td>
<td>β/α ratio</td>
</tr>
<tr>
<td>−3,500 β-MHC pGL3 + α-MHC pRL</td>
<td>236,500 ± 119,200</td>
<td>74,250 ± 36,940</td>
</tr>
<tr>
<td>−2,000 β-MHC pGL3 + α-MHC pRL</td>
<td>208,800 ± 49,050</td>
<td>127,400 ± 43,560</td>
</tr>
<tr>
<td>−914 β-MHC pGL3 + α-MHC pRL</td>
<td>145,700 ± 68,530</td>
<td>56,160 ± 12,640</td>
</tr>
<tr>
<td>−408 β-MHC pGL3 + α-MHC pRL</td>
<td>280,300 ± 58,860</td>
<td>143,100 ± 68,050</td>
</tr>
<tr>
<td>−215 β-MHC pGL3 + α-MHC pRL</td>
<td>38,160 ± 18,630</td>
<td>126,800 ± 112,900</td>
</tr>
<tr>
<td>Mutant −3,500 β-MHC pGL3 + α-MHC pRL</td>
<td>19,070 ± 7,756</td>
<td>359,400 ± 154,800</td>
</tr>
<tr>
<td>Mutant −408 β-MHC pGL3 + α-MHC pRL</td>
<td>0.000 ± 0.000</td>
<td>34,790 ± 19,880</td>
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</table>

Values are means ± SE. Each rat heart was injected with 40 µl of PBS containing 8 µg of α-myosin heavy chain (α-MHC) pRL construct and an equal molar amount of either the β-MHC pGL3 constructs. At 7 days after injection, hearts were removed and extracted, and the 2 luciferase activities were measured using a dual-luciferase assay. Despite variability in DNA uptake by tissue, data show that β- and α-MHC genes are regulated by thyroid hormone. All tested β-MHC pGL3 constructs are highly expressed in hypothyroid state and are dramatically downregulated in hyperthyroid hearts. α-MHC pRL construct shows opposite response in all experiments.

Fig. 3. Activity of different-sized β-MHC promoter fragments in euthyroid hearts. Seven different-sized β-MHC promoter segments (−215, −408, −914, −2,000, −2,500, −2,900, and −3,500 bp) were tested. All these constructs were injected in equimolar amounts equivalent to 10 µg of −3,500-bp β-MHC firefly luciferase construct. Activity is expressed as β-to-α ratios of reporter gene expression. Data suggest presence of a positive regulatory element located between −2,900 and −3,500 bp upstream of transcription start site. Values are means ± SE; n = 10/group. *P < 0.05 vs. all.

Fig. 4. Ratios of β-MHC firefly luciferase to α-MHC Renilla luciferase activity in different thyroid states. Equimolar amounts of different-sized β-MHC promoter fragments linked to a firefly reporter gene were tested along with same α-MHC Renilla luciferase construct. In euthyroid state (NC), α-MHC promoter is 2–8 times more active than β-MHC promoter. In hypothyroid state (PTU), this pattern is reversed; i.e., activity of β-MHC promoter is 1.5- to 8-fold higher than activity of α-MHC promoter. In hyperthyroid state (T3), β-MHC gene is suppressed, whereas α-MHC gene is activated to become ~40 times higher than β-MHC activity. Values are means ± SE; n = 10/group.
gene regulation in the euthyroid state, appear to have little or no influence on regulation of the gene in the hyper- or hypothyroid state (Fig. 4). An explanation for this finding could be that thyroid hormone regulation of the gene is so prevailing over the effect of any other transcription factors that either the lack of or the abundance of thyroid hormone masks the operation of other transcriptional regulators.

Effect of a triple base mutation within the putative TRE on β-MHC promoter activity and thyroid responsiveness. A putative TRE has been proposed to be contained within the basal β-MHC promoter at position −55 to −60 with the sequence GGTGGG (10). This sequence partially overlaps with an E box (CAGGTC). We were interested in how a mutation in this element would affect the in vivo β-MHC transcriptional activity and the responsiveness to thyroid hormone. With the E box left intact, a triple G sequence at position −56 was mutated to a triple T. This mutation was introduced into the −408- and −3,500-bp β-MHC promoter reporter constructs. Injecting the mutant −408-bp β-MHC firefly luciferase construct along with the α-MHC Renilla luciferase construct resulted in background levels of firefly luciferase activities in all thyroid states, whereas the Renilla luciferase activities were unaffected and followed the typical regulation pattern in a given thyroid state (Fig. 5). Consequently, the β-to-α ratios of reporter gene activities were essentially zero in each of the three thyroid states.

Surprisingly, when the mutant −3,500-bp β-MHC firefly luciferase construct was injected into hearts along with the α-MHC Renilla luciferase construct, firefly luciferase activities were not null; they were significantly decreased but readily detectable (Fig. 5). Even more surprising was the fact that the ratios of reporter gene activities in the different thyroid states still followed, albeit at an ~100-fold lower level, the wild-type pattern induced in PTU hearts and suppressed in T₃ hearts. These results suggest the presence of an upstream element on the β-MHC promoter sequence that is able to interact with elements in the basal promoter to partially rescue or stabilize the transcriptional activity, which was severely reduced by the sequence mutation. Our results also imply that the bulk of the thyroid hormone regulation of the β-MHC gene is contained within the first 215 bp of the promoter sequence, but such regulation is probably not restricted to the putative TRE site at −54 to −56 bp of the promoter sequence.

**DISCUSSION**

The effect of thyroid hormone on endogenous expression of the β- and α-MHC genes has been well established (20, 27, 32, 33). The transcriptional regulation of the α-MHC gene in response to thyroid hormone alteration can be explained by the existence of at least three positive TREs that are contained in the promoter sequence of the gene (15, 19, 38). The mechanism of regulation of the β-MHC gene by thyroid hormone changes is more elusive and not well defined. In the present study we examined transcriptional regulation of the β-MHC gene in physiological in vivo settings involving euthyroid animals as well as animals spanning the spectrum of thyroid states.

Using a gene injection approach, we established an experimental setup that allows us to study transcriptional activity of the β-MHC gene in vivo. A deletion analysis of the β-MHC promoter revealed the importance of upstream sequences for full transcriptional activity of the gene in the euthyroid state and that most of the thyroid hormone regulation of the gene is contained within the first 215 bp of the promoter sequence. A mutation in the region of a putative TRE, located in the basal promoter, annulled transcriptional activity in the context of a −408-bp promoter segment. When the same mutation was tested in the context of a long (−3,500-bp) promoter segment, some of the transcriptional activity and the thyroid responsiveness of the gene was retained.

Sequence analysis and comparison to published TRE motifs revealed the existence of four TRE half-sites within the first 215 bp of the promoter sequence (Fig. 6). The functional significance of these TREs is not

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**Fig. 5.** Effects of a mutation in putative thyroid response element (TRE) on β-MHC promoter activity in different thyroid states. A: β-to-α ratios of reporter gene expression of wild-type (wt) −3,500-bp β-MHC and −408-bp β-MHC promoters. B: β-to-α ratios of reporter gene expression of mutant (Mut) −3,500-bp β-MHC and −408-bp β-MHC promoter. TRE mutation had a detrimental effect on −408-bp β-MHC promoter; its activity was comparable to that of noninjected tissue. In contrast, activity of TRE-mutated −3,500-bp β-MHC fragment was measurable but very low, ~100 times less than that of wild type. Interestingly, TRE-mutated −3,500-bp β-MHC still showed T₃ responsiveness. Values are means ± SE; n = 10/group.
The responsiveness of the tested human β-MHC gene; i.e., they have been reported that several genes that are negatively regulated by T₃ share common features; i.e., they contain several TRE half-sites in the basal promoter region, and there is variable spacing between them (8, 14, 31). These genes include thyrotropin-releasing hormone (18), thyroid-stimulating hormone-α (hTSH-α), human thyroid-stimulating hormone-β (β-TSH) (7), α-TSH (9), the epidermal keratin gene family (36), epidermal growth factor receptors (41), and rat growth hormone (4, 15).

Exact identification of the regions of thyroid hormone responsiveness of the β-MHC promoter has been attempted mainly in tissue culture systems. Only one publication attributed the strong regulation of the gene by thyroid hormone to a negative TRE located in the basal promoter (10). Six nucleotides, located between the TATA box and the CAAT element, were identified as a putative TRE (10). Mutation of this element severely inhibited transcriptional activity and abolished T₃ responsiveness of the tested human β-MHC promoter segment in vitro (10). This particular TRE sequence is most similar to a half-site TRE described in the human α-MHC gene promoter, which is also located close to the TATA region (9).

In our hands, a similar, although less severe, mutation of the corresponding sequence in the rat β-MHC promoter nullified transcriptional activity in vivo in any given thyroid state when tested in a short (~408-bp) β-MHC promoter sequence (Fig. 5). When the same mutation was tested in the longest (~3,500-bp) β-MHC promoter construct, transcriptional activity was reduced ~100-fold compared with the wild-type construct, yet not nullified. Thus the presence of upstream regulatory sequences was able to restore some of the in vivo transcriptional activity that was lost through the mutation. Interestingly, the thyroid hormone responsiveness was preserved in the mutated 3,500-bp construct and was similar in relative magnitude to the wild-type construct, although overall transcriptional activity was reduced by two orders of magnitude (Fig. 5). Our data suggest that thyroid hormone regulation of the rat β-MHC promoter in vivo is contained within the first 215 bp of the promoter sequence, but the exact location(s) remains to be defined. The data also indicate that thyroid hormone is able to mask the effect of other regulatory elements on transcriptional activity of the gene, since the enhancer properties of the upstream promoter sequences are absent when the thyroid hormone state of the animal is manipulated. It is conceivable that T₃ binding interferes with the formation of the activating complex for initiating transcription, thereby causing the suppression of transcriptional activity. It has been shown that TREs are repeats of the half-site consensus motif (A/G)GG(A/T)CA (30). Spacing is critical in determining the specificity of the response, and several other receptors such as retinoid X and retinoic acid receptors (RXRs and RARs) of the steroid receptor superfamily share a similar binding motif (31). Thyroid hormone receptors (TRs), liganded or not, can bind to TREs as monomers, homodimers, or heterodimers (14). They can heterodimerize with RXRs and RARs (14, 31). These protein-protein interactions (RAR/RXR-TR) can inhibit or induce the regulation by T₃.

Deletion analysis of the β-MHC promoter in normal control hearts revealed the presence of an enhancer region contained within ~2,900 to ~3,500 bp of the upstream sequence of the gene. This upstream enhancer might likely also be the factor that compensates for some of the activity that was lost by mutating 3 bp in an obviously critical region of the basal promoter. A mechanism in which an upstream sequence forms a loop and therefore again comes close to some elements in the basal promoter is not uncommon, and in fact, this is how most enhancers work (2). This and the above-discussed results point to a model in which the interaction of transcription factors with sequences in the basal promoter is brought to full activity in the euthyroid state by additional interactions with upstream sequences (and possible proteins bound to those sequences). In the absence of thyroid hormone, this interaction could become impossible, whereas with an abundance of thyroid hormone the interaction with upstream sequences might have so little effect that it is not measurable.

In previous studies of the β-MHC promoter in which transgenic mice were used, it was found that 600 bp of promoter sequence were not enough to confer thyroid responsiveness and, in particular, induction of β-MHC gene expression on reduction of thyroid hormone levels in heart muscle (37). However, the same studies also emphasized the importance of upstream sequences for full transcriptional activity of the gene (37). Finally, our results demonstrated that expression of the β-MHC gene in normal control and altered thyroid states is regulated to a great extent at the transcriptional level. Even though post- and pretranslational processes undoubtedly play a role in the regulation of β-MHC gene expression, our data underline the critical importance of the regulation at the transcriptional level.
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