Cardiac myosin heavy chain gene regulation by thyroid hormone involves altered histone modifications

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Submitted 30 June 2010; accepted in final form 7 September 2010

Haddad F, Jiang W, Bodell PW, Qin AX, Baldwin KM. Cardiac myosin heavy chain gene regulation by thyroid hormone involves altered histone modifications. Am J Physiol Heart Circ Physiol 299: H1968–H1980, 2010. First published September 10, 2010; doi:10.1152/ajpheart.00644.2010.—The antithetical regulation of cardiac α- and β-myosin heavy chain (MHC) genes by thyroid hormone (T3) is not well understood but appears to involve thyroid hormone interaction with its nuclear receptor and MHC promoters as well as cis-acting noncoding regulatory RNA (ncRNA). Both of these phenomena involve epigenetic regulations. This study investigated the extent that altered thyroid state induces histone modifications in the chromatin associated with the cardiac MHC genes. We hypothesized that specific epigenetic events could be identified and linked to cardiac MHC gene switching in response to a hypothyroid or hyperthyroid state. A hypothyroid state was induced in rats by propylthiouracil (PTU), whereas a hyperthyroid (T3) was induced by T3 treatment. The left ventricle was analyzed after 7 days for MHC pre-mRNA expression, and the chromatin was assessed for enrichment in specific histone modifications using chromatin immunoprecipitation quantitative PCR assays. At both the α-MHC promoter and the intergenic region, the enrichment in acetyl histone H3 at K9/14 (H3K9/14ac) and trimethyl histone H3 at K4 (H3K4me3) changed in a similar fashion. They were both decreased with PTU treatment but did not change under T3, except at a location situated 5’ to the antisense intergenic transcription start site. These same marks varied differently on the β-MHC promoter. For example, H3K4me3 enrichment correlated with the β-promoter activity in PTU and T3 groups, whereas H3K9/14ac was repressed in the T3 group but did not change under PTU. Histone H3K9me was enriched in chromatin of both the intergenic and α-MHC promoters in the PTU group, whereas histone H4K20me1 was enriched in chromatin of β-MHC promoter in the normal control and T3 groups. Collectively, these findings provide evidence that specific epigenetic phenomena modulate MHC gene expression in altered thyroid states.

gene transcription; epigenetic regulation; chromatin immunoprecipitation quantitative polymerase chain reaction; noncoding RNA; Sprague-Dawley rats

TWO MYOSIN HEAVY CHAIN (MHC) isoforms are expressed in the myocardium, designated as the α- and β-MHC. Cardiac MHC phenotype is a major determinant of contractility, mechanical performance, and energy turnover of the heart (59). For example, hearts rich in α-MHC expression have high contractility, whereas those rich in β-MHC expression have slow speed of contraction but are more energy efficient (1). The normal control adult rodent heart expresses predominantly the α-MHC gene, but this can be readily altered by several pathophysiological conditions. For example, hypothyroidism, diabetes, pressure overload, and caloric deprivation enhance the β-MHC gene expression and repress the α-MHC expression, whereas thyroid hormone treatment produces the opposite effect (4, 48). During these MHC transformations, the MHC genes are regulated in an antithetical fashion; i.e., as one isoform’s transcription increases, the other decreases in a coordinated fashion.

Cardiac α- and β-MHC isoforms are the products of two distinct genes that are situated in tandem in a head-to-tail position on the chromosome in the order of β → α (43) and are separated by a 4.5-kb intergenic space. The intergenic region between α- and β-MHC is transcriptionally active on both strands (22). The upper strand transcribes a transcript initiated from within the α-MHC gene promoter, and its product merges with the α-MHC gene (22). The lower strand transcribes a noncoding RNA (ncRNA) that is overlapping to the β-MHC gene (antisense), which extends into its promoter. Our previously reported data suggest that the intergenic transcription is contributing to the coordinated antithetical regulation between these two closely linked genes (21, 22). When the intergenic transcription is activated, it negatively influences the upstream β-MHC sense transcription. The exact mechanism of how the antisense transcription inhibits the corresponding sense gene is not clear; however, it may involve epigenetic processes such as chromatin remodeling and histone modifications (27, 58, 66). In fact, there is increasing evidence that long ncRNAs, similar to the antisense β-RNA, can function to recruit histone-modifying enzymes to the corresponding chromatin milieu to regulate gene transcription (13, 44, 52).

In the nucleus of a cell, the eukaryotic genome is packaged into a dense structure called chromatin, consisting of DNA, histones, transcription factors, and other nuclear proteins. The fundamental repeating unit of chromatin is the nucleosome, in which 146 DNA base pairs are wrapped left-handed around an octamer core of histones (39). The NH2-terminal tails of the histones are subject to posttranslational modifications including acetylation and methylation of lysines (K) and arginines (R), phosphorylation of serine (S) and threonines (T), ubiquitination, and sumoylation and biotinylation of lysines, as well as ADP ribosylation (49). Site-specific combinations of histone modifications correlate well with particular biological functions, such as transcription, silencing, or DNA repair; which leads to the concept of a “histone code” (34, 49). Both positive and negative regulation of eukaryotic transcription have been shown to be mediated, in part, by two opposing enzymatic activities: histone acetylases and histone deacetylases (15). Generally, lysine acetylation mediated by histone acetyltransferases (HATs) demarcates transcriptionally active regions. In contrast, lysine deacetylation catalyzed by histone deacetylases (HDACs) results in hypoacetylated histones that are usually associated with transcriptionally inactive chromatin (35). Histone tail methylation may have different consequences depend-
ing on the affected residues. For example, histone H3 methylation at K4, K36, or K79 is associated with active chromatin, whereas histone H3 methylation at K9 or K27 and histone H4 methylation at K20 are associated with a repressive chromatin milieu (18, 42, 73). Histone modifications involve a dynamic process that can be altered rapidly depending on cellular events, to ensure tight control of gene expression. Thus it became of interest to examine specific histone modifications in the chromatin of the cardiac MHC gene locus in the left ventricle in response to hypothyroid (PTU) and hyperthyroid (T₃) states to better understand the chromatin environment in states whereby the MHC transcription is dynamically altered.

The mechanism of T₃ action on target genes occurs via the thyroid receptor (TR), a transcription factor that binds to a thyroid response element (TRE) located on promoters of target genes. Several functional TREs were identified on the α-MHC gene promoter, whereas the site of action of T₃ on the β-MHC promoter is not clear, but it is thought to occur via a negative TRE located near the TATA box (14, 48). Cardiac α-MHC gene expression is upregulated by T₃, whereas β-MHC transcription is repressed by T₃. Furthermore, the effect of T₃ on gene expression via its nuclear receptor is complex and involves cooperation of many processes, including epigenetic events such as histone modifications and chromatin remodeling (42, 63). The question becomes to what extent one can attribute the altered T₃ and histone modifications of cardiac MHC genes in response to altered T₃. The dynamic nature of MHC gene expression in the absence of T₃. However, TSA potentiating T₃ activation of the α-MHC gene when administered along with T₃ (11). Treatment with trichostatin A (TSA), a histone deacetylase inhibitor, was not effective in altering cardiac MHC expression in the absence of T₃. However, TSA potentiated T₃ activation of the α-MHC gene when administered along with T₃ (11). These findings support the notion that MHC gene transcription is regulated via altered histone acetylation state. Therefore, it is logical to identify specific histone modifications in the chromatin of these two linked genes under both hypothyroid and hyperthyroid states, presenting the two extremes of the cardiac MHC gene expression.

This study provides a new approach to understanding the role of specific histone modifications in the antithetical regulation of cardiac MHC genes in response to altered T₃. The dynamic state of MHC gene expression in the heart, and particularly in response to altered T₃ state, implies the ongoing function of various gene regulatory mechanisms. We utilized chromatin immunoprecipitation (ChIP) assays to detect specific epigenetic marks of histones associated with the α- and β-MHC genes as well as the α-β intergenic region in euthyroid, hypothyroid, and hyperthyroid hearts. We examined modifications that are associated with chromatin of active genes (H3K9/14ac and H3K4me3), as well as modifications found in repressive chromatin (H3K9me1/2/3 and H4K20me1). We hypothesized that a ChIP approach could identify specific epigenetic events that could be linked to cardiac MHC gene switching in either a hypothyroid or hyperthyroid state.

Our results suggest that histone H3K9/14ac plays a role in the active chromatin of the intergenic region as well as in the α-MHC gene promoter in the euthyroid state. Histone H3K4me3 is enriched in active promoters of the cardiac MHC gene locus. Histone H3K9me is enriched in chromatin of the intergenic and α-MHC promoters in the hypothyroid state, whereas histone H4K20me1 is enriched in chromatin of β-MHC promoter under repressive euthyroid and hyperthyroid conditions. These findings suggest that the mechanism of cardiac MHC gene regulation is complex and involves combinatorial control of several histone modifications identified with divergent enrichment throughout the cardiac MHC gene locus. These results are discussed in the context of constructing a working model whereby the cardiac MHC genes are regulated by epigenetic regulation involving recruitments of specific histone-modifying enzymes via T₃-TR-TRE interactions or via interplay with ncRNA such as intergenic sense and antisense RNA.

**METHODS**

**Animal procedures.** Female Sprague-Dawley rats (~180–200 g) were used for all experiments. Animals were randomly assigned to either normal control (NC), hypothyroid propylthiouracil (PTU)-treated, or hyperthyroid triiodothyronine (T₃)-treated groups (n = 5/group). Hypothyroid state was induced by daily intraperitoneal (IP) injections of PTU (12 mg/kg body wt). A hyperthyroid state was induced by daily IP injections of T₃ (150 µg/kg body wt). These pharmacological treatment regimens of T₃ and PTU were utilized on the basis of previous studies where we showed that they are effective in making the animal hyperthyroid and hypothyroid based on plasma T₃ analyses (Ref. 25; Haddad F, unpublished data). Animals were housed in groups of four in a temperature- and light-controlled environment (i.e., 12:12-h light-dark cycle). All animals in a given experiment were provided with food and water ad libitum, and all procedures were approved by the Institutional Animal Care and Use Committee. After 7 days of daily treatment, and 6 h after the last T₃/PTU injections, rats were euthanized and the heart was rapidly removed. The left ventricle was dissected, weighed, and frozen at −80°C for later analysis.

**RNA analysis.** Total RNA was extracted from frozen left ventricular tissue using the Tri Reagent protocol (Molecular Research Center). Extracted RNA was DNase-treated using 1 unit of RNase-free DNase (Promega) per microgram of total RNA and was incubated at 37°C for 30 min, followed by a second RNA extraction using Tri Reagent LS (Molecular Research Center). Total RNA concentration was determined using optical density at 260 nm (OD260) and the factor of 40 µg/ml for an optical density of 1. The integrity of the isolated RNA was determined by gel electrophoresis whereby a good-quality RNA results in three bands: 28S, 18S, and 5S, whereas degraded RNA produces smeared bands. Only good-quality RNA was utilized for subsequent analyses. Total RNA was used in RT-PCR to determine the relative expression of specific mRNAs, pre-mRNAs, antisense β-RNA, and intergenic sense RNA (22). All RT-PCR reactions were performed with the One-Step RT-PCR kit (Qiagen) using 100 ng of DNase-treated RNA per reaction and an optimized number of cycles so that the signal was in the linear range of detection. These One-Step RT-PCR analyses were performed as described previously and are thought to accurately amplify specific strands of RNA when both sense and antisense strands are expressed (22, 23). RT-PCR products were run on a 2.5% agarose gel (1X Tris-acetate-EDTA buffer) and stained with GelGreen (Biotium, Hayward, CA). At the completion of electrophoresis, a digital image was taken of the UV-exposed gel, and the band intensity was determined by volume integration with local background correction using Image-Quant Software (GE Healthcare).

**MHC mRNA isoform distribution.** The MHC mRNA isoform distribution was evaluated by RT with oligo(dt)/random primers followed by PCR with primers targeting cardiac α- and β-MHC mRNAs, as described previously (22).

**Chromatin isolation from ventricular tissue.** Frozen ventricular tissue was thawed on ice, minced, and then washed in ice-cold PBS.
All solutions were supplemented with protease inhibitors [leupeptin, 4-(2-aminoethyl)benzenesulfonyl fluoride, and aprotinin; each at 1:1,000]. Minced tissue was then incubated for 10 min in 1% formaldehyde to cross-link chromatin-DNA. Cross-linking was stopped by addition of glycine to 0.125 M for 5 min. This solution was exchanged with cold PBS and then repeated a second time to remove all the formaldehyde. Tissue samples were then homogenized in PBS (20 volumes of the muscle weight) with a Dounce homogenizer. The homogenate was then pelleted by centrifugation at 1,500 g for 10 min. The pelleted muscle tissue was resuspended in cold SDS lysis buffer (1% SDS, 10 mM EDTA, 50 mM Tris, pH 8.1) and sonicated (VibraCell, model VCX 130) to fragment the DNA. Samples were centrifuged at 12,000 g for 10 min to remove insoluble material. To ensure effectiveness of sonication, an aliquot of the supernatant was reverse cross-linked by incubation at 65°C overnight and RNase treated (RNase A). The protein was then digested (proteinase K) and run on a 2% agarose gel to confirm the size of DNA fragments, which were between 200 and 1,000 bp. This aliquot was also used to measure the DNA concentration of the chromatin-DNA using SYBR Green I. A Stratagene Mx3000p real-time PCR machine was used in the quantitative plate read mode to accurately measure DNA concentration, along with a serial dilution of calf thymus DNA (Sigma) that was used as a standard.

**Chromatin immunoprecipitation.** For each muscle sample, 10 μg of DNA were used to perform chromatin immunoprecipitation (ChIP). Chromatin isolation and ChIP reactions were largely based on the EZ-ChIP protocol by Millipore, with some modifications as described previously (51). Normal rabbit IgG (catalog no. 12-370) and specific antibodies for dimethyl histone H3 at lysine 9 (H3K9me3; ab9398), trimethyl histone H3 at lysine 9 (H3K9me3; ab8898), and monomethyl histone H3 at lysine 9 (H3K9me1; ab9045), trimethyl histone H3 at lysine 3 (H3K36me3; ab6002), monomethyl histone H4 at lysine 3 (H4K36me1; ab9045), trimethyl histone H4 at lysine 3 (H4K36me3; ab8898), and monomethyl histone H4 at lysine 20 (H4K20me1; ab9051) were purchased from Millipore (Billerica, MA). Specific antibodies for trimethyl histone H3 at lysine 4 (H3K4me3; ab8580), trimethyl histone H3 at lysine 9 (H3K9me3; ab9045), and monomethyl histone H4 at lysine 20 (H4K20me1; ab9051) were purchased from Abcam (Cambridge, MA). All the above specific antibodies were rabbit polyclonal IgG isotype. The normal IgG IP was included as a negative control to determine nonspecific immunoprecipitation for each PCR target.

After overnight incubations of chromatin DNA with either normal serum (IgG) or specific antibodies at 4°C, chromatin-DNA immunocomplexes were bound to protein A immobilized on resin (protein A Ultralink resin; Pierce). After several washes, these complexes were eluted from protein A resin beads (Pierce) using elution solution (0.1 M NaHCO3, 1% SDS), and cross-links were reversed by incubation at 65°C overnight.

After RNase treatment and protein K digestion, the DNA was purified using a spin column (QiAgQuick PCR purification kit). Input DNA material was reverse cross-linked and purified from one-tenth of the chromatin used for IP. For each sample, input DNA, normal IgG ChIP DNA, and specific antibody ChIP DNA were eluted in nucleasefree water to serve as a template for quantitative polymerase chain reaction (qPCR) analyses.

**Quantitative PCR analysis for ChIP DNA.** Immunoprecipitated DNA for specific genes was analyzed by qPCR using Brilliant II Fast SYBR green qPCR Master Mix and MX3000p (Stratagene/Agilent Technologies). SYBR green quantitative real-time PCR analyses were carried out using primers specific for the α- and β-MHC genes and the intergenic region corresponding to the antisense β-RNA and intergenic α-MHC RNA (22), as well as for the β-actin gene. With the ChIP assay, primers can be targeted to any genomic region. Some histone modifications have been shown previously to peak immediately downstream of the transcription start sites (TSS) of active genes (5, 20, 71). Thus, for each of the genes of interest, we designed primers targeting both 5’- and 3’-flanking regions of TSS (see Table 1 for primer information). Before use on ChIP DNA, all primers were validated for use in qPCR, and the reaction conditions were optimized for a high efficiency (>95%). A single band was obtained at the end of 40 PCR cycles, as determined by electrophoresis on agarose gels stained with GelGreen. PCR efficiency for each primer was determined using four different concentrations of input DNA.

For all the tested samples, the normal IgG precipitated negligible levels of DNA for the analyzed target genes, which resulted in either no threshold cycle (Ct) values or a very high Ct value similar to those obtained in negative control consisting of PCR reactions with no template. For each sample, specific ChIP DNA was normalized to input DNA using the 2^−ΔCt method (ΔCt = input DNA Ct − ChIP DNA Ct) for data analysis.

**Statistical analyses.** Data are means ± SE. Differences between the three groups (NC, PTU, T3) were analyzed using one-way ANOVA with a Newman-Keuls post hoc test using GraphPad Prism software. Statistical significance was set at P < 0.05.

### Table 1. Primers used for ChIP qPCR

<table>
<thead>
<tr>
<th>Primer Designation</th>
<th>Sequence</th>
<th>Product Size, bp</th>
<th>Position on MHC Gene Locus</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>Fwd: CCGCCGCGTGAGCTTTCCATGCC</td>
<td>101 bp vs. β-MHC TSS</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Rev: CCGCCGCGTGAGCTTTCCATGCC</td>
<td>244 bp vs. β-MHC TSS</td>
<td></td>
</tr>
<tr>
<td>B</td>
<td>Fwd: GCAGCGCCGAGCATGCACAGA</td>
<td>100 bp vs. α-MHC TSS</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Rev: GCAGCGCCGAGCATGCACAGA</td>
<td>100 bp vs. α-MHC TSS</td>
<td></td>
</tr>
<tr>
<td>C</td>
<td>Fwd: TCGCGCGCCGTTGACAGATGT</td>
<td>100 bp vs. α-MHC TSS</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Rev: TCGCGCGCCGTTGACAGATGT</td>
<td>100 bp vs. α-MHC TSS</td>
<td></td>
</tr>
<tr>
<td>D</td>
<td>Fwd: TCGCGCGCCGTTGACAGATGT</td>
<td>100 bp vs. α-MHC TSS</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Rev: TCGCGCGCCGTTGACAGATGT</td>
<td>100 bp vs. α-MHC TSS</td>
<td></td>
</tr>
<tr>
<td>E</td>
<td>Fwd: CAGCGCGCGCCGTTGACAGATGT</td>
<td>100 bp vs. α-MHC TSS</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Rev: CAGCGCGCGCCGTTGACAGATGT</td>
<td>100 bp vs. α-MHC TSS</td>
<td></td>
</tr>
<tr>
<td>F</td>
<td>Fwd: CGCGCGCGCCGTTGACAGATGT</td>
<td>100 bp vs. α-MHC TSS</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Rev: CGCGCGCGCCGTTGACAGATGT</td>
<td>100 bp vs. α-MHC TSS</td>
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</tr>
<tr>
<td>G</td>
<td>Fwd: CGCGCGCGCCGTTGACAGATGT</td>
<td>100 bp vs. α-MHC TSS</td>
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<tr>
<td></td>
<td>Rev: CGCGCGCGCCGTTGACAGATGT</td>
<td>100 bp vs. α-MHC TSS</td>
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</table>

The β-actin primers were used for both pre-mRNA and chromatin immunoprecipitation (ChIP)-quantitative polymerase chain reaction (qPCR) analyses. Primers C, D, and G were also used in RT-PCR to determine β-myosin heavy chain (β-MHC) pre-mRNA, antisense β-RNA, and α-MHC pre-mRNA expression, respectively. TSS, transcription start sites. See Fig. 2G for a schematic of the cardiac MHC gene locus with the location of the designated primers.
RESULTS

Heart weight and body weight. Seven days of PTU treatment had no effect on body weight, whereas the left ventricle weight was significantly lower than the control (PTU: 427 ± 12 mg; NC: 540 ± 13 mg) and resulted in a 19% decrease in left ventricle weight-to-body weight ratio ($P < 0.05$). Daily T$_3$ treatment resulted in a 12% decrease in body weight ($P < 0.05$), whereas the left ventricle was enlarged by 36% relative to body weight (data not shown). These responses are consistent with previous findings of hypothyroid and hyperthyroid treatments (16, 37). Thus the effectiveness of T$_3$ and PTU treatments in creating a hyperthyroid and hypothyroid state in the present study was verified using heart weight and heart weight-to-body weight ratios.

Left ventricular RNA analyses. Total RNA concentration in left ventricular tissue decreased by 8% ($P < 0.05$) in the PTU-treated rats, whereas it was increased by 18% in the T$_3$-treated animals ($P < 0.05$) (data not shown). Normal control left ventricles have a predominant $\alpha$-MHC mRNA expression (87.9 ± 2.7% of total MHC mRNA). PTU was associated with a significant decrease in $\alpha$-MHC expression and a simultaneous increase in the $\beta$-MHC mRNA (Fig. 1). Also, in response to T$_3$, the $\beta$-MHC mRNA became repressed, whereas the $\alpha$-MHC mRNA became almost exclusively expressed (Fig. 1). These results demonstrate the antithetical regulation of cardiac MHC genes by thyroid state. These responses are a hallmark of altered thyroid state; and they validate the effectiveness of the treatment in altering the cardiac MHC transcription. As a marker of transcription, we report the expression of the pre-mRNA for the $\alpha$-MHC gene and the $\beta$-MHC gene, as well as the antisense $\beta$-RNA and the intergenic sense $\alpha$-RNA. These data are reported in Fig. 1, and they show that the pre-mRNA for $\alpha$- and $\beta$-MHC follows the same pattern as the mRNA. In addition, we show that the antisense $\beta$-RNA and the sense intergenic RNA transcripts occurring in the intergenic region between $\beta$ and $\alpha$ also have the same pattern of expression as the $\alpha$-MHC gene (Fig. 1). Importantly, the $\beta$-actin pre-mRNA expression remained unchanged across the three groups (data not shown).

Histone modifications analyses of specific markers of either active or repressed chromatin states. The association of histones with specific posttranslational modifications was assessed at the intergenic region, as well as at the $\alpha$-MHC gene, $\beta$-MHC gene, and $\beta$-actin gene, using ChIP assays (see Table 1 for primer information). As a negative control for each sample, we carried out the ChIP reactions with normal rabbit serum or normal rabbit IgG at the same concentration used for the specific antibody. We examined the following histone marks: acetyl histone H3 lysine 9 and 14 (H3K9/14ac) and trimethyl histone H3 at lysine 4 (H3K4me3). Both of these posttranslational modifications of histone H3 are markers of an active chromatin state and are associated with transcriptionally...
active genes. In contrast, as markers for repressed chromatin, we analyzed monomethyl, dimethyl, and trimethyl histone H3 at lysine 9 (H3K9me1, H3K9me2, H3K9me3) and mono-methyl histone H4 at lysine 20 (H4K20me1). ChiP with antibodies against histones with specific modifications resulted in 300- to 10,000-fold enrichment over precipitation using normal serum IgG, which confirmed specificity of the ChiP reactions.

**Histone modifications at the β-MHC gene.** ChiP results demonstrated that the chromatin associated with the β-MHC gene is less enriched in H3K9/14ac and H3K4me3 in the T3-treated heart (Fig. 2, A–D), which is consistent with the β-MHC transcriptional repression. Association of the β-MHC gene with H3K9/14ac was not altered in the PTU heart, whereas association with H3K4me3 was significantly increased (Fig. 2, A–D). This latter observation is consistent with activation of the β-MHC gene in the left ventricle of the PTU-treated rat. Examining the β-MHC promoter association with histones of repressive chromatin showed that the β-MHC gene association with histone H3K9me3 was not altered in response to altered T3 state (data not shown).

**Histone modifications at the intergenic region of the cardiac MHC gene locus.** ChiP results demonstrate that the chromatin associated with the intergenic region is depleted of H3K9/14ac and H3K4me3 in the PTU heart (Fig. 3, A–D), which correspond to repression of the intergenic sense and antisense transcription in the hypothyroid state (Fig. 1). In contrast, in the T3 heart, the chromatin association with H3Ac and H3K4me3 in the intergenic region was position dependent. For example, in the 3'-flanking region relative to TSS of the antisense β-RNA, using primer D (Table 1), there was enrichment of these two histone marks relative to control (Figs. 3, A and C). In contrast, in the 5'-flanking region between sense and antisense intergenic TSS, using primer E (Table 1), the chromatin association with H3K9/14ac and H3K9me3 was lower than control (Figs. 3, B and D). The reason for this difference is not clear, but it could have to do with total histone H3 density being significantly lower near the TSS of active promoters (40). Examining the intergenic promoter’s association with histones of the repressive chromatin demonstrated a significant increase in the intergenic promoter association with histone H3K9me3 (Fig. 3E) and H3K9me2 (data not shown) in the PTU group. This observation is consistent with the function of this specific histone modification and the repressed transcriptional activity of the intergenic promoter in the PTU group (Fig. 1). This increase in H3K9me was also observed in other locations of the promoters, i.e., in the 3'-flank of the β-anti-
sense TSS using primer D (Table 1 and data not shown). There was no alteration in intergenic promoter chromatin association with the repressive histone mark H4K20me1 (Fig. 3).

**Histone modifications at the β-α intergenic region.** ChIP was performed with specific antibodies against specified histone modifications: H3K9/14ac (A and B), H3 K4me3 (C and D), H3K9me3 (E), and H4K20me1 (F). ChIP qPCR targeted the β-α intergenic region of the cardiac MHC gene locus in either the 5’ (primer E)- or 3’-flanking region (primer D)-flanking region relative to antisense β TSS (see Table 1 for primers information). Immunoprecipitated DNA qPCR data are expressed to input DNA within the same reaction. The designated primer used in qPCR is indicated in parentheses on the y-axis. G: the designated primer location relative to cardiac MHC genes. Values are means ± SE; n = 6/group. *P < 0.05 vs. NC. #P < 0.05, T3 vs. PTU.

**Histone modifications at the α-MHC gene.** Chromatin immunoprecipitation results demonstrate that the chromatin associated with α-MHC gene is less enriched in H3K9/14ac and H3K4me3 in the heart of PTU group when targeting either 3’- or 5’-flanking regions (Fig. 4, A–D), which is consistent with the α-MHC repression in the PTU heart. To our surprise, however, we also found that T3 treatment resulted in depletion of H3K9/14ac and H3K4me3, markers of active genes, both 5’ and 3’ to TSS (Fig. 4, A–D) (see DISCUSSION). Examining the α-MHC promoter association with histones of the repressive chromatin demonstrated that the association with dimethyl and trimethyl histone H3K9 was increased in the heart of the PTU group whereby the α-MHC was repressed (data not shown and Fig. 4E, respectively). There was no observed change with association of the chromatin with repressive histone mark H4K20me1 (Fig. 4F).

**Histone modifications at the β-actin gene.** The β-actin gene transcriptional activity remained unchanged after 7 days of altered thyroid state (data not shown). Therefore, it represents a good control gene to use to ascertain the state of histone modifications in its chromatin with the presumption that if a certain histone modification was critical to induce altered transcription, it should not vary in the chromatin of the β-actin gene. ChIP results show that the histone modifications within the chromatin associated with the β-actin remained unaltered in the altered thyroid state (Fig. 5). For all the antibodies we tested, the antibody specifically immunoprecipitated the chromatin of β-actin, since we obtained 300- to 1,000-fold over background precipitation with normal serum IgG. Interestingly, the chromatin of the β-actin gene was associated with both active (H3K9/14ac and H3K4me3) and repressive histone marks (H3K9me and H4K20me1) based on immunoprecipitation data (Fig. 5). However, theses associations appeared to remain the same in magnitude in response to altered state, which is concordant with unaltered β-actin gene activity. Although unexpected finding, the β-actin gene association with repressive histone marks is not unique. For example Vakoc et al. (64, 65) and others (6, 69) have reported that the chromatin of some actively transcribed genes was found enriched with repressive histone marks such as H3K9me3 and H4K20me1 chromatin.

**DISCUSSION**

Gene regulation is governed by the combinations of transcription factor and cofactor expression patterns and their interactions with the binding sites on promoter regulatory regions. Furthermore, the chromatin structure regulates the accessibility of binding sites by transcription factors and cofactors, and thus can play a major role in gene regulation. The chromatin structure is thought to play a major role in the regulation of the cardiac MHC gene locus during development and in response to altered thyroid hormone, in which there is an
antithetical regulation of the cardiac MHC genes (30, 31, 48). Two characteristics of the cardiac MHC genes make the locus likely to be highly regulated by epigenetic mechanisms: 1) the presence of ncRNA transcription and 2) its high sensitivity to thyroid hormone due to the presence of TREs on the gene promoters. Both of these characteristics are thought to involve epigenetic regulation of gene expression.

Epigenetic regulation of gene expression. Epigenetic phenomena such as histone modifications and chromatin remodeling involve the collaboration of numerous components of the epigenetic machinery. The existence of opposing chromatin-modifying enzymes, along with cross talk between different epigenetic systems, demonstrates the inherent complexity in dynamic gene regulation by epigenetic modifications. Transitions between different chromatin states are dynamic and depend on a balance between factors to sustain a silent state (for example, HDACs and histone H3K9 methyltransferases) and those that promote a transcriptionally active state (for example, HATs and histone H3K4 methyltransferases). Disturbances of any of these components can shift the balance between an active and a silent chromatin conformation, resulting in an altered transcriptional state (34).

Noncoding RNA is a probable guide for histone-modifying enzymes to specific target sites. Previously, we have shown that the intergenic region between the two cardiac MHC genes is transcriptionally active on both strands (22). On the upper strand it transcribes the sense intergenic RNA within the α-MHC promoter, which also is positively coregulated with the α-MHC pre-mRNA. On the opposite strand, it transcribes the antisense β-RNA, which is also coregulated with the β-MHC pre-mRNA (22). These intergenic transcripts are noncoding regulatory RNAs that are found abundantly in both the mammalian and complex organism transcriptome (46). We proposed that these noncoding intergenic transcripts are regulatory, and their main role is to maintain a tight coordinated regulation of the cardiac MHC gene expression. Their exact mode of regulation by ncRNA is not clear, but it is believed to be via epigenetic processes involving histone modification and chromatin remodeling. In fact, recent evidence indicates that ncRNAs play a role in guiding chromatin-modifying enzymes to specific sites to alter nearby gene transcription (45, 47). Thus the goal of this current study was to explore histone modifications in different regions of the cardiac MHC gene locus under states of either active or repressed intergenic transcription, such as in euthyroid, hypothyroid, and hyperthyroid states. We have targeted the α-MHC promoter, the intergenic region, and the β-MHC promoter for qPCR after ChIP with antibodies to histones with specific modifications, to examine their association with corresponding chromatin and their relationship to ncRNA transcription.

![Fig. 4. Histone modifications at the α-MHC gene. ChIP was performed with specific antibodies against specified histone modifications: H3K9/14ac (A and B), H3 K4me3 (C and D), H3K9me3 (E), and H4K20me1 (F). Each ChIP used 10 μg of chromatin DNA isolated from left ventricular tissue of NC, PTU-treated, and T3-treated rats and an optimized amount of antibodies (3–7 μl, depending on the antibody). Real-time SYBR green qPCR targeted the α-MHC gene in either the 5′ (primer F) or 3′-flanking region (primer G or H)-flanking region vs. TSS (see Table 1 for primer information). Immunoprecipitated DNA qPCR data are expressed relative to input DNA within the same reaction. The designated primer used in qPCR is indicated in parentheses on the y-axis. G: the designated primer location relative to cardiac MHC genes. Values are means ± SE; n = 6/group. *P < 0.05 vs. NC. #P < 0.05, T3 vs. PTU.]
Effect of T₃ on cardiac MHC gene expression: a novel perspective. Previous studies have demonstrated the powerful effect of thyroid state on the heart, in particular on the MHC phenotype (4, 48). High T₃ levels stimulate the expression of the β/MHC while repressing that of the α/MHC gene. Low T₃ levels induce the opposite changes (see Fig. 6 for a schematic presentation of the cardiac MHC gene locus in both hypothyroid and hyperthyroid states). Although T₃ is known to be a potent regulator of the cardiac MHC genes, in this study we examined its effect from an epigenetic perspective. The goal was to identify alterations in histone modifications in the chromatin of the cardiac MHC gene locus when the animals are subjected to either a hypothyroid (PTU) or a hyperthyroid (T₃) state.

T₃ exerts its action via its nuclear TRs, transcription factors belonging to the superfamily of nuclear hormone receptors. These receptors bind to TREs in the promoters of target genes and can regulate both positive and negative transcription states depending on the TRE. It has been shown that both liganded and unliganded TRs are bound to TREs and are involved in the regulation of gene transcription. The dual regulatory activity of the TR has been attributed to the TR’s ability to associate with proteins that possess regulatory function directly affecting the chromatin structure (50, 68, 72). The α/MHC gene is positively regulated by T₃, and this positive regulation is attributed to the presence of several TREs in both the promoter and intergenic region (17, 22, 32). The molecular mechanism of T₃-dependent gene activation is relatively well understood. For example, NCoR and SMRT can play the role of coactivators for thyroid-stimulating hormone TSHα, TSHβ, and thyrotropin-releasing hormone (TRH) genes (60), whereas coactivators and corepressor release play a role in T₃-dependent repression of TSHα (61, 67). Thus, coactivator-associated changes in histone modifications and alterations in chromatin structure may be involved in T₃-mediated negative regulation. Despite this evidence for other genes (phospholamban, TSHα, and TSHβ), until now it was not possible to identify the negative TRE on the β/MHC promoter, although there was a report the β/MHC gene promoter attributed T₃ negative regulation to a negative TRE located near the TATA box (14) (Fig. 6). However, that could not be proven since its mutation eliminated all basal activity of the promoter. Thus, based on the current literature, it appears that the effect of T₃ on the β-MHC promoter is not well characterized and may well be an indirect effect via other T₃-induced transcription factors or events. One possible scenario of T₃ effects is as illustrated in Fig. 6. In this model, T₃ stimulates intergenic promoter activity via recruitment of chromatin-activating complexes. The intergenic promoter initiates sense and antisense ncRNAs. The antisense ncRNA is pro-
posed to guide chromatin repressing complexes to be recruited at the β-promoter to alter its chromatin structure and repress its activity (45).

In the present study, we used the ChIP assay to explore chromatin enrichment with specific histone modifications targeting strategic regions of the cardiac MHC gene locus. Results are summarized in Table 2 in the form of directional changes of transcription as well as histone modifications. We discuss the results with a potential role of T₃-TR as well as ncRNA on histone modifications and in the context of ascertaining agreement with the proposed model in Fig. 6.

**Histone acetylation in chromatin of cardiac MHC genes: response to altered T₃.** Acetylated histones normally associate with chromatin of active genes (34, 49). ChIP analyses of histone acetylation using a specific antibody against H3K9/14ac showed that the β-MHC gene acetylation was depleted in response to T₃ treatment, a finding that correlates well with the repression of β-MHC transcription (Fig. 2, A and B, and Table 2). T₃ is associated with an increased antisense ncRNA, in which case antisense ncRNA is proposed to recruit HDACs to deacetylate the histone H3 at the β-promoter. In the NC state, there is a significant ongoing antisense ncRNA transcription (Fig. 4C), which should recruit HDACs to reduce the levels of H3K9/14ac at the β-promoter (Fig. 6). However, the level of H3K9/14ac was still high in the β-promoter in the euthyroid state, comparable to the hypothyroid state (PTU) (Fig. 2, A and B). This is an abnormality in the response that does not fit our model of ncRNA recruitment of HDACs. One possible explanation for this paradoxical response involves the possibility that there may be a threshold for how much ncRNA is being transcribed to effectively recruit HDACs. For example, with T₃ treatment, there is a modest increase in antisense ncRNA transcription that could have brought the levels above threshold. On the other hand, the presence of high levels of histone H3K9/14ac associated with the β-MHC gene in the euthyroid heart could be due to some level of constitutive expression of the β-MHC gene in euthyroid hearts as shown by Danzi et al. (12) and Fig. 4B of this study. In response to T₃ treatment, our data show complete repression of β-MHC transcription; at the same time there was deacetylation of histone H3 at K9/14 within the promoter region. Furthermore, this abnormality could be due to other unknown reasons. Clearly, more research is needed to address this issue.

Examination of histone H3 acetylation of the α-MHC gene and the intergenic region demonstrated that in the hypothyroid state there is a significant depletion in histone acetylation of the intergenic region and α-MHC gene promoter (Figs. 3 and 4), which correlated well with the corresponding transcriptional activity (Fig. 1 and Table 2). In the absence of T₃ ligand, TR-TRE on the intergenic and alpha promoters can recruit HDAC repressors to deacetylate the histones in nearby chromatin. However, the histone H3K9/14ac directional shift was not concordant with the pre-mRNA in the T₃ group (Table 2). Surprisingly, in the T₃-treated group, histone acetylation at the intergenic region and in the proximal α-MHC promoter was decreased (Table 2) despite its increased transcriptional activity. These discrepancies could have to do with histone acetylation kinetics or thyroid hormone kinetics. The action of T₃-TR in recruiting HAT to the TRE vicinity may be transient and lost after 6 h of treatment. In fact, studies on molecular interactions between ligands, nuclear receptors, and gene promoters in living cells show that nuclear receptors induce transient and highly dynamic changes in the chromatin structure (2, 26, 28, 53). More research is needed in the future on the time course of T₃ actions posttreatment to better understand the time course of the response of modifying enzymes and how these relate in time to altered gene transcription. In the intergenic region near *primer D*, which is ~1.5 kb 3' to antisense ncRNA TSS, directional shift of H3K9/
Table 2. Summary of directional changes in pre-mRNA and noncoding RNA abundance and enrichment of specific histone modifications on the cardiac MHC gene locus in response to PTU and T3 treatments

<table>
<thead>
<tr>
<th>Gene Promoter Variable</th>
<th>PTU Effects</th>
<th>T3 Effects</th>
</tr>
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<tbody>
<tr>
<td>β-MHC</td>
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<td></td>
</tr>
<tr>
<td>Pre-mRNA</td>
<td>↑</td>
<td>↓</td>
</tr>
<tr>
<td>H3K9/K14ac</td>
<td></td>
<td></td>
</tr>
<tr>
<td>H3K4me3</td>
<td>↑</td>
<td>↓</td>
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<tr>
<td>H3K9me2</td>
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</tr>
<tr>
<td>H3K9me1</td>
<td>↑</td>
<td>↓</td>
</tr>
<tr>
<td>H4K20me1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Intergenic region</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sense ncRNA</td>
<td></td>
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<tr>
<td>Antisense ncRNA</td>
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<tr>
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<td>H3K9me1</td>
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<td>H4K20me1</td>
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<tr>
<td>α-MHC</td>
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<td>Pre-mRNA</td>
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<td>H4K20me1</td>
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</table>

Arrows indicate directional changes in pre-mRNA and noncoding RNA (ncRNA) abundance and enrichment of specific histone modifications on the cardiac MHC gene locus in response to propylthiouracil treatment (PTU) and thyroid hormone (T3) treatments. NC, normal control; see text for definition of histone marks. *P < 0.05, T3 vs. PTU (not significant for T3 vs. NC). †Unexpected result; opposite to expectation based on known function of histone modification and gene activity. ‡Position-dependent effect; there was a significant enrichment in histone modification with this mark at the center of the intergenic region, 5′-flanking region of intergenic TSS of both sense and antisense, using primer E in Table 1.

14ac matched perfectly with ncRNA activity (Fig. 3A), which also raises the possibility that the effect may be dependent on position vs. TSS and T3-TR-TRE kinetics in response to T3 injections.

Histone methylation in chromatin of cardiac MHC genes: response to altered T3. Unlike histone acetylation, which is always linked to active chromatin, histone methylation can have either a positive or a negative influence on transcription depending on the site of methylation on the histone tail. For example, methylation of histone H3 at K4 is thought to be involved in gene activation, whereas methylation of histone H3 at K9, as well as decreased methylation of histone H3 at K4 (42). In contrast, transcription activation by liganded TR was coupled with a significant decrease in histone H3 methylation at both K9 and K4 (42). This latter finding is unexpected, since the H3K4me mark is thought to be associated with active chromatin. However, it appears that there is an interplay among various histone modifications to achieve local control of gene transcription. In fact, according to the histone code hypothesis (34, 57), it is the overall pattern of histone modifications that determines the outcome on chromatin structure and function. Thus a single modification can either inhibit or activate transcription, depending on the context of nearby histone modifications.

In the present study, we determined the change of repressive histone mark, histone H3 methylation at K9 in response to altered thyroid hormone. For the β-MHC gene, there was no histone H3K9/14ac (Figs. 3 and 4). This would be possible if T3-TR-TRE were to recruit both HAT and HMT at K4 associated with active chromatin, whereas an unliganded TR on a positive TRE could recruit HDAC and histone demethylases for K4.

Previously Li et al. (42) demonstrated that, in addition to histone acetylation and deacetylation, other histone modifications, including methylation and phosphorylation, are also involved in transcriptional control by TR. For example, in Xenopus oocytes, gene repression by unliganded TRs was associated with increased methylation of histone H3 at K9 as well as decreased methylation of histone H3 at K4 (42). In contrast, transcription activation by liganded TR was coupled with a significant decrease in histone H3 methylation at both K9 and K4 (42). This latter finding is unexpected, since the H3K4me mark is thought to be associated with active chromatin. However, it appears that there is an interplay among various histone modifications to achieve local control of gene transcription. In fact, according to the histone code hypothesis (34, 57), it is the overall pattern of histone modifications that determines the outcome on chromatin structure and function. Thus a single modification can either inhibit or activate transcription, depending on the context of nearby histone modifications.
significant change in chromatin enrichment with histone H3 methylation at K9 in response to altered thyroid state (Table 2). In contrast, the associations with H3K9me2 and H3K9me3 were higher in chromatin associated with the α-MHC gene and intergenic region of the PTU ventricle. These observations suggest recruitment of repressive histone methyltransferases on the chromatin by unliganded TR-TRE to repress nearby genes. In the hyperthyroid state, there was no change in enrichment of H3K9me at the α-promoter relative to the euthyroid state. This lack of response may mean that the T₃ response has a lower threshold that is already met in the euthyroid state.

In addition to H3K9me, we explored chromatin enrichment with repressive histone mark H4K20me1. Histone H4K20me1 has been implicated in both gene repression as well as gene activation (73). ChIP results demonstrated no change for the α-MHC promoter and intergenic region regardless of T₃ state (Figs. 3 and 4), whereas at the β-MHC promoter, the chromatin was depleted from monomethyl histone H4 at K20 in the PTU group (Fig. 2F), and thus it negatively correlated with the β-MHC gene activity (Fig. 7A). It is curious that the enrichment of histone H4K20me1 at the β-MHC promoter positively correlated with antisense ncRNA abundance (Fig. 7B). Is it possible that antisense ncRNA is recruiting chromatin-repressing complexes such as methyltransferases targeting H4K20. Chromatin enrichment of H4K20me1 was suggested as an early event in the initiation of the X chromosome inactivation (38). This mechanism also involves antisense transcription (41, 54) similar to that observed for the β-MHC gene (22).

Perspectives and Conclusions

The regulation of transcription by thyroid hormone involves cooperation among many processes, including histone modifications, chromatin organization, and dynamic alterations in nucleosome structure as well as interconnected networks of signaling within the cells. Using the ChIP assay, we have identified specific histone modifications associated with the chromatin of the MHC genes in response to altered thyroid state. Results were fit into a working model whereby the cardiac MHC gene locus is subject to regulation by chromatin remodeling. Chromatin structure can be influenced by ncRNA as well as by T₃-TR-TRE interactions. We propose that T₃ action is mainly on the α-promoter/intergenic region, whereas its action on the β-promoter occurs indirectly via ncRNA regulation. Future studies need to amplify these findings by examining other modifications and recruitment of modifying enzymes to the promoters. Identifying a mechanism of T₃-dependent MHC gene expression may have important implications in treating thyroid disorders as well as during heart failure and could lead to identification of specific therapeutic drug targets.

ChIP assays examine chromatin structure in a single time frame. Although the information obtained from the ChIP assay is valuable and represents a current state of chromatin, one should keep in mind that the turnover rate of histone modifications can also influence data interpretation (9). Histone modifications are reversible, and some can exist only transiently for a short period of time and thus can be absent at the specific time frame of the analysis.

A shortcoming of ChIP assays on chromatin prepared from cardiac muscle tissue is the fact that the myocardium is a heterogeneous muscle whereby the majority of the cells are nonmuscle cells (fibroblasts, interstitial, and endothelial cells) (33). However, nonmuscle cells do not express the MHC gene under any condition. Therefore, we expect chromatin associated with these MHC genes in nonmuscle cells to be repressive and not responsive to altered thyroid state. Thus, if we detected a change in histone modifications, we assumed that it is reflecting chromatin MHC genes in myocytes. Consequently, to be accurate, one must consider the current reported ChIP results in the context of cardiac tissue and not pure myocardial cells. Future studies should be carried out on highly enriched fractions of myonuclei along with cardiac tissue to validate our speculation.

In conclusion, our results identify specific dynamic histone modification events, along the cardiac MHC gene locus, that are associated with the antithetical regulation of α- and β-MHC genes. These histone modifications could be the result of T₃-TR-TRE on the α-promoter, or the result of regulatory ncRNA. Thus both systems could guide and recruit chromatin modifying complexes as a function of thyroid state.

ACKNOWLEDGMENTS

We greatly thank Dr. Clay Pandorf for critical reading of the manuscript. We greatly thank Li-Ying Zhang, Ming Zeng, Alvin Yu, Sandy Liu, Daniel Jemineme, Phuc Tran, and Marinelle Camillon for excellent technical assistance.

GRANTS

This research was supported by National Heart, Lung, and Blood Institute Grant HL-073473 (to K. M. Baldwin).

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

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