Intergenic transcription and developmental regulation of cardiac myosin heavy chain genes

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Haddad F, Qin AX, Bodell PW, Jiang W, Giger JM, Baldwin KM. Intergenic transcription and developmental regulation of cardiac myosin heavy chain genes. Am J Physiol Heart Circ Physiol 294: H29–H40, 2008. First published November 2, 2007; doi:10.1152/ajpheart.01125.2007.—Cardiac myosin heavy chain (MHC) gene expression undergoes a rapid transition from β- to α-MHC during early rodent neonatal development (0–21 days of age). Thyroid hormone (3,5,3′-triiodothyronine, T3) is a major player in this developmental shift; however, the exact mechanism underlying this transition is poorly understood. The goal of this study was to conduct a more thorough analysis of transcriptional activity of the cardiac MHC gene locus during the early postnatal period in the rodent, in order to gain further insight on the regulation of cardiac MHC genes. We analyzed the expression of α- and β-MHC at protein, mRNA, and pre-mRNA levels at birth and 7, 10, 15, and 21 days after birth in euthyroid and hypothyroid rodents. Using novel technology, we also analyzed RNA expression across the cardiac gene locus, and we discovered that the intergenic (IG) region between the two cardiac genes possesses bidirectional transcriptional activity. This IG transcription results in an antisense RNA product as described previously, which is thought to exert an inhibitory effect on β-MHC gene transcription. On the second half of the IG region, sense transcription occurs, resulting in expression of a sense IG RNA that merges with the α-MHC pre-mRNA. This sense IG RNA transcription was detected in the α-MHC gene promoter, approximately 1.8 kb relative to the α-MHC transcription start site. Both sense and antisense IG RNAs were developmentally regulated and responsive to a hypothyroid state (11, 14). This novel observation provides more complexity to the cooperative regulation of the two genes, suggesting the involvement of epigenetic processes in the regulation of cardiac MHC gene locus.

gene transcription; antisense RNA; pre-mRNA; thyroid hormone; reverse transcriptase-polymerase chain reaction; bidirectional promoter; epigenetic regulation; Sprague-Dawley rats

CARDIAC MUSCLE EXPRESSES TWO myosin heavy chain (MHC) isoforms designated as α (high ATPase) and β (low ATPase), which are encoded by two distinct genes. These two genes are located in tandem in close proximity on the same chromosome, in the order of their expression during development (25). The MHC is the molecular motor driving muscle contraction, and its phenotypic composition regulates the intrinsic contraction properties of the heart (1, 10, 27, 38). The pattern of cardiac MHC isoform expression is highly dynamic in that it can be changed totally in either direction in certain pathophysiological states (6, 30–32, 39). For example, while α-MHC normally predominates in the adult rodent, hypothryoidism and diabetes are associated with a switch in cardiac MHC gene expression from a predominant α- to a predominant β-MHC (6, 30). In contrast, thyroid hormone (3,5,3′-triiodothyronine, T3) treatment increases α-MHC expression while repressing β-MHC expression.

While the exact molecular mechanism causing such antithetical coordinated regulation of these two genes remains unclear, T3 manipulation has been shown to be a major regulator of MHC gene expression, and its regulation is thought to occur mainly via transcriptional processes involving T3 interaction with its receptor bound to the promoter of target genes (9, 12). Several thyroid-responsive elements have been located on the promoter of the α-MHC gene, whereas the localized action of T3 on the β-MHC promoter remains poorly defined (3, 7, 20, 37, 40, 41). In previous studies an antisense β-MHC RNA product was discovered, and this product was implicated in the antithetical regulation of cardiac MHC expression in response to both hypothyroidism and diabetes (14). This antisense RNA transcript was shown to have its origin in the intergenic (IG) region linking the two genes, and it extended upstream, thereby overlapping the entire β-MHC gene including the promoter region. In addition, expression of the antisense RNA was shown to be strongly correlated with transcriptional activity of the α-MHC gene, and it was inversely correlated to transcriptional activity of the β-MHC gene (11, 14). More recently, we reported findings (16) suggesting a similar regulatory scheme in response to aortic constriction-induced pressure overload in which the β-MHC gene is upregulated while the α-MHC gene is downregulated.

In addition to the above-reported schemes of cardiac MHC switching in response to hormonal and mechanical stimuli, these isoform expression profiles are developmentally regulated. In rodents, both isoforms are expressed in the fetal heart. In late fetal life, β-MHC expression predominates in the ventricle. Shortly before birth and through the first 3 wk of postnatal life, the heart is subjected to a strong developmental cascade affecting both its growth and contractile phenotype. During this period, α-MHC expression gradually increases, whereas β-MHC becomes repressed such that the α-isoform becomes the sole isoform expressed in the ventricles. These developmental regulations are strongly influenced by the thyroid state of the neonate (24). Both cardiac growth and the myosin isoform transition can be blunted if rodents are made thyroid deficient (24, 26). This developmental transition of myosin isoform expression is probably the result of a surge in circulating levels of T3 that occurs in the first 2–3 wk after birth (24).

The goal of the present study was to investigate cardiac MHC isoform regulation in developing rodent ventricles, mak-
ing use of newly available sequence databases and better molecular biology tools. Modern technical advancements allow one to analyze transcriptional activity (marked by RNA expression) and strand-specific RNA expression corresponding to different regions of the cardiac gene locus. These analyses include sense and antisense RNA expression in the IG spacer linking the two cardiac MHC genes. Thus, with the presently available information that was not available a few years ago, the goal of this study was to conduct a more thorough analysis of transcriptional activity of the cardiac MHC gene locus during the early postnatal period in the rodent. We examined the expression of the two cardiac MHC isoforms (α- and β-MHC) at the protein, mRNA, and pre-mRNA levels at birth and 7, 10, 15, and 21 days after birth. This temporal assessment made it possible to capture dynamic changes in gene expression during a critical stage in myocyte gene remodeling that had not been assessed before these experiments. Furthermore, in view of previous findings on the involvement of antisense RNA with cardiac MHC regulation (11, 14), we tested the general hypothesis that the inherent developmental program involving cardiac MHC transition in the normal postnatal cascade is coordinated by antisense β-MHC RNA expression. In addition, in these same neonatal hearts, we have analyzed both sense and antisense RNA expression in the IG region between the two cardiac genes. Not only did these investigations of transcriptional activity across the cardiac MHC gene locus reveal the expression of antisense RNA as reported previously, but to our surprise, we detected sense RNA resulting from sense transcription in the α-MHC gene promoter, at approximately −1.8 kb relative to the α-MHC transcription start site (TSS). This unique observation provides additional complexity to cardiac MHC gene regulation.

Previously, we showed that an antisense β-MHC RNA may play a critical role in cardiac MHC isoform switch in the adult heart in response to diabetes and to a hypothyroid state (11, 14). It is not clear whether the normal developmental MHC cascade is also influenced by antisense RNA regulation and how the sense IG RNA might fit into play. We propose that both the antisense and sense RNA, which originate in the IG region, tightly link the two cardiac MHC genes and account for their reciprocal regulation. The presented results and discussion suggest that the developmental cascade is not established by mere independent regulation of the two genes, but as one gene is upregulated, the other is repressed in a well-coordinated fashion as the myocardium undergoes a marked increase in mass.

METHODS

Animal care and experimental design. Nine timed pregnant rats (gestational day 14) were purchased from Taconic Farms (Germantown, NY) and housed in individual cages in light- and temperature-controlled quarters. After birth the neonates were randomly redistributed to ensure a similar number of neonates (N = 10) per female parent. The neonates were then allocated into two experimental groups: 1) normal control (NC) and 2) propylthiouracil (PTU) treated to induce thyroid deficiency. At the time of birth, some animals were killed (0 days, n = 8) and were used as the reference base for the variables examined in the study. Neonates from the two groups were then studied at 7, 10, 15, and 21 days of age. For groups designated to be hypothyroid (PTU treated), both the mother and the neonates began receiving daily intraperitoneal injections of PTU (12 mg/kg body wt) from postnatal day 1 until the end of the experiment. This dose was selected on the basis that in the adult rat 12 mg/kg approximates three times the dosage necessary to completely block conversion of L-thyroxine (T4) to T3 (18). The protocols used in the present study were approved by our Institutional Animal Review Committee.

Tissue preparation and biochemical analyses. At the designated time points, the rats were weighed and euthanized with a lethal injection of Nembutal (50 mg/kg). Blood was withdrawn from the left ventricle into EDTA-containing tubes, and then the sample was centrifuged at 1,000 × g for 10 min at 4°C. The resulting plasma was stored at −20°C until being analyzed for T3. The hearts were quickly removed, and the ventricles were trimmed of connective tissue, weighed, frozen on dry ice, and stored in a freezer (−80°C) until being processed for protein and RNA analyses.

Thyroid hormone analysis. Plasma total T3 concentrations were assayed with a commercially available RIA kit (ICN Pharmaceuticals). Measurements were performed on groups at 7, 10, 15, and 21 days of age. Insufficient blood was obtained for the animals at the time of birth for a complete analysis; therefore, the 0 day time point data were not reported.

MHC protein analyses. A preweighed portion of the left ventricle sample was homogenized in 20 volumes of a solution that contained (in mM) 250 sucrose, 100 KCl, 5 EDTA, and 10 Tris-Cl, pH 7.0. The homogenate protein was diluted 10-fold with a storage buffer containing 50% glycerol, 50 mM Na2P2O7, 2.5 mM EGTA, and 0.5 mM β-mercaptoethanol (pH 8.8) and was stored at −20°C until subsequent analysis. Cardiac MHC protein isoforms were separated by electrophoresis using an SDS-PAGE technique according to the procedures described by Reiser et al. (35). At the end of the run, the gels were stained with Coomassie blue to visualize the protein bands. Band identification was based on comparisons to known α- and β-MHC bands, such that the atria and soleus muscle MHC profiles were used as positive identification for α- and β-MHC, respectively. The gel was scanned with laser scanning densitometry for image analyses. The calculation of MHC composition as percentage of total was based on the integration of the area under the peak of the density of the line drawn across the MHC bands (Image Quant software, GE Healthcare).

MHC mRNA analyses. Total RNA was extracted from frozen ventricles with the Tri Reagent protocol (Molecular Research Center). The extracted RNA was DNase treated, using 1 unit of RQ1 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). The RNA concentration was determined by optical density at 260 nm (OD260), and its high purity was confirmed based on an OD290/OD280 ratio of ~2. The RNA integrity was assessed by gel electrophoresis and ethidium bromide staining of 0.5 µg of total RNA on the gel. Only RNA samples that were not degraded were utilized for the RNA analyses. At first, a two-step RT-PCR was carried out with a mix of random primers and oligo(dT) in the reverse transcription step. The PCR reactions were directed to amplify the specific MHC mRNA isoform (α or β) along with an external control fragment that was added to the cDNA. The external control DNA was a heterologous fragment that was synthetically modified so that it can be amplified with either the α-MHC or the β-MHC primer sets to give PCR products that are either 244 or 224 bp, respectively, which can be separated from the PCR product of the coamplified endogenous MHC bands (~545 bp). The exogenous control PCR signal was used to correct for the PCR reaction efficiency. This RT-PCR procedure was designed to determine cardiac MHC mRNA composition, and the results are expressed as a percentage of the total MHC mRNA pool.

Sense and antisense primary transcript analyses. In addition to the above procedure, a strand-specific RT-PCR approach was utilized to analyze sense and antisense primary transcripts (RNA) corresponding to several locations on the cardiac MHC gene locus. These included sense and antisense β- and α-MHC RNA as well as IG RNA. RT-PCR reactions were performed with the One Step RT-PCR Kit (Qiagen) so that the reactions were performed in one reaction tube. In this assay
system, the reactions were carried out with 100 ng of total RNA in a 25-μl reaction. In the reverse transcription (RT) step, only one primer was included. The forward primer in the RT reaction was used to target the antisense RNA, whereas the reverse primer was used to target the sense RNA. The RT reactions were carried out at 50°C for 30 min, followed by denaturing of the RT enzyme for 15 min at 95°C. After this step, the missing PCR primer was added to the reaction tube, and this step was followed by PCR for 25–30 cycles depending on the target abundance (see Supplemental Table 1 for primer information). For each run, a representative sample from each group was included. Conditions were optimized so that the product was in the linear range of detection. PCR products were separated by gel electrophoresis on a 2.5% agarose gel using 1× Tris-acetate-EDTA (TAE) buffer. Gels were stained with ethidium bromide, and then the gel was exposed to a UV light source to capture the image with a digital camera. Band intensity was analyzed with Image Quant software. For these one-step RT-PCR reactions, we carried out two types of negative controls for each RNA sample. 1) To ensure that the product was RNA specific and not from DNA, before the RNA was added to the reaction the reaction tubes were heated to 95°C for 15 min to denature the RT enzyme. After this denaturing, the RNA was added to the reaction along with the PCR primers to undergo the PCR reaction. All the utilized RNAs were found to be negative for these controls, proving that the DNase treatment was effective in removing the traces of genomic DNA. 2) The second negative control was aimed to provide evidence of the RNA strand specificity of the RT-PCR reactions. In the RT step, we either omitted the RT primer altogether or used a nonspecific primer not related to the target RNA in question. After denaturing the RT reactions, both PCR primers were added and the PCR was carried out in the same way as for the other reactions that included specific RT primers in the RT step. For all the primers used in this study, the results of these controls were negative, which validated the strand specificity of the RT-PCR reaction (15).

Quantitative real-time RT-PCR. In addition to the end point PCR used in this study, we performed real-time PCR (SYBR Green, using Stratagene Mx3000p) to determine quantitative expression of α-MHC pre-mRNA, β-MHC pre-mRNA, and antisense β-MHC RNA at 10 and 21 days of postnatal development of the eutherian rat, a period in which the most significant changes occurred. These selected points enabled us to both validate the data generated by end point PCR and obtain a quantitative relationship among antisense β-MHC RNA, sense β-MHC, and sense α-MHC pre-mRNA at the two stages of developments, i.e., 10 and 21 days of age. For these analyses, a two-step RT-PCR system was used. RT was performed with 1 μg of total RNA, 2.5 pmol of specific primers, and Superscript II reverse transcriptase (Invitrogen) in a 10-μl reaction volume at 50°C for 30 min. This was followed by a denaturing step at 95°C for 5 min. For the RT, the primer used to target the antisense RNA was the forward PCR primer, whereas the reverse PCR primer was used as RT primer to target the sense RNA. Real-time PCR involved the use of full-velocity SYBR Green premixed reagents (Stratagene), and the reaction conditions were optimized to give efficiencies of 100 ± 5% based on standard curve analyses. PCR was carried out for 40 cycles with annealing and extension temperatures both at 60°C, followed by a melting curve analysis. For each primer set, PCR specificity was evaluated based on the presence of a single product at the end of the 40 PCR cycles, as determined by melting curve analyses showing a single peak at the product melting temperature, as well as by examination of the products after gel electrophoresis on 2% agarose gel and ethidium bromide staining. Only primers resulting in a single product were utilized. For each PCR primer target, each sample was performed in duplicate (570 nl cDNA/25 μl reaction) along with a standard curve, which was based on different cDNA amounts per reaction, ranging from 10 to 1,000 nl. Standard curves were generated via regression analyses whereby the x-axis represented the log of initial cDNA amounts expressed as nanoliters and the y-axis represented the threshold cycle (Ct) or the cycle number at which fluorescence reached a value above an arbitrary set value. The standard curve was utilized to calculate the efficiency based on the slope, and it was also utilized to ensure linearity of the amplification with different initial amounts of target cDNAs. To compare initial amounts of specific RNA in the two samples, the 2^(-ΔCt) method was utilized (34), which assumes a PCR efficiency of 100%; Ct is the threshold cycle, or the cycle number at which amplification fluorescence reaches a value above a preset threshold. In these two-step RT-PCR reactions, negative control RT-PCR reactions were performed in which the RT reactions were carried out under the same conditions as described above except that primers were omitted from the RT reaction. Under these conditions, we can quantitatively monitor how much from the obtained PCR signal was nonspecific to the targeted strand, that is, amplified in absence of the RT primer. Also, as additional negative controls, water and non-reverse-transcribed RNA were used as templates in the real-time PCR, and these generated no detectable fluorescent amplification signals (Ct > 37).

DNA plasmid construct injection in young mature rat ventricles. To determine whether transcriptional activity occurs in the IG region in the sense direction independently of the α-MHC proximal promoter, a truncated α-MHC promoter DNA fragment, representing IG DNA from −2.6 kb to −945 bp relative to the α-MHC TSS, was studied in a reporter gene assay system. A high-fidelity PCR reaction using Ultra pfu DNA polymerase was carried out to amplify the DNA fragment of interest, using the 5-kb intergenic fragment pRL construct as a template for this amplification (14). The PCR-generated fragment was ligated into pGL3 basic in front of firefly luciferase (Fluc) reporter gene. The generated construct was sequenced across the entire length of the insert to ensure 100% identity to the original product (Univ. of California, Irvine sequencing facility). For these studies, an additional group of young mature female rats (~150 g body wt) were used to perform direct gene transfer into the myocardium. Animals were assigned to three groups: euthyroid (NC), hypothyroid (PTU), and hyperthyroid (T3 treated) (n = 8/group). The DNA injection into the myocardium was performed under general anesthesia via a subdia- phragmatic approach exactly as described previously (46, 47). Each injection consisted of 40 μl of PBS containing 2 pmol of each of the test and reference plasmids. The cardiac myosin light chain (MLC2) gene promoter (a gift from Dr. K. Esser, University of Kentucky) driving the expression of a Renilla luciferase (Rluc) gene was used as a reference. We showed previously (11) that the activity of the MLC2 plasmid construct is not responsive to altered thyroid state. The animals were euthanized on the seventh day after direct DNA transfer, and the apex was removed for analysis. Tissue was processed as described previously for reporter gene assays (46, 47). The Fluc activity (test promoter) was normalized to Rluc activity, to correct for variation in plasmid uptake.

Statistical analyses. All data are reported as means ± SE. Developmental effects as well as PTU effects on the studied parameters were analyzed by two-way ANOVA, which determined whether the variables were significantly affected by either age or by PTU treatment and whether there was an interaction effect between age and treatment. When effects were detected for a given variable, a Bonferroni post hoc test was used to determine statistical differences between NC and PTU at a given age. Relationships between variables were analyzed with linear regression and correlation analyses. Statistical significance was set at P < 0.05. All statistical analyses were performed with the Prism GraphPad software package.

RESULTS

Plasma T3 levels. Plasma T3 levels demonstrated a significant rise during the 21-day time interval investigated. Between

1 The online version of this article contains supplemental material.
7 and 21 days of age, T₃ levels increased ~3.3-fold (Fig. 1), which is consistent with the early postnatal surge of thyroid hormones (44). In contrast, T₃ levels remained markedly depressed throughout the developmental period in the PTU-treated group (Fig. 1).

Body weight, heart weight, and heart weight-to-body weight ratios. Body weight (BW) increased approximately eightfold from birth to 21 days of age in the euthyroid group of neonates, with the greatest gain occurring between days 15 and 21 (Supplemental Fig. 1A). This response was markedly reduced in the hypothyroid group, because BW increased only approximately fourfold during the first 21 days. The divergence in BW between the two groups occurred primarily at the stage between 15 and 21 days. Heart weight (HW) responses paralleled the BW response, as evidenced by the relatively constant HW-to-BW ratios in the euthyroid groups (Supplemental Fig. 1, B and C). In the hypothyroid groups it is obvious that the lack of T₃ severely blunted both absolute and relative heart growth, especially between 10 and 15 days (Supplemental Fig. 1). These contrasting responses in body and heart growth patterns between the two groups are hallmarks of a hypothyroid vs. euthyroid state during neonatal development, and they are consistent with the differential patterns of T₃ levels between the two experimental groups presented in Fig. 1.

Relative distributions of MHC protein isoforms. In this study we determined the relative distribution of the cardiac MHC protein isoforms during development as expressed as the percentage relative to the total MHC protein that was present in the gels. As presented in Fig. 2, at birth the proportion of β-MHC expression slightly exceeded that of α-MHC (%β-MHC: 54.3 ± 3.3, %α-MHC: 45.7 ± 3.3). Under euthyroid conditions there was a progressive increase in relative α-MHC protein expression, while that of β-MHC progressively decreased such that by 21 days α-MHC was exclusively expressed. In the hypothyroid state, the opposite pattern occurred in that between 7 and 15 days of age there was a marked decrease in α-MHC and a marked increase in β-MHC protein expression, with the latter becoming the isoform exclusively expressed (Fig. 2). This pattern of response indicates that during early postnatal development, not only does the euthyroid heart increase in size but also α-MHC expression increases to become exclusively expressed by day 21. In contrast, the hypothyroid heart loses its α-MHC expression, whereas β-MHC expression increases to become the only isoform expressed by day 15.

Relative distribution of MHC mRNAs. In the euthyroid state the overall patterns of postnatal change of cardiac MHC mRNA expression were similar to those seen for the MHC protein. That is, the relative content of α-MHC mRNA was increased, while that of β-MHC was decreased relative to the total MHC mRNA abundance (Fig. 3). This suggests that pretranslational processes were operating in conjunction with the translational processes that were occurring to enhance the α-MHC protein pool in the rapidly growing heart. In contrast, it is most likely that the loss in β-MHC protein was impacted by the progressive reduction in the β-MHC mRNA pool such that by 21 days both β-MHC mRNA and protein pools became

**Fig. 1.** Plasma thyroid hormone (3,5,3'-triiodothyronine, T₃) levels in normal control (NC) and propylthiouracil (PTU)-treated neonatal rats as they matured from 7 to 21 days. Each point represents mean ± SE; n = 6/data point. Two-way ANOVA analyses results demonstrated an age and PTU treatment effect (P < 0.05), and the interaction between these variables was significant. *P < 0.05 for NC vs. PTU at corresponding age (Bonferroni post hoc test).

**Fig. 2.** Cardiac myosin heavy chain (MHC) protein isoform distribution. A: representative gel image for MHC isoform separation by PAGE electrophoresis according to the method described by Reiser et al. (35). B: % α-MHC protein distribution in ventricles during 21 days of postnatal development in euthyroid (NC) and hypothyroid (PTU) rats. C: % β-MHC protein distribution in ventricles during 21 days of postnatal development in euthyroid (NC) and hypothyroid (PTU) rats. For B and C, each point represents mean ± SE; n = 6/data point. Two-way ANOVA analyses results demonstrated age and PTU treatment effect (P < 0.05), and the interaction between these variables was significant. *P < 0.05 for NC vs. PTU at corresponding age (Bonferroni post hoc test).
fully repressed. However, in the hypothyroid state, by 10 days of age there was full repression of α-MHC mRNA, whereas it took a little longer to repress the β-MHC protein because of a possible longer turnover rate compared with the mRNA. It is also interesting that during the hypothyroid state there was a possible longer turnover rate compared with the mRNA. It is took a little longer to repress the β-MHC protein did not peak until day 15. These collective observations suggest that in order for β-MHC to become the sole isoform expressed in the hypothyroid state it is necessary to increase pretranslation and translation events for this gene to increase its protein expression and to complete the remodeling pattern.

**Sense and antisense RNA expression of cardiac MHC genes.** In this study we analyzed the expression of α-MHC pre-mRNA, β-MHC pre-mRNA, and β-MHC antisense RNA in the ventricular tissue. These RNAs represent the primary transcription products transcribed on the cardiac MHC gene locus and thus are used as approximate markers for transcriptional activity. MHC RNA products were analyzed by strand-specific RT-PCR and were expressed in arbitrary units (Fig. 4).

In the euthyroid state there was a net 45% increase in α-MHC sense RNA expression from 0 to 21 days of age (Fig. 4A). In contrast, there was a significant decline in sense β-MHC RNA expression, and this decline was relatively steady from 7 to 21 days, at which time it became undetectable under the applied experimental conditions (Fig. 4B). There was a rapid rise in β-MHC antisense RNA expression, which appeared as a mirror image of the β-MHC sense pre-mRNA response, but in the opposite direction (Fig. 4C). In the hypothyroid state, these developmental changes in MHC RNA expression were reversed early on. For example, in the PTU neonates both α-MHC pre-mRNA and antisense β-MHC RNA decreased to become undetectable by day 10, whereas β-MHC pre-mRNA increased throughout this period (Fig. 4). The inverse relationship between β-MHC antisense RNA and β-MHC pre-mRNA expression is best demonstrated by linear regression analyses of these two variables between 0 and 21 days. A strong negative relationship was found between these two variables in the euthyroid neonates, with a Pearson correlation coefficient of \(-0.8 (P < 0.0001; Fig. 5)\). These results on the antisense β-MHC RNA regulation during development, its response to PTU, and its relationship to the β-MHC sense pre-mRNA fit the hypothesis that the antisense β-MHC RNA is likely involved in the negative developmental regulation of the β-MHC sense gene expression during the early postnatal period.

Furthermore, there was a significant positive relationship between antisense β-MHC RNA and α-MHC pre-mRNA \((R = +0.58, P < 0.05)\). However, this relationship was not as strong as anticipated based on previously published work on diabetic and PTU-treated adult rats (11, 14). This positive relationship between the α-MHC sense pre-mRNA and the antisense β-MHC RNA agrees with the proposed model, which suggests that the promoters of the α-MHC and the antisense β-MHC genes, being positioned in a head-to-head fashion, share some important regulatory elements. Our data show that both the α-MHC pre-mRNA as well as the antisense β-MHC RNA are quite influenced by thyroid hormone as they are turned off in the PTU state (Fig. 4). However, in the euthyroid state, it appears that the antisense β-MHC RNA expression follows different kinetics from the α-MHC pre-mRNA expression; the former exhibited a steeper rise during the early developmental neonatal stages (10–21 days). The difference in the magnitude of the response as well as the relatively lower correlation coefficient \(R = +0.58\), suggest that during development α-MHC pre-mRNA and β-MHC antisense transcription may be regulated by both independent processes and common regulatory schemes.

While the directional change in mRNA and pre-mRNA expression are well matched during the developmental shifts and in response to PTU, the quantitative relationship may be difficult to interpret. For example, the relative α-MHC mRNA expression almost doubled from 0 to 21 days (Fig. 3B), whereas for the same period, the α-MHC pre-mRNA increased.
only by 45% (Fig. 4A). This difference in magnitude may be due to a different turnover rate for the two types of molecules. On the other hand, this difference may be due to different assay systems. The mRNA is determined as a percentage of the total MHC mRNA pool, whereas the pre-mRNA is determined as a RT-PCR signal per unit of total RNA.

The above findings on the early neonatal development of the myocardium demonstrate a substantial increase in cardiac mass that is also associated with a dynamic remodeling of the cardiac MHC isoforms from ~50% to 100% α-MHC protein. This remodeling appears to be in part driven by well-coordinated transcriptional, pretranslational, and translational processes as indicated by pre-mRNA, mRNA, and protein levels, respectively. Transcriptional regulation appears to be coordinated via a complex mechanism involving the antisense β-MHC RNA inhibiting the β-MHC gene transcription.

Quantitative cardiac MHC RNA analyses at postnatal days 10 and 21. Since significant developmental changes in MHC RNA expression occurred between 10 and 21 days of age, real-time PCR analytical methods were applied to analyze heart MHC primary transcriptional products at these two time points, to ascertain better quantitative relationships for cardiac MHC RNA products. Results of these analyses demonstrated that as the animals matured from 10 to 21 days of age, α-MHC pre-mRNA expression increased 1.4-fold, whereas β-MHC antisense RNA expression increased 5-fold. In contrast, β-MHC pre-mRNA expression decreased 84% (Fig. 6A). These results suggest that between 10 and 21 days of postnatal development antisense β-MHC transcription as well as that of sense β-MHC pre-mRNA were more responsive to developmental regulation compared with the α-MHC gene. At 10 days of age the ratio of β-MHC antisense to β-MHC sense pre-mRNA was 1.25.
whereas at 21 days of age this ratio became $\sim 38$. This large increase in antisense transcription may be the direct result of the surge in $T_3$, which appears to be rather effective in inhibiting $\beta$-MHC gene transcription as the animal matures from 10 to 21 days of age. In addition, real-time PCR quantitative analyses of cardiac MHC gene expression in the euthyroid state showed that at 10 days of age antisense $\beta$-MHC RNA was less abundant than $\alpha$-MHC pre-mRNA, as demonstrated by a ratio of 0.34 (Fig. 6B). At 21 days of age, this relationship was reversed: antisense $\beta$-MHC RNA was 78% more abundant than $\alpha$-MHC pre-mRNA, as demonstrated by a ratio of 1.78 (Fig. 6B). In summary, these real-time PCR data, which examined the quantitative relationship between MHC RNA expression at 10 days and 21 days of age, were in general agreement with the data generated by end point RT-PCR, thus validating the collection of data presented in this report. Furthermore, these quantitative measurements highlighted the potency of the antisense transcription and its responsiveness to development and inverse relationship to the sense $\beta$-MHC gene transcription.

**Sense intergenic transcription in the $\alpha$-MHC promoter.**

Strand-specific RT-PCR is a useful tool, very sensitive and accurate, to detect RNA, which is an indicator of transcriptional activity. Initial analyses scanning the cardiac MHC IG spacer for transcriptional activity revealed bidirectional transcriptional activity. Specifically, antisense RNAs can be detected across the first half of the IG region, whereas sense IG RNA was detected in the second half of the IG region (see Fig. 7A). These findings are very significant in terms of implicating the IG spacer of the cardiac MHC gene locus in bidirectional transcriptional activity potentially affecting the regulation of both adjacent genes. For the complete analyses of the pattern of expression of the sense IG RNA during the postnatal time course and in response to PTU, we targeted a region located at position $\sim 1201\sim 927$ bp relative to the $\alpha$-MHC TSS, using a strand-specific one-step RT-PCR method. The results of these analyses, shown in Fig. 7B, revealed that the IG sense RNA was expressed at a relatively high level in the neonatal animals, and such expression reached a maximum at 7 days and was followed by a gradual decrease between 7 and 21 days of age (Fig. 7B). In the PTU-treated

![Fig. 6. SYBR Green real-time PCR quantitative MHC RNA analyses. A: $\alpha$-MHC pre-mRNA, $\beta$-MHC pre-mRNA, and antisense (AS) $\beta$-MHC RNA expression in ventricles in 21- vs. 10-day-old rats. Data are shown as ratio based on the $2^{-\Delta\Delta CT}$ method (23, 34). *$P < 0.05$, mean is different from 1. B: ratio of antisense $\beta$-MHC RNA to $\beta$-MHC pre-mRNA and antisense $\beta$-MHC RNA to $\alpha$-MHC pre-mRNA at 10 and 21 days of age. #$P < 0.05$, 10 days vs. 21 days. Each bar represents mean ± SE; $n = 6$ each.](http://ajpheart.physiology.org/)

![Fig. 7. Bidirectional transcription in the intergenic (IG) spacer based on RNA analyses. A: schematic representing the cardiac MHC gene locus and the sites of sense (S) and antisense (AS) RNA detection across the IG region. Square brackets indicate the site of RT-PCR target of the sense IG RNA analyzed in B. B: a representative gel image is shown for the RT-PCR product for RNA target at position $\sim 1201\sim 927$ bp relative to the $\alpha$-MHC TSS, using a strand-specific one-step RT-PCR method. The results of these analyses, shown in Fig. 7B, revealed that the IG sense RNA was expressed at a relatively high level in the neonatal animals, and such expression reached a maximum at 7 days and was followed by a gradual decrease between 7 and 21 days of age (Fig. 7B). In the PTU-treated](http://ajpheart.physiology.org/)
neonates the sense IG RNA expression rapidly decreased, and by 10 days of age, it was reduced to trace levels (Fig. 7). These initial analyses show that sense IG RNA is downregulated by PTU treatment in a similar way as sense α-MHC pre-mRNA. Sense IG RNA can also be detected in adult rat ventricles in the euthyroid state, and its expression is repressed by PTU and further augmented by T₃ treatment (data not shown).

To confirm the existence of sense transcriptional activity in the distal promoter of the α-MHC gene, a truncated α-MHC gene promoter spanning the IG region between −2670 and −945 bp relative to the α-MHC TSS was tested by direct gene transfer into young mature rat ventricles. This truncated promoter expressed activity above a promoterless construct (pGL3 basic; Promega). Importantly, reporter expression response to altered thyroid state was identical to that of the endogenous sense IG RNA. That is, the reporter activity driven by the −2670/−945 bp IG promoter fragment was downregulated in the PTU group and upregulated in the T₃-treated group (P < 0.05; Fig. 8).

**DISCUSSION**

Previous studies established that cardiac α- and β-MHC gene expression are developmentally and hormonally regulated, and this regulation normally occurs in the opposite direction for the two genes (24); however, the exact mechanism of this developmental regulation is not clear. Making use of new available tools, this study was designed to conduct more thorough analyses of transcriptional activity of the cardiac MHC gene locus during the early postnatal period in the rodent, in order to gain further insight on the regulation of cardiac MHC genes. The present study investigated the dynamic regulation of the cardiac MHC genes at the transcriptional, posttranscriptional, and translational levels. In this study we depict for the first time the dynamic state of transcriptional activity for the cardiac MHC gene locus during the critical stage of postnatal development. In doing so, several new observations were revealed. First, we have shown that β-MHC gene transcription, as presented by the pre-mRNA, was more responsive to postnatal development compared with the α-MHC gene (see Fig. 4). Second, the high responsiveness of the β-MHC gene may be linked to the equally responsive antisense RNA expression, and this is highlighted by the presence of a tight negative correlation between the antisense RNA expression and the β-MHC expression in the normal developing heart (Fig. 5A). PTU treatment blunted the β-MHC antisense RNA expression, and this was associated with increased β-MHC gene expression, which further highlights the negative impact of the antisense on β-MHC transcription. Third, the analyses revealed a novel observation of bidirectional IG transcriptional activity located at the center of the IG spacer. This involved expression of an antisense RNA extending upstream to become complementary of the β-MHC gene and proceeding into its promoter, as well as expression of a sense RNA merging into the α-MHC gene. Finally, we have shown that a truncated α-MHC promoter fragment, consisting of IG DNA from −2.6 kb to −945 bp relative to the α-MHC TSS, is transcriptionally active and is regulated by altered thyroid state in the same way as the endogenous RNA expression (Fig. 8).

Our results at the pre-mRNA and mRNA levels are consistent with transcriptional/pretranslational regulation of these genes during development in both the euthyroid and hypothryroid states. The question is how the coordinated timely expression of these two genes is established and whether the IG bidirectional transcription is involved in this cooperative regulation.

*Thyroid hormone involvement.* While the molecular mechanism(s) regulating this postnatal transition from β- to α-MHC expression has been elusive, T₃ is thought to play a major role in this regulatory process. In fact, this early postnatal developmental stage coincides with the animal’s surge in circulating T₃ levels (4, 24, 30), which was further verified in this study (Fig. 1). However, while thyroid action can be explained via its own effect on the α-MHC gene promoter, which is based on the existence of several thyroid-responsive elements (3, 20, 37, 40, 41), its action on the β-MHC gene promoter has not been well defined. A negative thyroid-responsive element was proposed to be the site of action on the β-MHC gene, but this has
not been clearly delineated. Thyroid action on cardiac gene transcription is not limited only to α- and β-MHC genes but is also linked to the transcription of antisense β-MHC RNA (Fig. 4C) as well as the newly discovered sense IG RNA originating in the α-MHC promoter and merging into the α-MHC gene. Recently, we showed (11) that the IG antisense promoter is sensitive to T₃, and this was attributed to at least one thyroid-responsive element that may interact with the retinoic acid receptor (RAR). Mutation of this element resulted in reduced responsiveness to T₃, whereas the α-MHC pre-mRNA and the antisense β-MHC RNA increase, whereas the β-MHC pre-mRNA decreases. These responses are consistent with either a direct or indirect action of T₃ on the individual promoters, because circulating T₃ is increased during this early postnatal development. Direct action of T₃ is the result of its interaction with a thyroid receptor bound to a thyroid-responsive element on the target gene promoter to either increase or decrease its activity, whereas in an indirect T₃ action T₃ may create a primary change elsewhere, and this response, in turn, affects the target gene promoter activity via a different set of transcription factors.

Potential role of intergenic region in cardiac MHC postnatal regulation and in response to T₃. Because of the head-to-tail tandem position of the two cardiac MHC genes and their conserved gene order, orientation, and tightly linked regulation, one may question whether these spatial characteristics are important for their cooperative gene regulation. Analyses of RNA expression in the rat ventricle using one-step RT-PCR and specifically targeting different regions of the IG spacer between the two genes (β and α) allowed us to detect expression of sense RNA in the α-MHC gene promoter region (Fig. 7). This IG sense RNA can be detected upstream up to approximately −1,800 bp from the α-MHC TSS. This is a novel observation and raises an important question regarding its significance in the overall regulation of the cardiac MHC genes. While the antisense RNA has been detected in the first 2 kb of the IG spacer (3’ flank of β-MHC gene) as well as throughout the β-MHC gene into its promoter (16), the IG sense RNA was detected along the α-MHC gene promoter, where it becomes undistinguishable from the α-MHC RNA once it meets the α-MHC gene downstream, past the TSS (Fig. 9). Preliminary analyses of the 5’ end of these sense IG transcripts revealed the existence of several TSSs with the most 5’ end mapped at −1,833 bp relative to the α-MHC gene start site (data not shown). The significance of these numerous start sites is not clear, but it is not surprising given the fact that the antisense RNA has at least two TSSs (14). These sense and antisense TSSs are separated by 318 bp of DNA sequence that is part of a highly conserved region (ECR1 in Fig. 9). This region contains several T₃ receptor (T₃R), RAR, and GATA factor binding sites (Fig. 9).

These observations on including sense and antisense IG RNA detection (Fig. 7) and IG promoter activity (Fig. 8) are intriguing and confirm that the regulation of cardiac MHC gene expression is much more complicated since it involves a hidden layer of IG transcription. It is of interest to note that despite the strong depression of sense IG RNA expression in the absence of thyroid hormone, its expression was not correlated with circulating T₃ levels. Between the ages of 7 and 21 days the sense IG RNA expression tended to decrease at a time when T₃ levels continue to rise sharply (Fig. 7). The reason for this response in normal developing rodents is not clear but may suggest that this sense IG RNA activity depends on other factors in addition to thyroid hormones. Further studies are necessary to fully understand its regulation in neonatal and adult ventricles as well as its role in the regulation of the adjacent cardiac MHC genes.

The presence of these bidirectional IG transcription patterns is intriguing and may be implicated in the control of the cardiac MHC locus. The strategic location of the bidirectional IG transcription (central to the locus) and the correlation between its products (sense and antisense RNA) and MHC gene products suggest that their activation causes inhibition of the upstream gene (β-MHC) while simultaneously causing an activation of the downstream gene (α-MHC). There is evidence that cis-transcription, whether on the same or on the opposite DNA strand, can affect the expression of the adjacent genes, either by promoter interference (42) or by altering chromatin structure (13, 19, 29). These types of regulations are known as epigenetic processes consisting of structural adaptation of chromosomal regions so they eventually achieve an altered activity state.

It is of interest to note that a DNase-hypersensitive (DHS) site has been discovered in the middle of the IG region between the two cardiac MHC genes (17). Such hypersensitivity coincides with regions of histone acetylation and DNA demethylation, thereby demarcating areas of high transcriptional activity (8). In the report by Huang and Liew (17), DHS sites were mapped in the α-MHC gene promoter in hearts from fetal and adult hamsters. A DHS site was mapped at −1.9 kb of the α-MHC promoter in both fetal and adult hearts, even though the fetal heart does not express the α-MHC gene. Another DHS site was mapped corresponding to the proximal promoter only in the adult heart, which expresses a high level of the α-MHC gene. Examination of the sequence at the −1.9 kb DHS site revealed the existence of a highly conserved GATA binding site. Thus GATA was implicated in the regulation of the α-MHC promoter (17). Pertinent to these current findings, the −1.9 kb region corresponds to the IG bidirectional promoter in which the conserved GATA site is found on the evolutionarily conserved region (ECR1) depicted in Fig. 9 along with T₃R/RAR binding sites (Fig. 9C). According to DHS principles, this region located at the center of the IG spacer demarcates an open chromatin state in both the adult and fetal heart. This condition is consistent with the establishment of a permissive state for subsequent activation of gene transcription (22).

Recently, a microRNA originating from α-MHC gene intron 27, miR-208, was implicated in T₃ signaling and regulation of the switch from α-MHC to β-MHC during stress-induced cardiac growth (43). Therefore, it would be of interest to investigate, in future research, any possible relationship between the sense IG RNA and this microRNA.

Phylogenetic analyses of intergenic region using Mulan multiple sequence local alignment. Comparative genomics is a valuable tool to gain insight on regulatory regions and is based on the notion that functionally important sequences are conserved through evolution among species (2, 45). The entire IG sequence between the β- and α-MHC genes was subjected to comparative analyses using the computational resources publicly available at http://mulan.dcode.org (33). Mulan is dynamically interconnected with the multiTF utility (http://
multitf.dcode.org) that identifies transcription factor binding sites (TFBSs) that are shared among all the input species involved into the alignment. Thus it is primarily designed to identify potential functional domains in a sequence based on sequence conservation. Analyses of the rat sequence against the cardiac MHC IG sequences from four other mammalian species (mouse, hamster, rabbit, and human; all extracted from GenBank via Blast searches) show that regulatory elements are conserved mainly in two ECRs (Fig. 9): 1) a proximal domain (~350 bp), ECR2 in Fig. 9, consisting of the already characterized α-MHC promoter (28), and 2) a distal region, ECR1 in Fig. 9, located between −1.3 and −2 kb relative to the α-MHC TSS (Fig. 9). A significant portion of ECR1 is situated between the sense and antisense IG RNA start sites (Fig. 9). The high conservation (>80% similarity across 5 species) and the strategic position of ECR1 (distal region) establish it as a good candidate for a promoter region for both sense and antisense IG transcription. Close examination of the rat ECR1 sequence using the TESS web tool (http://www.chil.upenn.edu/cgi-bin/tess/tess) to analyze the sequence and predict TFBSs revealed the presence of several cis-regulatory elements such as binding sites for T3R, retinoic acid receptor (RAR), and GATA transcription factor. These potential regulatory elements are found on the upper (sense) as well as lower (antisense) DNA strand. It is interesting that the T3R/RAR binding site is conserved in several locations such as those shown on the alignment for the region located between −1967 and −1862 bp in Fig. 9C, which implicates functional significance of these sites. The abundance of T3R/RAR/RXR binding sites in this region is consistent with observed responsiveness to T3, which affects both sense and antisense IG RNA transcription. In addition, given the important role of the thyroid receptor in chromatin remodeling (49), the IG region may be implicated in epigenetic regulation of the cardiac MHC gene locus, which involves
histone modifications to either repress or activate transcription. While these observations are based on computational analyses of the sequence, the biological function of these associated binding sites and their role in cardiac MHC gene regulation remain to be determined in future studies.

In light of these collective observations we believe that the IG region plays a key role in controlling the cardiac MHC gene locus. Gene-to-gene cross talk is likely established via the cis-intergenic transcriptional activity in the sense and antisense directions. These two genes are physically and functionally linked. In particular, it appears that in order for the β-MHC gene to be regulated, a feedback from the IG antisense RNA is needed.

**β-MHC promoter activity in transgenic mice reveals abnormalities of regulation during development.** In the literature there are several lines of evidence that show that the developmental regulation of the β-MHC gene is not manifest in the isolated promoter as found in transgenes whereby the promoter is linked to a reporter gene used in transgenic animals. It has been reported that the transferred β-MHC promoter does not respond well to this developmental regulation when studied in transgenic animals (21, 36). Specifically, these investigators reported that for the majority of the generated lines reporter expression driven by the β-MHC promoter transgene remained high during the early stages of development, even though the endogenous β-MHC mRNA was rapidly downregulated.

In another study (48), the hamster β-MHC gene promoter (−2.5 kb) was used to drive the expression of green fluorescent protein (GFP) in transgenic mice, in order to biologically mark cardiac myocytes in vivo in the whole animal as they proceed through various developmental stages. It was found that GFP expression, which is a marker for the transferred β-MHC promoter activity in transgenic animals, was persistently expressed in the heart throughout postnatal life, including the adult state, i.e., it was not subjected to the developmental regulation observed for the endogenous β-MHC gene, which is consistent with the findings reported here.

Together, these findings support the idea that the 2.5 kb β-MHC promoter is not sufficient to confer developmental regulation, i.e., it requires other regulatory mechanisms. Our data on the developmental regulation of the antisense RNA and its inverse relationship with the β-MHC pre-mRNA (see Fig. 5) are consistent with the model proposed here that the IG transcription is important in the downregulation of β-MHC gene transcription. This regulation is important developmentally, and it is also consistent with the response to altered thyroid state and diabetes in adult rodents (11, 14).

We speculate that the antisense RNA is acting to interfere with the sense gene via transcription interference. Therefore, when the interference was not part of the scheme, as found in the transgenic promoter, the developmental downregulation of β-MHC gene expression failed to occur. In future studies, the role of the IG region in cardiac MHC gene regulation may be best studied in targeted mutation, whereby the consequence of deletion of a 500-bp region encompassing both sense and antisense IG TSSs can be evaluated in terms of its effects on the regulation of cardiac MHC genes.

Collectively, the results of this study are consistent with the notion that the developmental regulation of the two cardiac MHC genes is the result of cooperative functional linkage between the two genes. Cross talk between the two genes during development is likely established via the sense and antisense IG RNA that originate in the IG region between the α- and β-MHC genes. Thus the cardiac MHC IG bidirectional transcription serves a relevant molecular function in modulating the transcription of cis-linked α- and β-MHC genes and plays a critical role in their being reciprocally regulated in expression during development. The detection of the sense IG transcription in the distal region of the α-MHC promoter is a novel intriguing observation that also provides more complexity to the mechanism of cardiac MHC gene regulation. Its exact relationship and role in cardiac MHC gene regulation remain to be determined. Furthermore, a recent report (5) showed that the 5′ end of several of the MHC mRNAs is diverse. Multiple start sites for the mouse α-MHC mRNA were found; they were mapped at −65, −31, +1, and +5 bp relative to the already known TSS (+1, located 30 bases downstream from the TATA box). Consequently, future studies are necessary to examine the nature of these multiple start sites in the rat, along with ascertaining whether the distal TSS mapped at −1830 bp is related to a splice variant of the α-MHC mRNA, thus providing more to the diversity of the 5′ untranslated region of this mRNA.

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H40 DEVELOPMENTAL REGULATION OF CARDIAC MHC GENE


