Regulation of antisense RNA expression during cardiac MHC gene switching in response to pressure overload


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Haddad, F., A. X. Qin, P. W. Bodell, L. Y. Zhang, H. Guo, J. M. Giger, and K. M. Baldwin. Regulation of antisense RNA expression during cardiac MHC gene switching in response to pressure overload. Am J Physiol Heart Circ Physiol 290: H2351–H2361, 2006. First published January 13, 2006; doi:10.1152/ajpheart.01111.2005.—Hypertension affects the physiodynamics and energetic properties of the working heart. The α-MHC isoform is characterized by a higher ATPase activity and faster shortening speed than the β-MHC isoform (1, 12). Thus hearts rich in the α-isofrom have a high intrinsic contractility, whereas those rich in the β-MHC have a lower contracility but a higher economy of tension development (1). In the adult rodent heart, the α-MHC is the predominant isoform expressed in the ventriciles, accounting for ~85–90% of the total MHC protein pool, whereas the β-MHC accounts for the remaining 10–15% (9). In cardiac myocytes of different mammalian species, the expression of the two MHCs is developmentally regulated (17), and it can be altered by a variety of pathophysiological conditions, including abnormal thyroid status (6, 14, 17, 29), diabetes (4), and hemodynamic overload (13, 18).

One of the unique features of the cardiac MHCs is that the α- and β-MHC genes are located in tandem on chromosome 15 in the rat, and each gene is 25 kb separated by 4.5 kb of intergenic region. They are in the order of functional significance. In a recent study (8), we have shown that a naturally occurring antisense RNA is transcribed from the DNA strand that is opposite to the MHC genes, with an initiation site located in the intergenic region between the β- and α-genes. Transcription occurs in the direction of the β-MHC gene, thus creating a β-antisense RNA. This antisense β-transcript is associated with the MHC isoform gene switching in the heart in response to diabetes and hypothyroidism (8). Interestingly, in the normal control heart we showed that both the α- and β-MHC genes are actively transcribed based on primary transcript (pre-mRNA) analyses; however, the antisense β-RNA is also highly expressed and appears to be involved in the repression of the βMHC gene expression into mRNA via a complex, less understood mechanism (8). Diabeties and hypothyroidism are associated with a decrease in the antisense expression and a concomitant increase in the β-MHC mRNA. Furthermore, we have shown a significant positive correlation between α-MHC gene transcription and that of the antisense β-transcript, which suggests that the α- and antisense β-transcriptions are coregulated likely via some common regulatory elements located on the intergenic region between the α- and β-MHC genes (8).
Given this proposed novel mode of coordinated regulation of cardiac MHC gene expression, the primary objective of the present study was to address the question whether this mode of regulation observed in diabetes and hypothyroidism, also applies to MHC regulation in the hypertensive heart. Cardiac pressure overload is associated with an increase in muscle mass, as well as a significant phenotype shift in MHC mRNA and protein composition (7). The end result is that the hypertensive heart becomes larger, and the β-MHC mRNA and protein composition is enhanced beyond that of a normal control heart.

Previous studies suggest that the increase in β-MHC protein expression in pressure-overloaded hearts is partly the result of a pretranslational mechanism based on significant correlations between β-MHC protein and β-MHC mRNA expression (7). Furthermore, based on promoter-reporter studies reporting significant increase in β-MHC promoter activity in pressure-overloaded hearts (32) and the fact that this occurs without any obvious change in the α-MHC promoter activity, we hypothesized that the increase in β-MHC expression is due to transcriptional activation of the β-MHC gene independently of the involvement of the newly discovered antisense β-MHC mRNA, which appears to play a critical role in the MHC shift occurring in thyroid deficiency and diabetes. Our results support the idea that the observed increase in β-MHC gene expression in the AbCon heart is the result of not only transcriptional activation of the β-MHC gene but that it also involves regulation by the antisense β-MHC transcript via a complex mechanism that might influence transcriptional and posttranscriptional processes within the cardiac MHC gene locus. The exact mechanism of the antisense β-RNA regulation has not been determined at the molecular level of analyses, but it could be implicated in a universal mode of coordinated regulation between two adjacent genes of the same family.

METHODS

Animal model. Twelve young adult female Sprague-Dawley rats (~150 g body wt, purchased from Taconic Farms, Germantown, NY) were used as subjects for these experiments. Animals were assigned to one of two groups: the normal control group (NC; n = 6) and the hypertensive group (n = 6). Hypertension was induced via the abdominal aortic constriction model (AbCon). Rats were anesthetized with a mixture of ketamine, acepromazine, and xylazine (40, 1, and 4 mg/kg, respectively). The abdominal aorta was surgically isolated just rostral to the left renal artery. A 2-0 silk suture was tied tightly around the mid–rostral to the left renal artery. A blunt 22-gauge needle placed along the side of the aorta. The abdomen was closed. The abdominal aorta was surgically isolated just rostral to the left renal artery. A 2-0 silk suture was tied tightly around the mid–rostral to the left renal artery. A blunt 22-gauge needle placed along the side of the aorta. The abdomen was closed. The abdomen was closed. The abdomen was closed.

At the end of 12 days, the animals were euthanized with Nembutal (32). The experimental procedure was carried out for 12 days. In a previous study, the AbCon procedure was associated with MHC phenotype remodeling consisting of a previously known α- to β-shift in MHC. However, these data will not be able to determine how the shifts occurred. Is it via change in the α-MHC mRNA, or change in the β-MHC mRNA expression, or both? One must analyze MHC mRNA expression for each isoform in a constant amount of total RNA to answer this question.

Specific MHC RNA analyses. Complete DNA sequence data on the rat cardiac MHC genes and the intergenic region is available in GenBank (chromosome 15, contig NW_047454). Specific RT primers were designed to target the exonic sequences from the 5' and 3' ends of the α- and β-MHC genes. RT reactions using these exonic RT primers generate cDNA from both the pre-mRNA and the mRNA. These cDNAs were amplified by PCR using primers specific for the mRNA (exonic primers) or the pre-mRNA (which includes at least 1 intronic primer). In addition, RT primers targeting the antisense RNA were designed based on the positive-strand sequence of the MHC gene locus and they targeted different sites such as the 5' end, the 3' end of the β-MHC gene, and the intergenic region between the β- and α-MHC genes. The cDNA product complementary to the antisense RNA was amplified using PCR primers targeting intergenic sequences. In all these reported RT-PCR amplification results, the PCR primers' target was located within a short distance from the specific RT primer to ensure high amplification efficiency. DNA sequence and other details on the primers used can be found in Table 1. Specific RT reactions used 2 μg of total RNA in 20 μl of total volume in presence of 5 pmol of specific primers using Superscript II reverse transcriptase (Invitrogen). PCR amplifications used Biolase DNA polymerase (Bioline) in the presence of magnesium (at an optimized concentration ranging from 1.5 to 2 mM), 0.2 mM dNTPs, and 0.6 μM PCR primers and were carried out on a Robocycler (Stratagene). The annealing temperature was adjusted based on the PCR primers' optimal annealing temperature. The amount of cDNA and PCR cycles was adjusted so that the accumulated product was in the linear range of the exponential curve of the PCR amplifications. PCR products were separated by electrophoresis on agarose gels and stained with ethidium bromide. The ultraviolet light-induced fluorescence of stained DNA was captured by a digital camera, and the band
<table>
<thead>
<tr>
<th>RT Primer Name and Sequence: 5′ → 3′</th>
<th>Location/Orientation</th>
<th>Target</th>
<th>PCR Primers</th>
<th>PCR Product Size, bp</th>
</tr>
</thead>
<tbody>
<tr>
<td>3′ β-MHC antisense: CTGCTAAAGGGCTTGTTTT</td>
<td>3′ end of the β-gene, 3′ UTR/antisense</td>
<td>1) 3′ end β-pre-mRNA</td>
<td>β-pre-mRNA Fwd: ATCCCAAGGGCTCACTA-ATCTCA</td>
<td>308</td>
</tr>
<tr>
<td>2) 3′ end β-mRNA</td>
<td>β-pre-mRNA Rev: CTCAGGGCTTCACAGGCA-TCC</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5′ β-MHC antisense: GCAGGCAATAACAGAATAA</td>
<td>5′ End of the β-gene 3′ kb from TSS/antisense</td>
<td>1) 5′ end β-pre-mRNA</td>
<td>β-mRNA Fwd: GGAGTCGACCCTACAGGAGA</td>
<td>308</td>
</tr>
<tr>
<td>2) 5′ end β-mRNA</td>
<td>β-mRNA Rev: CTGAGGGCTTCACAGGCA-TCC</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3′ end β-MHC sense: TGAAGAAAGGACGACGAC</td>
<td>2.4 kb Upstream of the 3′ end of the β-MHC gene/sense</td>
<td>Antisense β-RNA overlapping the 3′ end of the β-gene</td>
<td>β-pre-mRNA Fwd: GGGTGCGCTACCTTATTA-TCC</td>
<td>425</td>
</tr>
<tr>
<td>5′ end β-MHC sense: TCACGTAGTACCAGGTTATTATTG</td>
<td>154 bp From the 5′ end of the β-MHC gene (intron 2/sense)</td>
<td>Antisense β-RNA overlapping the 5′ end of the β-gene</td>
<td>β-pre-mRNA Rev: GTACGGCCTTGCCACAC</td>
<td>488</td>
</tr>
<tr>
<td>3′ α-intergenic sense RT: CCCTAAGGCTATTCT</td>
<td>1.5 kb Downstream from the 3′ end of the β-MHC gene/sense</td>
<td>Antisense β-RNA in the intergenic region</td>
<td>β-Intergenic region Fwd: TACAGGCTTACAGGCTGAAG</td>
<td>399</td>
</tr>
<tr>
<td>5′ α-MHC antisense: GCAAAGTCAGCATTACAT</td>
<td>3′ end of the α-gene, 3′ UTR/antisense</td>
<td>1) 3′ end α-pre-mRNA</td>
<td>α-pre-mRNA Fwd: TCTCCTGCTCAAATTACAGCAGTCA</td>
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<td>α-pre-mRNA Rev: ATAGAAGACGCGGCTC-TTCTG</td>
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<td></td>
<td></td>
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<td>Antisense α-RNA overlapping the 3′ end of the α-gene</td>
<td>α-pre-mRNA Fwd: TCTCCTGCTCAAATTACAGCAGTCA</td>
<td>399</td>
</tr>
</tbody>
</table>

MHC, myosin heavy chain; UTR, untranslated region; TSS, transcription start site; Fwd, forward; Rev, reverse; RT, reverse transcription; PCR, polymerase chain reaction.

Table 1. RT and PCR primers and their specific target, with PCR product size, for all the MHC RNA data reported in this study
cycler system and the FullVelocity SYBRgreen QPCR Master Mix reagents from Stratagene (La Jolla, CA). For each primer set to be used in quantitative PCR, a standard was prepared as follows: 1 µl of cDNA was amplified using these primers for 30 cycles. The PCR product was separated on a 2% agarose gel, and the band was excised and purified using the Qiagen gel extraction kit. A serial dilution was prepared from the purified PCR product to make a standard curve for quantitative PCR reaction. Primer concentration was optimized to yield an efficiency of 95–100% using standard dilutions from 100 to 1,000,000 copies per reaction. All standards and unknown samples were run in duplicates. The threshold cycle number was generated automatically by the provided software with the MX3000p, and this was plotted against the log of the copy number. The data were analyzed by linear regression, and the unknown copies were generated based on corresponding threshold cycle number using the regression line of the standard curve during the same run.

Statistical analyses. Data are reported as means ± SE. The effect of AbCon was studied for each region of the heart independently of the other. For each experimental group (NC vs. AbCon) were analyzed using an unpaired t-test. For multiple comparisons, a one-way analysis of variance with Newman-Keuls post hoc test was utilized. Relationships between two variables were assessed using linear regression and correlation analyses (GraphPad Software). Statistical significance was set at P < 0.05.

RESULTS

Body weight and heart weight. Body weight was not different between the NC and the AbCon group (190 ± 3 vs. 183 ± 3 g, respectively). However, absolute heart weight and the relative heart weight (heart weight-to-body weight ratio) were significantly elevated by 39% (P < 0.05) and 44% (P < 0.05), respectively, in the AbCon group relative to the NC group. This is a signature response in this model whereby the increased cardiac mass is considered to be a compensatory adaptation of the heart muscle to increased work demand. This hypertrophic response is due to the increased mean blood pressure and increased afterload that is imposed, and the heart undergoes remodeling in an effort to reduce the wall stress (30).

MHC mRNA analyses. Throughout this paper we report the various results based on analyses from the apex, base, and septum regions of the heart to ensure that the AbCon hypertension affects the cardiac MHC shifts in a global way. To initially assess the shift between the α- and β-isofrm, cardiac MHC mRNA composition was analyzed using the standard competitive MHC PCR approach that was developed by our laboratory (32). These analyses showed that after 12 days of AbCon, the MHC isoform composition is altered to be significantly enriched in the β-MHC mRNA by 104% in the base, by 91% in the apex, and by 68% in the septum relative to control (Fig. 1). Comparisons of the β-MHC mRNA induction between different regions of the hearts revealed that the septum response was slightly lower compared with the base response, while the apex response was not different from either the base or the septum. The reason for this difference in the magnitude of septum response at this time is unclear and could be the result of one of three possibilities. 1) The β-MHC percent composition in the control septum was slightly higher than the control base and apex (27% vs. 21%; P = 0.052, 1-way ANOVA); 2) it is possible that β-MHC mRNA response has to do with the regional effect of the afterload on the left ventricle such that the stimulus is less at the septum level compared with base and apex; or 3) it is possible that in a time course of 12 days, the heart has not reached a steady state, and this response might be identical in all three regions of the heart if analyzed at a later time point.

These types of analyses on MHC composition are useful in determining the effectiveness of the AbCon in altering MHC gene expression; however, the altered composition could merely be the result of either β-MHC mRNA increase, α-MHC mRNA decrease, or a combination of the two. Thus each MHC isoform mRNA expression was studied as an independent entity, and its expression was normalized to the initial amounts of total RNA.

Expression of β-MHC transcripts in the AbCon heart. MHC gene expression was analyzed at the level of pre-mRNA and mRNA. The β-MHC pre-mRNA was studied as a marker for transcriptional activity of the β-MHC gene. The analyses of the pre-mRNA expression used RT-PCR techniques in which the pre-mRNA was targeted using gene-specific antisense RT primers to generate a cDNA followed by amplification using PCR primers. The RT primers were designed to target the pre-mRNA molecule (−25 kb), these distant analyses are
important to ensure that any observed change in the AbCon heart can be equally detected at the 5' end as well as at the 3' end of the long target RNA. At the same time, these analyses may be more complex and difficult to interpret for such long transcripts whereby processing may be occurring along with transcription. For example, enhanced intron removal during splicing of the primer targeted region would result in a decrease RT-PCR product, even though the actual number of transcripts is the same as that analyzed at different sites. Also, as more transcripts are being initiated at the 5' end, for example, in response to activation, fewer will be detected at the 3' end because transcription has not been completed yet. Results from the -MHC pre-mRNA analyses are reported in Fig. 2, and they show that whereas the pre-mRNA is increased at the 5' end of the gene in response to AbCon, it was not significantly induced at the 3' end. This discrepancy between the 5' end and 3' end response of the pre-mRNA was found in all three regions of the left ventricles that were analyzed (apex, base, septum). The increased -MHC pre-mRNA at the 5' end is consistent with an increased transcription of the gene in response to AbCon. It was not expected that the pre-mRNA as measured at the level of the last intron (3' end) was not increased in the AbCon heart as observed for the 5' end measurement. The reason for this difference is not clear but could involve limited elongation of the transcript as it is synthesized from the 5' end to the 3' end. That is, whereas transcription initiation is increased in the AbCon state, there is a limit concerning how many transcripts can be completed as they are being extended to the 3' end of the gene. On the other hand, it is possible that the splicing of the introns at the 3' end of the gene is enhanced in the AbCon heart so that the pre-mRNA may appear as not changing when studied at this level because it was spliced (loss of introns) at a higher rate.

In addition to the pre-mRNA analyses, we also determined the expression of -MHC mRNA product when targeted at both the 5' and 3' ends. Results of these analyses are reported in Fig. 3, and they show that -MHC mRNA expression was significantly increased in the AbCon ventricles regardless of

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**Fig. 2.** -MHC pre-mRNA expression in NC and AbCon heart. A: RT-PCR targeting the 5' end of the -MHC pre-mRNA in 3 cardiac regions. Gel images of PCR products obtained from both -specific RT reactions (RT-PCR) and RT reactions from RNA without primers (RT). Presented data are the net signal (difference in signal between both reactions). PCR was carried out on 1 μl cDNA that was diluted 15-fold for 28 cycles. B: RT-PCR targeting the 3' end of the -MHC pre-mRNA. -pre-mRNA expression in NC and AbCon heart, analyses in 3 cardiac regions. Also shown are representative gel images of PCR products obtained from both -specific RT reactions (RT-PCR) and RT reactions from RNA without primers (RT). Presented data are the net signal [difference in signal between both reactions in arbitrary scan units (ASU)]. PCR was carried out using 1 μl cDNA that was diluted 5-fold for 29 cycles. Values are means ± SE; n = 6 animals/group. *P < 0.05, NC vs. AbCon (unpaired t-test).

**Fig. 3.** -MHC mRNA expression in NC and AbCon heart. RT-PCR targeting the 5' end (A) and the 3' end (B) of the -MHC mRNA in 3 cardiac regions. Gel images of PCR products obtained from -specific RT reactions. PCR from RT reactions on RNA without primers gave no detectable product at the dilutions used for these amplification reactions. PCR was carried out on 1 μl cDNA that was diluted 40-fold for 24 cycles. Values are means ± SE; n = 6/group. *P < 0.05, NC vs. AbCon (unpaired t-test).
PCR was carried out on the site of analyses (5’ end vs. 3’ end) of the β-MHC mRNA molecule, and this was found in all three regions of the AbCon myocardium (see Fig. 3). It is of interest to note that the difference in the pre-mRNA between 5’ and 3’ end in the AbCon heart is not observed at the mRNA level. The pre-mRNA is much less abundant than the mature message and is transient in nature, whereas the mature message is more stable after completion. We postulate that newly formed mRNA represents a small percentage of the total mRNA population for a specific gene; therefore, it is less likely to detect any difference in the kinetics of its formation as for the corresponding pre-mRNA undergoing processing.

Expression of β-MHC antisense RNA. In view of our recent report (8) that a naturally occurring antisense β-MHC RNA is expressed in the rat myocardium, and that this antisense transcript appears to play an important role impacting the MHC isoform shifts in response to thyroid deficiency and diabetes, it was of interest to determine the expression of this antisense β-RNA in the AbCon myocardium. Thus the β-antisense RNA expression was analyzed in the AbCon heart by RT-PCR. Two regions were targeted by RT-PCR, the 3’ end of the β-MHC gene (2.4 kb from the 3’ end) and the intergenic region between β and α, ~1.5 kb downstream from the β-MHC gene (Table 1). As negative controls, PCR reactions were also carried with the same primers using reverse transcribed RNA in the absence of RT primers (ØRT). Results of these analyses show that the net 3’ end antisense products were reduced in all AbCon heart RNA with the net response being statistically significant in all regions of the heart (Fig. 4). These reductions ranged from 38 to 55% and were comparable in magnitudes between the 3’ end of the β-MHC gene and the intergenic region between the β- and α-genes.

Quantitative PCR using SYBRgreen real-time PCR to analyze β-MHC RNA expression. Real-time PCR technology was used to determine quantitative expression of the β-MHC pre-mRNA and antisense β-RNA in the NC vs. AbCon apex region. Both RNA species were analyzed at the 5’ end and 3’ end of the β-MHC gene for validation of the expression of the corresponding β-MHC pre-mRNA undergoing processing. The antisense RNA exhibited a significant reduction in the AbCon apex when analyzed at either the 5’ or 3’ end of the β-MHC gene (Fig. 5B). The

Fig. 5. Quantitative β-MHC RNA analyses in the apex region using real-time PCR and SYBRgreen fluorescence. A: β-MHC pre-mRNA expression targeted at the 5’ end and 3’ end. B: antisense β-RNA expression targeted at the 5’ end and 3’ end. C: β-MHC mRNA expression in NC and AbCon apex as analyzed using real-time PCR [quantitative PCR (QPCR)] and using the ethidium bromide gel (EtBr) end-point PCR method. In A, B, and C, values are reported as ratio of copy number to NC average, which makes the NC expression equivalent to 1 and the AbCon expression relative to NC. Values are means ± SE; n = 6 animals/group. *Mean is significantly different from 1; P < 0.05.  

Fig. 4. Antisense β-MHC RNA expression in NC and AbCon heart in 3 regions of the myocardium. RT-PCR targeting the antisense overlapping the 3’ end of the β-MHC gene (A) or the intergenic (IG) region between β and α (B). Gel images of PCR products obtained from both DNA strand-specific RT reactions (βRT-PCR) and RT reactions from RNA without primers (ØRT). Presentated data are the net signal (difference in signal between both reactions in ASU). PCR was carried out on 1 µl cDNA that was diluted 5-fold for 30 cycles. Values are means ± SE; n = 6 animals/group. *P < 0.05, NC vs. AbCon (Ab) (unpaired t-test).
mRNA expression was analyzed by real-time PCR only at the 3’ end. These results show that the AbCon apex expressed three times the level of β-MHC mRNA as the NC apex ($P < 0.05$; Fig. 5C). These results on β-MHC pre-mRNA, antisense RNA, and β-MHC mRNA expression in AbCon heart were equivalent to the ethidium bromide gel end point PCR results reported in Figs. 2, 3, and 4. The rationale to perform the real-time PCR was to validate the findings of the traditional PCR method concerning changes in RNA expression in response to AbCon. Therefore, only the apex RNA was analyzed for 5’ end and 3’ end β-MHC pre-mRNA and antisense RNA expression. Real-time PCR results can also be used to compare the expression of sense vs. antisense in the NC heart, which may not be possible in the traditional PCR method, because conditions (amounts of cDNA/reaction and PCR cycle number) are adjusted to bring the PCR product in the linear range of ethidium bromide detection. Real-time SYBRgreen detection has a wide linear range, and samples with a large difference in copy numbers can be run under similar conditions for quantitative analyses. The same PCR primer sets were utilized in the determination of the sense and antisense cDNA. Also a standard curve was generated during each run to determine the PCR reaction efficiency and to determine the unknown amounts based on regression analyses. The specific cDNA was synthesized using strand-specific RT primers that were designed with similar melting temperature and located at equal distances from the PCR primer targets, thus reducing variables that can possibly influence cDNA synthesis efficiencies for both the antisense and sense RNA targets. Reactions for standard dilutions were run in each real time PCR run and for each primer pair. Regression analyses were utilized to determine the slope of the standard curve. PCR results were considered valid when PCR amplification efficiencies were ≥95%. Our data show that, in the NC heart, the ratio of antisense β-RNA to sense β-pre-mRNA is 3.3 when analyzed at the 5’ end of the gene. In contrast, this ratio is 9.8 when analyzed at the 3’ end of β-MHC gene. In the AbCon heart, this ratio became significantly reduced at both the 5’ and 3’ ends due to decreased antisense expression. The reason for the difference in the ratio within the two regions relative to the end of the gene may have to do with more abundant RNA species when the latter is determined closer to the transcription initiation site. For example, the 5’ end of the β-MHC gene is closer to the initiation of β-MHC pre-mRNA, but is over 25 kb farther from the antisense RNA initiation site that is located in the β-α-intergenic region (8). Based on these results, one can conclude that the NC heart expresses more antisense β-RNA than sense β-pre-mRNA, whereas in the AbCon state, there is a reduced expression of the antisense β-RNA and an increased expression of the sense β-pre-mRNA. The exact quantitative relationship is difficult to determine and appears to depend on the position where the RNAs were targeted for amplification. This positional dependency reflects the dynamic state of the pre-mRNA as it is processed/spliced concomitantly as it is synthesized.

Expression of α-MHC transcripts in the AbCon heart. The α-MHC pre-mRNA and mRNA both were targeted at their 5’ and 3’ ends in their analyses for the same reasons as outlined for the β-MHC RNA species. The results of the 5’ end analyses suggested that there were small reductions in the net α-pre-mRNA in response to AbCon in all three regions of the heart. However, these reductions were not significant based on one-way analyses of variance. However, if only two groups were considered for t-tests, the AbCon base had significantly lower α-pre-mRNA expression ($P = 0.013$; Fig. 6). Analyses of the α-MHC pre-mRNA at the 3’ end showed that the α-pre-mRNA was significantly lower in AbCon base and apex regions but not in the septum. α-MHC mRNA analyses show that its expression is significantly reduced in the AbCon left ventricles when analyzed at the 5’ end in both the apex and base region but not in the septum. Analyses of the 3’ end of the mRNA showed a trend of a decrease, but these were not significant in any of the left ventricular regions (Fig. 7). Analyses targeting the antisense αMHC RNA showed that some small amounts of net antisense RNA expression can be detected; however, these amounts were not regulated in response to the AbCon condi-
tion (data not shown). Therefore, the functional significance of the antisense α-MHC transcripts is not clear and could represent only a “leakage” in the RNA machinery (3).

**DISCUSSION**

The aim of this study was to further evaluate the phenomena of MHC remodeling in response to pressure overload (hypertension) in light of the novel finding that a naturally occurring antisense β-MHC RNA is involved in regulating cardiac MHC gene expression in other models such as hypothyroidism and diabetes. Abdominal aortic constriction was used as a hypertension model, which proved successful in developing cardiac hypertrophy and MHC isoform shifts from an α- to β-isofrom after only 12 days of abdominal aorta banding. In an earlier study using reporter gene assay techniques and direct gene transfer in the myocardium, our laboratory showed that the βMHC promoter activity is increased in the AbCon apex, and the response was attributed to both distal and proximal regions of the promoter (32). In a recent paper (8), we described a coordinated regulation between the α- and β-MHC expression in hypothyroid and diabetic hearts. This coordinated regulation was attributed to an antisense β-MHC RNA, which appears to somehow inhibit β-MHC mature mRNA expression in the NC heart, and this inhibition is removed in the diabetic and thyroid-deficient hearts as a result of significant reduction in the antisense RNA expression (8). Thus it was of interest to analyze the β- and α-MHC pre-mRNA, mRNA, and antisense β-MHC RNA in the present study to determine how their expression relates to each other in the context of the hypertension-induced changes.

**Relationship between the α- and β-MHC genes in response to pressure overload.** Based on the discovery of the β-antisense RNA, the observed relationships between cardiac MHC pre-mRNA, mRNA, and antisense RNA, and in conjunction with the evolutionary conserved cardiac MHC gene organization, a model of coordinated regulation of cardiac MHC gene expression was proposed (8). This model, shown in Fig. 8A, basically proposes that the transcribed β-antisense RNA interferes with β-MHC gene expression 1) via interference with the normal transcription of the sense β-MHC gene and/or 2) via inhibiting the processing of the primary transcript (pre-mRNA) into mature mRNA. In addition, based on positive correlation...
between the antisense β-RNA and the α-MHC pre-mRNA, in conjunction with the proximity of the two promoter regions, we proposed that the antisense β-RNA transcriptional activity is linked to that of the α-MHC gene via some common regulatory elements in the intergenic region. The tight coordinated regulation between the α- and β-MHC gene, which is demonstrated by the rapid, simultaneous, and reversible shifts between α- and β-MHC gene expression to correspond to the functional demands of the myocardium, further corroborate this model.

To determine whether this “model of coordinated regulation of cardiac MHC genes” is also in operation in the AbCon model, we performed correlation analyses between the antisense β-MHC RNA and 1) the β-MHC mRNA, and 2) the 5′ end α-MHC pre-mRNA, using measurements obtained from all three regions of the heart under both NC and AbCon states. As presented in Fig. 8B, there was a significant inverse relationship between the antisense β-RNA and the β-mRNA. This negative correlation fits the proposed model shown in Fig. 8A, implying that the antisense β-RNA has a negative influence on the formation of β-MHC mRNA. Conversely, we observed that there was a significant positive relationship between α-pre-mRNA and the antisense β-RNA (Fig. 8C). This positive correlation, “although not necessarily cause and effect,” fits our model proposing a common regulatory process linking the α-MHC gene transcription to that of the antisense β-RNA (Fig. 8A). These observations, taken into context of the previous report on thyroid deficiency and diabetes, suggest that β-MHC antisense RNA may be a common mechanism in the regulation of cardiac MHC gene switching between α- and β-MHC genes.

The idea of negative regulation of gene expression by an overlapping antisense transcript is not new nor unique to the β-MHC gene, because it has been observed for several other genes, including the endothelial nitric oxide synthase (28), Hox A11 (2), atrial essential myosin light chain 1 (27), and collagen I α1-gene (5), and in the regulation of X inactivation (19). The proposed coordinated regulation between two adjacent genes via an antisense RNA is novel, and to date it has not been yet proposed for other genes. However, this may be an important mechanism for gene cross talk within gene clusters in order for the cell to simultaneously activate one gene while turning off another related gene. This idea is supported by the fact that the gene order, orientation, and spacing are conserved through evolution, suggesting functional significance.

In the context of these responses involving the heart, we have also noted that there is a similar phenomenon occurring in skeletal muscle involving fast MHC gene expression. In studying the fast MHC gene RNA expression in different models, there is an antisense IIA RNA expression associated with the IIA → IIX gene switching in slow muscle (vastus intermedius and soleus) undergoing slow-to-fast MHC transition such as during unloading (26), and an antisense IIX RNA expression associated with the IIB → IIX gene switching in fast muscles (white medial gastrocnemius) undergoing fast-to-slow transition such as occurring in an overload state (F. Haddad and K. M. Baldwin, unpublished observations). As in the case of the heart MHC genes, the skeletal fast type MHC IIA, IIX, and IIB genes are aligned in tandem on the same chromosome with much conserved gene order, orientation, and spacing throughout evolution. Taken together, it would appear that the classical gene switching that occurs among MHC isoforms involves a common phenomenon that implicates antisense RNA expression.

Validation of the antisense β-RNA results in the AbCon heart. The antisense β-MHC RNA expression was analyzed using specific RT-PCR methods and was found to be significantly decreased in the AbCon heart in all three separate regions (apex, base, and septum). The antisense RNA was analyzed in four separate regions across the MHC gene locus: in the intergenic region (Fig. 4B), in the region overlapping the 3′ end of the β-MHC gene (Fig. 4A), in the region overlapping the 5′ end of the β-MHC gene (Fig. 5B), and in the promoter region upstream of the β-MHC gene (data not shown). For all these analyses, the antisense was expressed in the NC heart, and the AbCon heart consistently expressed significantly less antisense β-RNA product. Real-time PCR analyses were performed to validate the quantitative comparisons between control and AbCon, and these results were well in correspondence to the end-point PCR as reported in the results above. In addition to quantitative validation of the results on the response of the antisense to AbCon, having detected the expression of the β-antisense RNA when targeted with specific primers at different sites in the MHC gene locus suggests that this antisense RNA is overlapping the entire β-MHC gene and extending into the promoter region as shown in the proposed model in Fig. 8A. In addition to the reported PCR results that were carried within short distance from the RT primers (for quantitative purpose), the antisense RNA extent was qualitatively assessed. The antisense RNA was targeted near the initiation start site of the β-MHC gene, and the PCR amplification targeted a region that is in the intergenic region, 26 kb from the RT primer target sequence, and we got a positive net response between a negative RT and the specific RT. These results were not used for quantitative assessment because the PCR amplification target is far away from the RT primer (~25 kb), making the reaction less efficient to occur. However, they confirm that the antisense exists as a long RNA, which overlaps the entire β-MHC gene.

Validation of the β-MHC pre-mRNA results. In this study, the pre-mRNA levels were used as a measure of transcriptional activation because it is the primary product of the RNA polymerase II activity, although these measurements may depend on the stability of targeted intronic sequence considering the fact that the pre-mRNA is processed quickly into mRNA as it is synthesized. It is intriguing that the β-MHC pre-mRNA expression in response to AbCon depended on the site of analyses. At the 5′ end, ~3 kb from the transcription start site, AbCon hearts expressed significantly more β-pre-mRNA. This response is consistent with a higher transcriptional activity of the gene. Analyses of the pre-mRNA targeted at the 3′ end of the β-MHC gene, e.g., at the site of the last intron, show that the β-MHC pre-mRNA expression was not appreciably altered in response to AbCon. We speculate that the pre-mRNA is rapidly processed to keep up with the high transcription rate, and this is consistent with the associated decrease in the antisense β-RNA, which we postulate to be an inhibitor of pre-mRNA processing. Thus, in the AbCon heart, intron splicing may be occurring at a rate faster than in the normal heart, which is consistent with the observed increase in the β-MHC m-RNA in the AbCon heart that exceeds the increase in the corresponding pre-mRNA. This difference in the stoichiometry
between pre-mRNA increase (100%) vs. the mRNA increase (225%) in the AbCon heart relative to NC was validated using quantitative PCR analyses (Fig. 5). This latter observation is also consistent with the model illustrated in Fig. 8A.

How does α-MHC gene regulation in the AbCon heart fit the model? In the model of hypertension, previous reports on the α-MHC gene regulation are equivocal. It has been reported to either decrease (22, 23) or not change (11, 13, 24). In the normal control heart in rodents, the α-MHC expression is predominant. In response to pressure overload, the heart hypertrophies and its MHC profile becomes richer in β-MHC expression. This can be possible by an increased β-MHC expression regardless of whether the α-MHC expression was maintained at control level or decreased. In this study, the results on the α-MHC were consistent with the literature, and they ranged between either no change or being decreased. For example, when analyzed at the 5′ end of the α-MHC gene, the pre-mRNA was not changed in the AbCon apex and septum, but it was significantly reduced in the AbCon base (Fig. 6A). The corresponding mRNA was significantly reduced in the AbCon apex and base but not in the septum (Fig. 7A). When analyzed at the 3′ end, the α-MHC pre-mRNA expression was reduced in the apex and base but not in the septum (Fig. 6B), whereas the corresponding mRNA expression was not changed in all three regions of the hearts (Fig. 7B). These results on the α-MHC suggest that the α-MHC expression is altered in response to AbCon, but it follows a different time course from that of the β-MHC and the antisense RNA regulation, which occurs very early after induction of pressure overload. We speculate that at a later time point after the pressure overload, the α-MHC expression will be significantly reduced in all regions of the hearts. Despite this varying response in α-MHC expression, the overall response is a reduction in its pre-mRNA and mRNA level. Statistical analyses examining the relationship between the antisense β-MHC RNA (at 3′ end of the β-MHC gene) and the 5′ end α-MHC pre-mRNA revealed a positive correlation that was statistically significant (P < 0.0001) (Fig. 8B). The Pearson correlation coefficient was 0.62, which translates into a coefficient of determination of ~0.4, which leads us to the following interpretation. In the AbCon heart, the α-MHC pre-mRNA and antisense β-RNA regulation is likely coordinated, and such coordination is postulated to occur, at least in part, via a common regulatory mechanism (Fig. 8A). But there are other independent regulatory factors that are affecting the β-antisense without altering the α. The nature of these factors remain to be determined in future studies.

Role of naturally occurring antisense RNA. It is important to note that the phenomenon of naturally occurring antisense RNA expression is not unique to the MHC gene family but appears to be prevalent in the mammalian genome. Recent reports have revealed a surprisingly large number of antisense transcripts (15, 34), but their function and mechanism of action are complex and remain largely speculative. The large number of bidirectionally transcribed genes is intriguing and substantiates the physiological importance of antisense regulation. Antisense gene products can exert their influence at any gene expression stage, including transcription initiation, elongation, RNA processing, RNA stability, and translation (21). Based on the literature, long naturally occurring antisense RNAs have been implicated in the regulation of gene expression via at least four different mechanisms as reviewed by Lavorgna et al. (16). 1) The first mechanism is transcriptional interference, whereby transcription of a gene encoded on the sense strand of the DNA inhibits concomitant transcription of the overlapping gene encoded on the opposite strand. When transcription occurs on both strands in opposite direction, there is attenuation of the polymerase II activity in one or both directions due to collision of large transcription complexes. 2) The second mechanism is RNA masking, whereby the formation of double stranded RNA may mask regulatory signals necessary for splicing, probably by blocking the accessibility of cis-regulatory elements. This mechanism may play a role in the alternative splicing between two gene variants, such as described for the thyroid receptor α-gene (10). 3) The third mechanism include double-stranded RNA-dependent mechanisms, whereby the double-stranded RNA in the nucleus triggers RNA editing [involving selective deamination of adenosine (A) to inosine (I) in the pre-mRNA] leading to nuclear RNA retention or RNA interference-dependent gene silencing. 4) The fourth mechanism is antisense-induced methylation, whereby the expression of the antisense transcript induces the methylation of the CpG island upstream of the sense gene, thus inhibiting its transcription. This latter mechanism is particularly true for antisense transcripts involved in gene imprinting (25, 31, 33) and thus is unlikely occurring in the cardiac MHC gene regulation because the effect is not total inhibition of the β-MHC mRNA expression. Although our results do not allow definition of the precise mechanism of action of this MHC RNA antisense, they suggest negative regulation of β-MHC gene expression by the antisense β-RNA, and this is supported by the negative correlation between the β-MHC mRNA and the antisense β-RNA. Direct sequencing of cDNA product failed to detect any deamination (A to I conversion) on the overlapping β-MHC RNA sequence (data not shown), which suggests that the third mechanism is unlikely to be occurring. This makes either the first and/or the second mechanism as possibly occurring in cardiac MHC gene regulation, and the presented data support either one. The first mechanism of transcription interference may be possible and can be occurring as follows: when the antisense transcription is attenuated in the AbCon heart, the sense β-MHC gene transcription is activated by having less interference from the opposite strand transcription. The second mechanism could involve the antisense RNA making double-stranded RNA with the sense pre-mRNA, thus masking the splicing recognition sites, and allowing less mRNA to be made. If this inhibition is occurring in the NC heart, processing may be activated in the AbCon heart expressing less antisense RNA. Two pieces of data support this notion: 1) the increase in mRNA in response to AbCon exceeds the increase in the corresponding pre-mRNA (difference validated with quantitative PCR), and 2) the pre-mRNA measurement at the last intron showed no change in the AbCon heart, suggesting a higher rate of intron removal at this site. While these ideas remain speculative in nature, clearly, a better understanding of the mode of action of the antisense RNA is needed especially in the context of maintaining tight coordinated regulation between two adjacent genes of related function. Future studies are needed to address this issue. For example, the consequences of targeted disruption of the antisense transcript on cardiac MHC gene expression should be examined to gain insight into its role in the cardiac MHC gene regulation. Disruption of the antisense can be achieved at the
transcriptional level by altering its basal promoter located in the β-α-intergenic region, or posttranscriptionally via the use of RNA interference system.

In summary, in this paper we reinvestigated a long-known phenomenon that involves MHC phenotype shifts in the pressure-overloaded heart. We investigated the mechanism of cardiac MHC shifts in view of the novel finding reported recently that describes the involvement of antisense RNA in the antithetical regulation of the cardiac MHC gene expression in thyroid deficiency and diabetes (8). Our results demonstrate that the increased β-MHC expression in the AbCon heart not only is the result of increased β-MHC transcription but also involves an increased accumulation of the β-MHC mRNA via a mechanism involving a reduced inhibitory effect of the antisense β-RNA via a complex mechanism of gene cross talk. Thus these findings add to an expanding database to suggest that the antithetical regulation of β/α-MHC gene switching is mediated by an intergenic mechanism involving naturally occurring β-antisense RNA transcription. The exact details of this mechanism at the molecular level remain to be elucidated.

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