The evolutionarily conserved order of the skeletal muscle myosin heavy chain (MHC) genes and their close tandem proximity on the same chromosome are intriguing and may be important for their coordinated regulation. We investigated type II MHC gene regulation in slow-type muscle fibers undergoing a slow to fast MHC transformation in response to inactivity, 7 days after spinal cord isolation (SI) in rats. We examined the transcriptional products of both the sense and antisense strands across the Ila-Ilx-Iib MHC gene locus. A strand-specific reverse transcription (RT)-PCR approach was utilized to study the expression of the mRNA, the primary transcript (pre-mRNA), the antisense RNA overlapping the MHC genes, and both the intergenic sense and antisense RNAs. Results showed that the mRNA and pre-mRNA of each MHC had a similar response to SI, suggesting regulation of these genes at the transcriptional level. In addition, we detected previously unknown antisense strand transcription that produced natural antisense transcription (NATs). RT-PCR mapping of the RNA products revealed that the antisense activity resulted in the formation of three major products: all, alle, and alleI NATs (antisense products of the Ila, Ilx, and Iib genes, respectively). The all NAT begins in the Ila-intergenic region in close proximity to the IIx promoter, extends across the 27-kb Ila MHC gene, and continues to the Ila MHC gene promoter. The expression of the all NAT was significantly up-regulated in muscles after SI, was negatively correlated with Ila MHC gene expression, and was positively correlated with Ilx MHC gene expression. The exact role of the all NAT is not clear; however, it is consistent with the inhibition of Ila MHC gene transcription. In conclusion, NATs may mediate cross-talk between adjacent genes, which may be essential to the coordinated regulation of the skeletal muscle MHC genes during dynamic phenotype shifts.

Skeletal muscle is highly adaptable when subjected to altered loading and hormone states. Its size, metabolic makeup, and contractile properties can all be altered to optimize function (1). Variability in contractile properties is achieved mainly by diversification in the motor protein myosin heavy chain (MHC), where different isoforms are encoded by distinct genes (1, 2). Of this family of eight MHC genes, six are tandemly linked and span ~420 kb in the rat on chromosome 10, with embryonic MHC situated at the most 5’ end, sequentially followed by Ila, Ilx, Iib, neonatal, and extraocular MHC. B (or type I) and a MHCs are located tandemly on separate chromosomes (chromosome 14 in the rat); they span ~50 kb and are separated by 4.5 kb. Interestingly, the genomic order and orientation on the chromosomes of the MHC genes are conserved in all mammalian species, leading researchers to suspect that this organization might be an important feature in the strategy for the coordinated regulation of these genes (2–5).

Types I, Ila, Ilx, and Iib, in respective order of increasing ATPase activity, are the four predominately expressed MHC isoforms in adult rat skeletal muscle. MHC gene expression is regulated at the transcriptional/pre-translational level (6–8). Such expression occurs in a way so that the isoform profile is dynamically altered to presumably confer optimal function in the animal in response to varying conditions (6–8).

In the adult rodent muscle, loading conditions, motor neuron innervation patterns, and hormone states determine the MHC isoform profile that is expressed in a muscle fiber. Alterations to these conditions can drive the expression profile toward either a faster or slower contractile phenotype (ι ↔ Ila ↔ Ilx ↔ Iib) depending on both the starting reference MHC profile of the muscle fiber and the newly imposed condition (1). For example, disuse, inactivity, lack of muscle innervation, and hyperthyroidism result in a shift of the MHC profile from slow (type I/Ila) to fast (type Ilx/Iib) MHC isoforms in slow muscle fibers (8–10). In contrast, increased loading state, chronic electrical stimulation, and hypothyroidism can cause the reverse transformations, i.e. a shift to expression of slower MHC isoforms in fast muscle fibers (1). The embryonic and neonatal MHC genes are also under regulatory control throughout the stages of development (11–13). Thus there is a precisely controlled expression of the six MHC genes that is managed in response to changing stimuli. It is not understood how this

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBankTM/EBI Data Bank with accession number(s) DQ872905 (Ila MHC), DQ872906 (Ilx MHC), DQ872907 (Iib MHC).

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2 The abbreviations used are: MHC, myosin heavy chain; NAT, naturally occurring antisense RNA transcript; VI, vastus intermedius; SI, spinal cord isolation; SOL, soleus; TSS, transcription start sites; UTR, untranslated region; Ct, cycle threshold; TBP, TATA-binding protein; RT, reverse transcription; CON, control; dsRNA, double-stranded RNA.
Role of Antisense RNA in Myosin Heavy Chain Gene Regulation

**MATERIALS AND METHODS**

**Animal Model**—Adult female Sprague-Dawley rats (240 ± 4 g) were used for all experiments. Slow muscle MHC remodeling was induced by SI. For the SI procedure the spinal cords of six animals were transected at both a mid-thoracic and the high sacral level, and a bilateral dorsal rhizotomy was performed between the two transection sites as described previously (9). The unique feature of the SI model is that the surgical treatment renders the motor neuron pools in the isolated region of the spinal cord inactive while maintaining an intact connection to the muscles of the leg. Thus, under these conditions the muscles fail to contract and generate force; they are more or less completely inactive. The animals were euthanized at the end of 7 days, and their VI and SOL muscles were isolated, quickly weighed (wet weight), and rapidly frozen in dry ice. Muscles were stored at −80 °C until used for RNA extraction. Age-matched normal control rats were used for the control muscles (CON, n = 6). A majority of the muscle atrophy during SI occurs during the first 7 days, and significant changes in MHC mRNA expression are also detected during this time period (8, 9). This study followed the National Institutes of Health Animal Care Guidelines and was approved by the University of California, Los Angeles, Animal Care and Use Committee.

**RNA Analysis**—Total RNA was extracted from frozen VI and SOL muscle using the Tri Reagent protocol (Molecular Research Center). Muscles of both legs of each rat were combined for RNA extraction. Extracted RNA was DNase-treated using 1 unit of RNase-free DNase (Promega)/µg 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 MHC mRNA isoform distribution was evaluated by RT with random primers followed by PCR with primers targeting the embryonic, neonatal, I, IIA, IIX, and IIB MHC mRNAs as described previously (25, 26). In these PCR reactions, each MHC mRNA signal was corrected to an externally added control DNA fragment that was co-amplified with the MHC cDNAs using the same PCR primer pair. This provides a means to correct for any differences in the efficiency and/or pipetting of each PCR reaction. A correction factor was used for each control fragment band on the ethidium bromide-stained gel to account for the staining intensity of the variably sized fragments (224–324 bp), as reported previously (25, 26).

Although the above RT-PCR can provide information on the MHC mRNA distribution pattern, it does not give information on how each isoform is regulated. Strand-specific RT-PCR was used to analyze the expression of specific MHC pre-mRNAs and mRNAs, as well as antisense RNAs (NATs) that are of opposite orientation to the MHC genes.

**PCR Primers**—Specific PCR primers were designed to target pre-mRNA and mRNA transcripts at the 5′ and 3′ ends of the type IIA, IIX, and IIB MHC genes as well as RNA transcripts at the intergenic regions. Primers targeting mRNA were located on two separate exons, whereas at least one of the primers targeting pre-mRNA was located on an intronic sequence. The skeletal muscle MHC gene locus sequence was obtained from NCBI Rat Genome Resources via BLAST analyses to previously
known MHC mRNA sequence. The rat skeletal muscle MHC genes are located on chromosome 10, Contig sequence accession number NW_047334. The specific MHC genes were identified based on the previously identified 3′-end untranslated region (UTR) of the mRNA, which are unique for each MHC gene (GenBank™ accession numbers X72589 (IIa), X72591 (IIx), and X72590 (IIb)). In GenBank™, only the coding sequence of the MHC mRNAs and the 3′ UTR sequences were annotated. Thus, the exon boundaries of the 5′ UTR were not known. This region, which confers isoform specificity for each MHC mRNA, contains exons 1 and 2 and part of exon 3. It was necessary to determine the intron/exon junctions to facilitate the design of primers at the 5′ end of each MHC gene. First, the transcription start site (TSS) was determined by comparison with known mouse MHC gene promoters and TSSs (GenBank™ accession numbers AF081358 (IIa), AF081359 (IIx), and M92099 (IIb)). Repeat sequences were determined based on alignment with genomic DNA sequence, and these were used in designing isoform-specific primers for the 5′-end mRNA analyses. Information on the PCR primers is reported in Tables 1 and 2. Sequences for cDNAs corresponding to 5′-end type II MHC mRNA can be found in the GenBank™ data base (IIa MHC, DQ872905; IIx MHC, DQ872906; IIb MHC, DQ872907).

One-step RT-PCR—RT-PCR reactions were performed with the One-step RT-PCR Kit (Qiagen), where the RT and PCR are performed in one reaction tube, with some modifications to the manufacturer’s protocol. Strand specificity of sense and antisense cDNA was established by the choice of the RT primer. The cDNA of the pre-mRNA and mRNA (sense strand) was synthesized by priming with the reverse PCR primer in the RT reaction, whereas the cDNA of NATs (antisense strand) was synthesized by priming with the forward PCR primer in the RT reaction. The missing primer was added before the PCR reaction at the end of 15 min of heating at 94 °C in order to denature the RT enzyme and activate the Taq DNA polymerase. In addition to reactions targeting the sense or the antisense RNA, two negative control reactions were performed on each sample. In the first negative control, the RT enzyme was first inactivated by incubation at 95 °C for 15 min, and then PCR primers were added and PCR performed. Under these conditions, formation of a product is indicative of either genomic DNA contamination in the RNA sample and/or reagent cross-contamination. Testing for the former is important when targeting unspliced pre-mRNA, which is indistinguishable from genomic DNA. None of the RNA utilized in this study yielded products in these reactions, thus confirming the effectiveness of the DNase treatment and the absence of cross-contamination. In the second negative control, primers were omitted from the RT reaction, i.e. the RNA was reverse transcribed in the absence of any specific primers. Both PCR primers were added before starting the PCR. Product formation in these reactions indicates that the reverse transcription has produced cDNA without the presence of specific primers, which would negate the strand specificity of the RT-PCR. This second negative control was performed because we had previously observed that under some RT-PCR conditions a fairly robust PCR signal can be detected despite the absence of RT primers in the RT reaction (27). Self-priming of the RNA can occur during the RT reaction to yield a cDNA that can be subsequently amplified by PCR. However, under the conditions we used, all PCR primers generated no detectable product for these negative controls, thus validating the strand specificity of the amplified RT-PCR product.

These one-step RT-PCR analyses were performed using 10–200 ng of total RNA and 15 pmol of specific primers in 25 μl of total volume and were carried out on a Robocycler (Stratagene). Conditions to be compared were run on the same samples under similar conditions (template amounts, PCR cycle numbers). RT reactions were performed at 50 °C for 30 min, followed by 15 min heating at 95 °C, followed by PCR cycling for a varied number of cycles (20–32 cycles). The annealing temperature was adjusted based on the PCR primers optimal annealing temperature. The amount of RNA and the number of PCR cycles were 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 band intensities were quantified by densitometry with ImageQuant software (GE Healthcare) on digitized images.

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 measure certain key transcripts in order to both validate the data generated by end-point PCR and obtain higher fidelity of some of the measured differences between control and treatment conditions. For these analyses, a two-step RT-PCR system was used. The RT was performed using 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. For the RT, the primer used to target the antisense RNA was the forward PCR primer, and the reverse PCR primer was used to target the sense RNA. Real-time PCR used 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 melting curve analysis. For each primer set, PCR specificity was judged 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

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sample was performed in duplicate (320 nl of cDNA/25 μl of reaction) along with a standard curve, which was based on different cDNA amounts per reaction, ranging from 10 to 1000 nl. Standard curves were generated via regression analyses whereby the x axis represented the log of initial cDNA amounts in nl, and the y axis represented the cycle threshold (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 was also utilized to ensure linearity of the amplification with different initial amounts of target cDNAs. To compare initial amounts of cDNA in the two samples, the \(2^{-\Delta\Delta Ct}\) method was utilized (28), which assumes a PCR efficiency of 100%. In these two-step RT-PCR reactions, negative control RT reactions were performed in which the RT reaction was performed under the same condition as described above except that primers were omitted from the RT reaction. PCR gave background signals that were below 4% of the signal obtained with normal reactions.

Genomic DNA PCR—Genomic DNA was extracted from rat tissue using the DNAeasy tissue kit (Qiagen). DNA was eluted with water and its concentration was determined by UV absorption at 260 nm (using a factor of 50 μg/ml per OD unit). Ten nanograms of genomic DNA were amplified in the presence of 15 pmol of primers in 25-μl reaction volumes using Biolase DNA polymerase (Bioline). All RT-PCR primers were tested with genomic DNA to ensure that they worked with similar efficiency.

DNA Sequence Analysis—Rat genomic sequence (Contig number NW 047334; location, 10q24) was aligned with human (Contig number NT 010718; location, 17p13) and mouse (Contig number NT 096135; location, 11 35 cM) using mVISTA (29) to determine intergenic sequence conservation across species. Transcription factor binding site predictions were performed with the program TESS (30) using the TRANSFAC data base.

Statistical Analyses—Data are reported as mean ± S.E. Differences between two groups (CON versus SI) were analyzed using an unpaired t test. Relationships between two variables were assessed using linear regression and correlation analyses (GraphPad Software, Inc.). Statistical significance was set at \(p < 0.05\).

RESULTS

Effects of SI on Muscle Mass—Seven days of SI resulted in a statistically significant decrease of 52 ± 4 and 54 ± 3% in VI and SOL muscle wet weight, respectively. Body weight was decreased by 20 ± 2%.

MHC mRNA Expression in the VI and SOL of SI Rats—The VI and SOL were chosen as the muscles of focus in this study because they are considered slow-type muscles with a predominance of slow-type muscle fibers. Slow-type muscles are highly sensitive to an unloading stimulus; they undergo rapid shifts in MHC composition from types I and IIa to IIx and Iib (6, 9). Fig. 1 shows the distribution of the MHC mRNAs, with the mRNA of each MHC gene expressed as a percent of the total. The VI has a unique MHC profile exhibited in the CON state; it expresses all of the four major MHC isoforms, and each isoform is responsive to inactivity. Conversely, the CON soleus expresses primarily types I and IIa MHC as well as lesser levels of embryonic MHC mRNA, whereas types IIx and Iib MHC have very low levels of expression. In comparing the SI to CON in the VI, the relative expression levels of types I and IIa MHC mRNA were decreased and that of type IIx was correspondingly increased (Fig. 1). The relative expression of Iib MHC mRNA did not change in response to SI. In the SOL, types I and IIa MHC mRNA expression decreased, whereas both IIx and Iib MHC mRNA levels were greatly increased in SI compared with CON. The embryonic MHC mRNA was expressed in both VI and SOL CON muscles. Its percent expression increased in the VI, whereas it decreased in the SOL in response to SI. In both VI and SOL, neonatal MHC mRNA was not detected in CON muscles, but trace amounts were detected in SI muscles. This MHC expression profile demonstrates a dramatic shift from slow to fast MHC isoforms in these slow muscles with 7 days of SI. These results are consistent with previous reports of MHC gene switching patterns during unloading and/or inactivity of target muscles (6, 8, 9).

Although this method of MHC mRNA distribution profile confirms phenotype shifts, it does not identify how these shifts
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TABLE 1
PCR primer sequences and their specific targets and PCR product size
The 5’-end primers were used in end point one-step RT-PCR to generate data shown in Figs. 2 and 3. 3’-end IIa pre-mRNA primers were utilized in real-time PCR to compare the NAT with pre-mRNA as shown in Fig. 6.

| Target | RT-PCR primers | PCR product size |
|--------|-----------------|-----------------|
| 5’-end IIa pre-mRNA | Fwd: TGCTTCCCCATGCGGCATATCTCATAT | 295 bp |
| 5’-end IIa mRNA | Rev: TCCCTCACTTCTTGGGCTGTTGCA | |
| all NAT in the intergenic region between IIa and IIx | Fwd: CGGCTGGCGCTTCTGGCAGAT | |
| 5’-end IIx pre-mRNA | Rev: ACATTCTCTGCCCTGCTTGCAGAT | |
| 5’-end IIx mRNA | Fwd: CGGCTGGCGCTTCTGGCAGAT | |
| xII NAT in the intergenic region between IIx and IIb | Fwd: CGGCTGGCGCTTCTGGCAGAT | |
| 5’-end IIb pre-mRNA | Rev: GATGCAATACCCGCTCCTGAAACAT | |
| 5’-end IIb mRNA | Rev: TGGGGAGGGGGAGGAAG | |
| bII NAT in the intergenic region between IIb and Neo | Fwd: AGGGTTGCTTCTTCAGCATGCG | |
| 3’-end IIa pre-mRNA | Rev: ACCGGCCGAUTCAAGCAGCTGAA | |

occur. In previous studies we determined that the inactivity-induced decrease in type I MHC is due to regulation at the transcriptional level (31, 32); however, the level of regulation for type II MHCs is not clear. Therefore, the next set of analyses were focused on assessments of type II MHC mRNA, pre-mRNA, and NAT expression using a gene-specific, strand-specific RT-PCR approach as explained under “Materials and Methods.”

IIa MHC RNA Expression in VI and SOL in Response to SI—Except where noted, the measurements reported below were taken at the 5’ ends of the IIa MHC pre-mRNA and mRNA transcripts. Also reported are measurements of NATs, products of transcription of the opposite DNA strand to the IIa gene, taken from within the intergenic region between IIa and IIx MHC genes. The IIa NAT, subsequently referred to as all NAT, was measured by strand-specific RT followed by PCR targeting an intergenic sequence (see Table 1 for the specific primers used). Fig. 2, A and B, shows that both IIa pre-mRNA and IIa mRNA decreased significantly in the VI muscle of SI rats compared with the CON group. This was also the case for the SOL muscle (Fig. 3, A and B). The all NAT was significantly higher in the SI than in the CON group (p < 0.05) (Figs. 2 C and 3C) and was inversely correlated to IIa mRNA (r = −0.74, p < 0.01 in VI; r = −0.60, p < 0.05 in SOL) in both the VI (Fig. 4A) and SOL (Fig. 4C).

We also performed real-time PCR with SYBR Green to more accurately evaluate the regulation of the IIa pre-mRNA and mRNA transcripts in response to SI. Fig. 5A shows a graphical representation of the real-time PCR amplification curves of these transcripts in representative samples that demonstrates the difference between SI and CON. Using the 2ΔCt method (28) for comparative quantification of IIa pre-mRNA and IIa mRNA in CON versus SI muscles, we found that the pre-mRNA decreased ~52% and the IIa mRNA decreased ~70% in the VI of SI rats as compared with CON (Fig. 5B). In the SOL, the pre-mRNA decreased ~70% and the IIa mRNA decreased ~80% in SI compared with CON (Fig. 5C). These results by real-time PCR were similar to those obtained by end-point PCR and thus validated those results.

all NAT Expression Levels as Compared with IIa Pre-mRNA—To gauge the relationship between all NAT and its sense (IIa pre-mRNA) counterpart in CON and SI muscles, we used a two-step quantitative RT-PCR method with PCR primers targeting the 3’ end of the IIa MHC gene, to which sense (pre-mRNA) and antisense (NAT) RNA transcripts correspond. For these comparisons, sense and antisense cDNA were amplified using the same PCR primer pair; however, strand specificity was established by the choice of the RT primer (see “Materials and Methods” for details). After cDNA synthesis, reaction products were amplified by the forward and reverse PCR primers using real-time PCR (SYBR Green) under optimized conditions to generate an efficiency of 100 ± 5%.

Using the 2ΔCt method (28) to compare antisense with sense RNA expression, we found that in the CON VI muscle, the all NAT to IIa pre-mRNA ratio was 4% on average. In the SI state, IIa pre-mRNA decreased by 56%, whereas the NAT expression increased by 2.2-fold, so that the ratio of NAT to pre-mRNA increased to 24% on average (Fig. 6A). SOL RNA analysis shows similar patterns for IIa pre-mRNA and NAT expression. In the CON SOL muscle, the NAT was expressed in trace levels, and its ratio to pre-mRNA was <1%. In the SI state, the IIa pre-mRNA decreased by 70%, whereas the NAT increased 5-fold so that in SI SOL muscle, the all NAT to IIa pre-mRNA ratio became 68% (Fig. 6B). These results on IIa pre-mRNA analysis at the 3’ end of the IIa MHC gene are consistent with IIa pre-mRNA regulation found at the 5’ end of the gene as reported above using both end-point PCR as well as quantitative RT-PCR. These results demonstrate that all NAT clearly has a greater prevalence in relation to its sense counterpart in the SI state as compared with CON and thus could have a significant impact on the regulation of IIa MHC gene expression.

Mapping of all NAT RNA Transcripts along the Ila MHC Gene and within the IIa-IIx MHC Intergenic Region—The above reported results demonstrate that the all NAT is both
expressed and regulated in slow muscle fibers. To further characterize the extent of its transcription, we designed a RT-PCR-based approach to target and amplify the αII NAT at sites corresponding to various regions of the IIa MHC gene. Strand-specific RT-PCR results demonstrated expression of αII NAT in SI SOL and SI VI muscles in the promoter region of the IIa MHC gene as far as 6 kb from the IIa MHC TSS (data not shown). Also, αII NAT was detected at every location we tested within the IIa MHC gene and in a large segment of the intergenic region flanking the 3′ end of the IIa MHC gene. Furthermore, cDNA synthesis using a RT primer from the first intron of the IIa gene followed by PCR targeting intergenic sequence resulted in a positive amplification of a PCR product that was increased in the SI state (data not shown). This observation, although not quantitative because of the low efficiency of the long range RT-PCR, clearly demonstrates that the antisense RNA is a long transcript (>27 kb), fully overlapping the IIa MHC gene.

It was also of interest to map sense and antisense RNA expression within the intergenic region between the IIa and IIx MHC genes. These analyses enabled us to determine the approximate start site of αII NAT transcription. Strand-specific RT-PCR was used with forward and reverse primer pairs spaced along the 2.7-kb intergenic region (see Table 2 for the primer sequences). As depicted in Fig. 7, various primer pairs covered the regions indicated by letters A through J, with A corresponding to the 3′ end of the IIa MHC gene and J corresponding to the 5′ end of the IIx MHC gene. This analysis, shown in the SOL muscle, revealed that the αII NAT was detected and up-regulated in SI as compared with CON at locations extending from the 3′ end of the IIa MHC gene through most of the IIa-IIx intergenic region (Fig. 7, regions A–G). The αII NAT could not be detected in region H (this was also confirmed with a separate primer pair nested within this region), the amplicon of which corresponds to 439 to 46 bp from the IIx MHC TSS, suggesting the αII NAT TSS is within this region. A NAT was detected in regions I and J, which overlap regions of the IIx MHC gene. We attribute this to a NAT to the IIx gene, which was also identified (see below). Also of note was a major decrease in the band intensity of the αII NAT at region F as compared with regions A–E. This observation is consistent with the existence of a major TSS for the IIa NAT within region F corresponding to −439 to −46 bp relative to IIx TSS. The second TSS, located within region H, may represent a minor NAT TSS based on transcript expression levels. Interestingly, in silico sequence analyses of the IIa-IIx intergenic region reveals that there is a TATA-binding protein (TBP) consensus sequence within region H on the reverse DNA strand that is 100% conserved between rat, mouse, and human. In the rat there is also a TBP consensus sequence contained within region

4 Primer sequences available upon request.

FIGURE 2. RNA analysis for the VI muscle. Bar graphs show mean ± S.E. of RT-PCR analyses: A, IIa MHC pre-mRNA; B, IIa MHC mRNA; C, αII NAT; D, IIx MHC pre-mRNA; E, IIx MHC mRNA; F, xII NAT; G, IIb MHC pre-mRNA; H, IIb MHC mRNA; I, bII NAT. AU, arbitrary units. *, significantly different from CON (p < 0.05).
Thus, all NAT transcription may be initiated from TATA-driven promoters within these regions. Their close proximity to the IIx MHC TSS also points to the intriguing possibility of co-regulation between the two divergently situated promoters. Supporting this hypothesis is the linear regression analyses showing that the all NAT was significantly correlated with IIx pre-mRNA in both the VI ($r = 0.70, p < 0.01$) and SOL ($r = 0.86, p < 0.01$) (see Fig. 4, B and D). The sense RNA is also shown for the same regions as the NAT. Region A corresponds to the pre-mRNA at the 3' end of the IIa MHC gene and is higher in CON than SI. Interestingly this IIa MHC transcript is detected at all regions probed in the IIa-IIx intergenic region, suggesting there is transcriptional read-through from the IIa MHC gene all the way through the 2.7-kb intergenic region and into the IIx MHC gene.

It is important to note these sense and antisense RNA transcripts detected in the intergenic region are specific to muscles expressing the IIa sense and NAT RNA. They could not be detected in RNA extracted from heart and liver, i.e. tissues that do not express the type II skeletal muscle MHC genes, but could be detected in medial gastrocnemius muscle RNA (data not shown). All of the PCR primers utilized for mapping RNA expression through the intergenic region amplified cDNA of the expected size. There was no noticeable problem to suggest low efficiency or mispriming of these primers, as all of the generated products produced single bands of equal intensity as confirmed by amplification of genomic DNA (Fig. 7, see gDNA products).

**IIx and IIb MHC RNA Expression in SI Rats**—The mRNA and pre-mRNA levels of the IIx MHC gene increased significantly in SI compared with CON in both the VI and SOL (Fig. 2 and 3, D and E). We discovered that there is also a NAT to the IIx gene, detected in the intergenic region between IIx and IIb genes (Figs. 2F and 3F) and also at the 3' and 5' ends of the IIx gene (data not shown). Thus, based on RT-PCR analyses this IIx NAT, named xII, appears to be complementary to the entire IIx MHC gene. Expression of the xII NAT was unchanged in SI compared with CON in the VI muscle (Fig. 2F), whereas it was significantly ($p < 0.05$) increased in the SOL with SI (Fig. 3F).

IIb pre-mRNA and mRNA was unchanged with SI in the VI (Fig. 2G and H), whereas both transcripts were strongly increased in the SOL (Fig. 3G and H). We also discovered a NAT to the IIb gene, named bII, which can be amplified using the strand-specific RT-PCR approach. In a similar pattern to the all and xII NATs, the bII NAT was detected in the 3'-flanking region of the IIb gene, in the intergenic region between the IIb and Neo MHC genes (Figs. 2I and 3I). The bII NAT was also detected at the 3' and 5' ends of the IIb MHC gene (data not shown), thus suggesting that it is also complementary to the entire IIb MHC gene. Expression of the bII NAT was significantly ($p < 0.05$) increased in both the VI and SOL in SI compared with CON (Figs. 2I and 3I).
adjacent IIx MHC gene. We also report the discovery of previous regulation of the IIa MHC mRNA is offset by up-regulation of the expression of these genes is coordinated such that down-regulation of the MHC gene family. These genes have apparently evolved to meet the functional demands of the muscle. The identification of each MHC gene, as is seen in other examples of RNA silencing. Also, complete transcriptional silencing does not occur in the IIa MHC and an increase in the proportion of the slower type-I and IIa MHC and an increase in the proportion of the faster IIx and IIb MHC (see Fig. 1). The NAT that is reversely complementary to the IIa gene, which we refer to as all, overlaps this gene entirely and is therefore complementary to the IIa pre-mRNA. Expression of all was increased in SI muscles as compared with CON, and there was a significant inverse relationship between IIa mRNA and all RNA (Fig. 4, A and C), suggesting an inhibitory role for all.

Potential Mechanisms of MHC Gene Regulation by Antisense Transcripts—Although the prevalence of overlapping sense transcripts in mammalian transcriptomes has been found to be high (18, 19, 33), reports of their regulatory function are very limited, and no generalizations concerning their mechanism of action can be made at the present time. Several models of NAT mechanistic action have been proposed based on analyses of eukaryotic systems: 1) double-stranded RNA (dsRNA)-dependent mechanisms; 2) RNA masking; 3) transcriptional interference; and 4) CpG island methylation (17, 34, 35).

The first proposed model encompasses dsRNA-dependent mechanisms that initiate what is collectively known as “RNA silencing,” such as RNA interference (RNAi) and RNA editing, in which NATs may play a role. The all transcript overlaps the entire IIa transcript, and therefore the IIa and all primary transcripts are complementary along their entire lengths. Thus, their potential for interaction and base-pairing is possible and could lead to either dsRNA-dependent mechanisms of inhibition or RNA masking. However, as indicated subsequently, these scenarios do not fit our observations. dsRNA is known to be highly susceptible to enzyme-mediated degradation, such as through RNA-silencing pathways. However, there is no indication that duplexes formed by natural antisense transcripts would be processed in this way. Such RNA-silencing pathways have only been observed to be activated by exogenous delivery of dsRNA, repetitive sequence elements, repetitive transposable elements, RNA editing, or dsRNA produced by RNA-dependent RNA polymerases (17, 36). Although SI resulted in reduced IIa MHC RNA transcripts as compared with CON, complete transcriptional silencing does not occur in the IIa MHC gene, as is seen in other examples of RNA silencing. Also,
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A.  

- CON pre-mRNA
- SI pre-mRNA
- CON mRNA
- SI mRNA

B. VI muscle

| SI/CON mRNA expression | pre-mRNA | mRNA |
|------------------------|-------|-----|
| CON                    | 0.5   | 0.3 |
| SI                     | 0.2   | 0.1 |

C. SOL muscle

| SI/CON mRNA expression | pre-mRNA | mRNA |
|------------------------|-------|-----|
| CON                    | 0.4   | 0.2 |
| SI                     | 0.1   | 0.05 |

FIGURE 5. IIa MHC transcripts measured by real-time PCR. A, a representative real-time PCR amplification plot from CON (open symbols) and SI (closed symbols) cDNA samples amplified over 40 cycles. Shown on the same graph are amplification curves for both pre-mRNA (squares) and mRNA (triangles). The cycle thresholds, taken at a fluorescence threshold of 0.06 (dRn), was 15.6 for CON and 18.0 for SI for the mRNA and 24.4 for CON and 26.5 for SI for the pre-mRNA. Ct values generated from plots like these were used to calculate the relative expression when comparing two samples as shown in B and C. IIa MHC pre-mRNA and mRNA expression in SI versus CON as calculated based on the 2^(-ΔΔCt) method (28) is shown for the VI muscle (B) and the SOL muscle (C). Data are means ± S.E., * significant change for SI versus CON based on performing a t test to determine whether the mean is different from a hypothetical value of 0 (p < 0.05).

in preliminary analyses of dsRNA of IIa MHC gene transcripts, we were not able to detect any dsRNA products; however dsRNA, particularly in the cell nucleus, is transient in nature and can easily escape detection. In addition, sequencing of 272 bp of the 3' end of the IIa pre-mRNA showed that there was no RNA editing in this transcript in either CON or SI samples, suggesting that any dsRNA that may be formed is not subject to RNA editing. The production of dsRNA by RNA-dependent RNA polymerases is also not likely, as mammals apparently lack this system (37). Thus, it remains to be determined whether any endogenous NATs can induce RNA silencing pathways.

The second model, RNA masking, pertains to post-transcriptional mechanisms whereby formation of a sense/antisense RNA duplex may mask regulatory sites on the transcript, thus disrupting the binding of factors that affect many steps of the mRNA life cycle, including pre-mRNA processing, export to the cytoplasm, translation, and subcellular localization. Such a mechanism has been reported to play a role in the α-thyroid hormone receptor RNA processing and alternative splicing (23). Based on the findings that both IIa MHC pre-mRNA and mRNA were decreased by similar amounts in response to SI, it appears that IIa MHC gene regulation is likely at the transcriptional and not at the post-transcriptional level. Thus, RNA masking mechanisms, which may inhibit formation of pre-mRNA into mRNA, do not fit the transcript profile observed in both the VI and SOL muscles in response to SI.

The third model, transcriptional interference, is based on the concept that convergent transcription by RNA polymerase II on two overlapping genes results in “transcriptional collision,” thus inhibiting transcript elongation (20, 21). The all transcript can be detected with a RT-PCR-based approach at numerous sites along the entire 27-kb length of the IIa MHC gene, from the IIa/Ix intergenic region to upstream of the IIa gene and into its regulatory promoter region. Given that there is transcriptional read-through on the antisense strand of the IIa promoter, it is possible that transcription of all causes interference of IIa transcription. Therefore, the data provided in this study are consistent with this model of regulation.

The fourth model, antisense RNA-mediated CpG island methylation, has primarily been associated with gene silencing at imprinted loci, although examples exist for nonimprinted loci (35). For example, transcription of an antisense-oriented gene across the α-globin (HBA2) gene promoter was found to be associated with HBA2 silencing, in cis (38). This silencing was associated with methylation of the HBA2 CpG island on the normal strand and chromatin remodeling at this site of DNA methylation (38). This was first observed in a human individual with a genetic disease and was replicated in transgenic mice, demonstrating the relevance of this model of NAT mediation of gene regulation in mammals. Our observations of transcription of the all NAT on the antisense strand of the IIa MHC promoter and the associated decrease in IIa MHC gene expression also fit with this mechanism of NAT-mediated transcriptional suppression.

An alternative explanation for the decrease in IIa mRNA transcripts must also be considered, namely that such a decrease is due to changes in the activity of trans-acting transcriptional activators and/or repressors of the IIa gene. Development of a reliable and accurate IIa MHC intramuscular gene injection model has proved problematic,5 possibly due to the absence of endogenous influences such as antisense RNA. Thus, the IIa pre-mRNA remains the best marker of endoge-

5 C. E. Pandorf, F. Haddad, A. X. Qin, and K. M. Baldwin, unpublished observations.
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Transcriptional activity, as measured by IIa pre-mRNA transcript abundance using real-time PCR, was observed to be matched by a substantial proportion of its complementary all NAT in SI-treated muscles. For example, in SOL and VI muscles of the CON group the IIa NAT represented only 1 and 4%, respectively, of the IIa pre-mRNA abundance (Fig. 6). In contrast, in the SI state, these ratios became 68 and 24%, respectively (Fig. 6), suggesting a high probability that the NAT serves a physiologically relevant function. This demonstrates that SI, which causes a shift from IIa to IIx MHC, induces a state in which two RNA molecules complementary to each other (and the IIa MHC gene) approach a relationship of high stoichiometry. The implication of these findings is proposed to be the notion that functional footprinting strategy was employed to provide insight on the sequence conservation of the IIa-IIx intergenic region, where regulatory sites for all transcription may reside. The rationale for this analysis resides in the notion that functional sequences are conserved over the course of evolution by selective pressure and that mutations within functional regions will accumulate more slowly than in regions without sequence-specific function (39). Our hypothesis that the noncoding intergenic DNA has a sequence-specific regulatory function is supported by comparison of rat, mouse, and human DNA sequences. Alignment of the rat IIa-IIx intergenic DNA with the mouse and human orthologous sequences revealed regions of high sequence conservation. In addition to the highly conserved 400 bp of the IIx proximal promoter region, two Ila-IIx intergenic regions shared more than 75% identity over at least 100 bp among the three species. These were located at ~1800 and ~1300 bp of the IIx MHC TSS. These highly conserved distal regions of the intergenic sequence raised the question of whether they are part of the regulatory regions driving the expression of the all NAT and/or the potential sites of common regulatory sequences driving the expression of both the IIx MHC and the IIa NAT. There is a TBP binding site (TATA box) located on the reverse strand at ~204 relative to the IIx TSS of the rat, which is also entirely conserved on the mouse and human sequences. This putative site on the reverse DNA strand for binding of TBP and other associated general transcription factors is 177 bp upstream of the IIx normal strand TATA box, which is consistent with RT-PCR analyses that probed for the region where the all TSS is located (see Fig. 7). Other transcription factors (MEF-2, MyoD, NFAT-1, Hb, C/EBP) also show a probability of binding to conserved regions on the reverse strand. Because of the high number of false-positive predictions in modeling transcription factor binding sites, only direct experimentation will identify the trans-acting proteins and their cognate cis-regulatory sequences that have functional sig-
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In *silico* analysis of the intergenic regions within 2 kb of the transcription start sites of both the IIx and IIb genes by other researchers also show a compelling pattern of conservation between human and mouse intergenic sequences (40).

Furthermore, evidence that the mechanism of NAT action on MHC gene regulation is evolutionarily conserved is supported by analysis of human skeletal muscle tissue. Human vastus lateralis muscle from spinal cord-injured subjects was compared with normal control subjects (41). In this situation, there was a decrease in IIa mRNA and a robust increase in aII NAT in the spinal cord-injured human subjects compared with normal control subjects. The evolutionary conservation of the intergenic region, the gene orientation, and detection of the all transcript within ~400 bp upstream of the IIx promoter all provide support for the intriguing possibility that sense and antisense transcription is activated by a bidirectional promoter. Transcription of an inhibitory molecule to IIa MHC gene expression linked to that of IIx MHC gene transcription provides a simple explanation for the IIa to IIx MHC “shift” that is reported here and elsewhere (6).

In the future it will be necessary to analyze the IIa-IIx intergenic bidirectional promoter activity functionally. We have attempted to characterize the IIx MHC gene expression with *in vivo* transient transfection assays using intramuscular injection of various IIx MHC promoter-reporter plasmid constructs. Unfortunately we were not able, consistently and reliably, to obtain promoter-reporter activity that reflected accurately the activity of the endogenous IIx MHC promoter, thus prohibiting, as of yet, further characterization of this promoter as well as any bidirectional activity that may be inherent in the IIa-IIx intergenic DNA. However, those negative results may hint at the complexity of IIx MHC transcriptional regulation, such as the requirement of the chromosomal/nucleosomal milieu that involves chromatin–gene interactions that are lacking in the transient gene promoter assay in which the transfected DNA remains episomal in the nucleus.

**NAT-mediated Regulation of Muscle Gene Expression**—The type of NAT-mediated regulation of the IIa MHC gene thus described herein may be common to all the MHC genes in response to any stimuli that causes MHC gene switching. These NATs may mediate cross-talk among individual members of the skeletal muscle MHC gene locus in order to orchestrate well coordinated MHC shifts in muscles undergoing dynamic MHC phenotype shifts. For example, resistance training involving rat fast-twist “white” skeletal muscle, which results in up-regulation of IIx MHC mRNA and down-regulation of IIb MHC mRNA, is associated with NATs (6). In this case, resistance training results in a decrease in xII NAT transcription as compared to that of IIx MHC gene transcription.

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6 F. Haddad, C. E. Pandorf, and K. M. Baldwin, unpublished observation.

7 F. Haddad and K. M. Baldwin, unpublished observations.
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MHC. Indicated by light gray shading, IIx MHC pre-mRNA, mRNA, and protein are less than that for IIa MHC, and the all NAT is correspondingly lower. The data reported herein suggest that the IIa MHC gene is negatively regulated by occurring antisense transcription (see Fig. 8). The data reported herein suggest that the IIa MHC gene is negatively regulated by occurring antisense transcription (see Fig. 8). The data reported herein suggest that the IIa MHC gene is negatively regulated by occurring antisense transcription (see Fig. 8). The data reported herein suggest that the IIa MHC gene is negatively regulated by occurring antisense transcription (see Fig. 8).

with CON and is associated with increased IIx MHC gene expression and decreased IIb MHC gene expression. Thus NAT-mediated shifts of IIa/IIx MHC and IIb/IIx MHC provide a means for the myofiber to shift MHC isoforms from a given reference isoform profile toward either a faster or a slower one depending on the stimulus.

Further work will be necessary to determine whether NATs are involved in regulation of other MHC genes, particularly during development, when the embryonic and neonatal MHC genes are initially expressed and subsequently repressed. High sequence homology among paralogous MHC promoters in this locus, in addition to high conservation of orthologous sequence among species, supports this notion (40). In addition to the cardiac MHC genes (14), other muscle-specific genes (myosin light chain 1 and troponin I) have been identified as having their expression regulated in light of the identification of this hidden layer of gene regulation involving NATs.

Summary and Conclusions—Our previous work on cardiac muscle, combined with the current findings in a different type of muscle tissue and with a different group of MHC genes, begins to reveal a unique model of gene regulation for the coordinated expression of MHC genes that involves naturally occurring antisense transcription (see Fig. 8). The data reported herein suggest that the IIa MHC gene is negatively regulated by NATs that originate in the IIa-IIx intergenic region, downstream of the 3’ end of the IIa MHC gene. The close proximity of the TSS for the all NAT to that of the TSS for the IIx MHC gene provides support for the intriguing possibility of cross-talk between the IIx and IIa MHC genes via the all NAT. The functional significance of the chromosomal juxtaposition, gene order, and orientation of the type II MHC genes thus described should be considered in future research on MHC gene regulation in light of the identification of this hidden layer of gene regulation involving NATs.

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