Journal of Cellular Biochemistry 94:848–855 (2005)

Regulation of Naturally Occurring Antisense RNA of Myosin Heavy Chain (MyHC) in Neonatal Cardiomyocytes

H.P. Luther,* H. Bartsch, I. Morano, S. Podlowski, and G. Baumann

Medical Clinic I, Department of Cardiology, Humboldt-University (Charité), Ziegelstr. 5-9, D10117 Berlin, Germany

Abstract Naturally occurring antisense RNA has been detected for a range of eukaryotic genes. Its abundance compared to levels of its complementary sense mRNA appears to be a factor indicating its possible regulatory function. In previous studies, we detected appreciable levels of antisense RNA against the two isoforms (α and β) of the heavy myosin-chain (MyHC) in the myocardium of rats. If this is to play a significant role in gene expression antisense levels should vary in response to external and internal cellular influences. Recently, a bidirectional promoter located in the α/β MyHC intergenic region was described, which was proposed to regulate coordinated transcription of α-MyHC sense and β-MyHC antisense. To study MyHC antisense regulation in neonatal heart, we investigated cultivated myocytes stimulated with either trijodthyronin (T3) as an inductor of α-MyHC or phenylephrine with stimulation of β-MyHC. RNA-quantification of sense and antisense transcripts of both isoforms was performed by real-time RT-PCR. Stimulation by T3 led to an induction of both sense and antisense of α-MyHC and to a decrease of β-MyHC sense and antisense. Phenylephrine increased sense and antisense β-MyHC but reduced antisense α-MyHC. The sense/antisense of α- and β-MyHC ratio was unchanged compared to control. Results indicate a coregulation of sense and antisense MyHC RNA under stimulation of T3 and phenylephrine in neonatal cardiomyocytes. J. Cell. Biochem. 94: 848–855, 2005.
© 2004 Wiley-Liss, Inc.

Key words: antisense RNA; heavy myosin chain (MyHC); rat cardiomyocytes

When investigating relative MyHC-isoform levels during ontogenesy in rats, we observed a greater abundance of α-MyHC sense mRNA compared to protein levels in the early neonatal phase [Luther et al., 1998]. We proposed posttranscriptional regulation might explain this non-coordinated expression, and we looked for naturally occurring antisense-mRNA which was first described by Boheler et al. [1992]. Indeed, we were able to detect antisense RNA for α-MyHC [Luther et al., 1997a] and more detailed investigations revealed antisense transcription of both α and β-MyHC isogenes. Both antisense transcripts were shown to have similar sizes of their sense counterparts (approximately 6,000 bp). Quantification revealed a twofold less abundance than their sense mRNA [Luther et al., 1998]. The high level of endogenous antisense RNA in the neonatal rat heart suggests this plays a role in the disproportionate expression of α-MyHC and possibly β-MyHC mRNA and their proteins in the growing heart.

If natural MyHC antisense RNA does play a significant role, then one would expect antisense RNA itself is regulated on external and internal cellular conditions which are known to affect MyHC expression. Since we did not find altered antisense transcription during ontogeny [Luther et al., 1997a], we investigated now neonatal cardiomyocytes which were exposed to T3 or phenylephrine in order to stimulate α-MyHC and β-MyHC. T3 is well

Grant sponsor: DFG; Grant number: Lu 694/1-1.

*Correspondence to: Dr. H.P. Luther, Medical Clinic I, Department of Cardiology, Humboldt-University (Charité), Ziegelstr. 5-9, D10117 Berlin, Germany.
E-mail: hanspeter.luther@charite.de

Received 30 July 2004; Accepted 3 August 2004
DOI 10.1002/jcb.20319

© 2004 Wiley-Liss, Inc.

characterized as a strong inductor of α-MyHC. The receptor of thyroxin and its biological active form T3 is known to bind directly regulatory motifs within the α- [Izumo and Mahdavi, 1988] and β-MyHC promoter [Edwards et al., 1994], resulting in trans-activation and trans-repression, respectively. Phenylephrine is a potent stimulator of β-MyHC [Waspe et al., 1990]. It acts as an α1-adrenergic agonist via the PKC-pathway. The zinc-finger early gene Egr-1 protein, which is upregulated upon α-adrenergic stimulation of heart cells [Iwaki et al., 1990], interacts with the promoter region of the *β-MyHC* gene increasing its transcription rate [Kariya et al., 1994]. The effect of phenylephrine on MyHC expression in cultivated neonatal cardiomyocytes, which are spontaneously beating, was shown to be independent of beating rate or systolic/diastolic calcium fluctuation [Luther et al., 1997b].

In recent years, there is an increasing attention for regulatory mechanisms in gene expression involving double-stranded RNA, such as RNA interference (RNAi) and endogenous antisense RNA. Recently, *cis*-encoded antisense RNA has been detected for an increasing number of genes [Yelin et al., 2003; Røsok and Sioud, 2004]. These antisense transcripts are thought to form duplexes with the complementary sense strands and hence modulate gene expression at transcriptional, posttranscriptional, or possibly translational levels [Knee and Murphy, 1997]. Although the precise molecular mechanisms involved in this control are as yet unknown, it seems likely that the amount of duplexed sense/antisense RNA dictates the degree of modulation. Whether the sense/antisense double-stranded RNAs evoke rapid endonuclease digestion similar to the cellular response in RNAi defense mechanisms is a matter for discussion.

MATERIALS AND METHODS

Heart Cell Culture

Cardiomyocytes from neonatal Wistar rats were isolated according to established protocol [Luther et al., 1997a] and cultivated for 2 days without serum. They were then separated into different samples and incubated with triiodothyronine (T3, 10 μM), or phenylephrine (100 μM) for 48 h. To perform the subsequent PCR experiments the cells were harvested and lysed, essentially as described.

RNA Preparation and Reverse Transcription

Total RNA was isolated, with minor modifications, according to the method of Chomczynski and Sacchi [1987]. DNA degradation was performed for 15 min at 37°C on 1 μg RNA samples with 1 μl DNase I (Ambion), 2 μl 10× DNase buffer and water to adjust to a final volume of 20 μl. The reaction was stopped by adding 2 μl DNase inactivation reagent for 2 min at room temperature.

Following digestion with DNase I 250 ng RNA was used for reverse transcription. In the reverse transcription (RT), reverse primers were used for subsequent sense amplification and forward primers for subsequent antisense amplification. RNA was denatured for 10 min at 70°C and then incubated for 60 min at 42°C in the presence of 0.5 mM dNTP, 10 μM dithiothreitol, 25 pmol of the respective primer, 1 U MMLV superscript reverse transcriptase (Invitrogen, Karlsruhe, Germany) in 50 mM Tris-HCl, pH 8.3, 75 mM KCl, and 3 mM $MgCl_2$ in a final reaction volume of 25 μl. The reaction was stopped at 70°C for 10 min.

Real-Time PCR

The GeneAmp® 5700 detection system (P.E. Applied Biosystems, Darmstadt, Germany) was used with SYBR Green I dye. This method is based on the phenomenon that unbound dye exhibits little fluorescence in solution. Binding with double-stranded DNA during the polymerization step increases the signal and falls off when the DNA is denatured. Fluorescence is monitored once each cycle after product extension and increasing signal depends on initial template concentration. Plotting the fluorescence signal as a function of temperature generates a specific melting curve. The shape of melting curves depends on the GC-content, length, and sequence. Unlike gel electrophoresis, melting curve analysis can distinguish products of the same length but different GC/AT ratio [Ririe et al., 1997]. Non-specific products usually melt at lower temperature in broader peaks [Morrison et al., 1998].

PCR was carried out in 25 μl reaction volumes containing 2.5 μl SYBR Green PCR reaction buffer (10×), 0.1 μl (5 U/μl) AmpliTaq Gold (P.E. Applied Biosystems), 0.5 μl 10 mmol dNTP, 3 μl 25 mmol $MgCl_2$, 200 nmol forward, and 200 nmol reverse primers, and 2.5 μl cDNA from the RT-PCR above, with the following cycle profile:

95°C for 10 min, then 40 cycles of 95°C for 15 s, 65°C for 1 min. All reactions were carried out in duplicate. Amplification efficiency was determined to be around 2 for all products what means doubling of the cDNA products after each cycle.

Ct-value is defined as the cycle number in which the fluorescent signal is first recorded as statistically significant above background. It was normalized against the external control of hypoxanthine-guanine-phosphoribosyltransferase (*HPRT*, a housekeeping gene) which is performed by subtraction resulting in ΔCt-values. Amplification of reverse transcribed HPRT mRNA was chosen as an external control since validation experiments had shown that this housekeeping gene is expressed at the very same levels for both sense and antisense RNAs (for more detailed description and discussion of real-time PCRs see Bustin, 2000).

Statistics

Values are based on data from $n = 6$ and expressed as means ± SEM. Significance was tested by ANOVA including Tukey's multiple comparison test.

RESULTS

PCR using the outlined primers (Table I) resulted in single products for α-MyHC (61 bp) and β-MyHC (60 bp) (see Fig. 1A). Analysis of melting curves revealed a sharp profile of each product (Fig. 1B). Product sizes and melting peaks were specific for α- and β-MyHC but identical for sense and antisense.

Theoretically, amplification of cDNA products can be described by the formula $P = m \times 2\Delta^{Ct}$, where P is the number of cDNA products synthesized, m is the number of cDNA molecules at the beginning of the reaction, and 2 is the amplification factor (doubling of the cDNA products after each cycle).

Sense MyHC level were changed by stimulation in an expected manner: sense α-MyHC was significantly extended by T3 (ΔCt-value + 2.0 ± 1.3, what is calculated as a 4.0-fold increase of transcript number) and was not changed by phenylephrine (Fig. 2a). Sense β-MyHC was significantly induced by phenylephrine ($\Delta Ct + 1.7 \pm 0.7 = 3.3$-fold) and significantly reduced upon T3 ($\Delta Ct - 2.1 \pm 0.9 = 4.3$-fold) (Fig. 3a).

The amount of antisense transcripts of both α- and β-MyHC were changed in similar quality and quantity like the corresponding sense transcripts. Antisense α-MyHC was increased by T3 ($\Delta Ct + 1.7 \pm 0.9 = 3.3$-fold) and not significantly affected by phenylephrine ($\Delta Ct + 1.0 \pm 0.7 =$ 2.0-fold) (Fig. 2b). Antisense β-MyHC was reduced by T3 ($\Delta Ct\ -2.3 \pm 1.2 = 4.9$-fold) and increased ($\Delta Ct + 1.5 \pm 0.8 = 2.9$-fold) by phenylephrine (Fig. 3b).

Calculating sense/antisense relation revealed in controls a higher value (i.e., relative higher antisense level) for α-MyHC than for β-MyHC in controls. T3 provoked a slight decrease of relative α-MyHC antisense compared to control or phenylephrine, but did not reach statistically significance. Relative β-MyHC antisense was unchanged under stimulation (Fig. 4).

DISCUSSION

For quantification of α-MyHC and β-MyHC sense and antisense transcripts, we applied the actual most accurate procedure, which is real-time PCR. The detection of the PCR products relied on SYBR Green I dye which quantitatively binds double-stranded DNA and is quantified by fluorescence measurement. Since a number of reaction steps performed in conventional PCR reaction (gel electrophoresis, densitometry, manual or automated analysis) are carried out by the thermal cycler/detector, a body of potential faults is excluded. The transcript specificity of PCR signals is based on the

TABLE I. Primers Used for Reverse Transcription and Real-Time PCR

	Position	α-MyHC (accession no. X 15938)	Position	β-MyHC (accession no. X 15939)	Position	HPRT (accession no. XM_343829.1)
Forward	4973–4992	5′-AACG CCCA AGCC CACT TGAA-3′	4942–4965	5′-GAGC CTCC AGAG TTTG CTGA AGGA-3′	304–325	5′-CTTG CTCG AGAT GTCA TGAA GG-3′
Reverse	5033–5042	5′-CATT GGCA CGGA CTGC GTCA-3′	5001–5020	5′-TTGG CACG GACT GCGT CATC-3′	380–400	5′-AATC CAGC AGGT CAGC AAAG A-3′
Product length		61		60		76

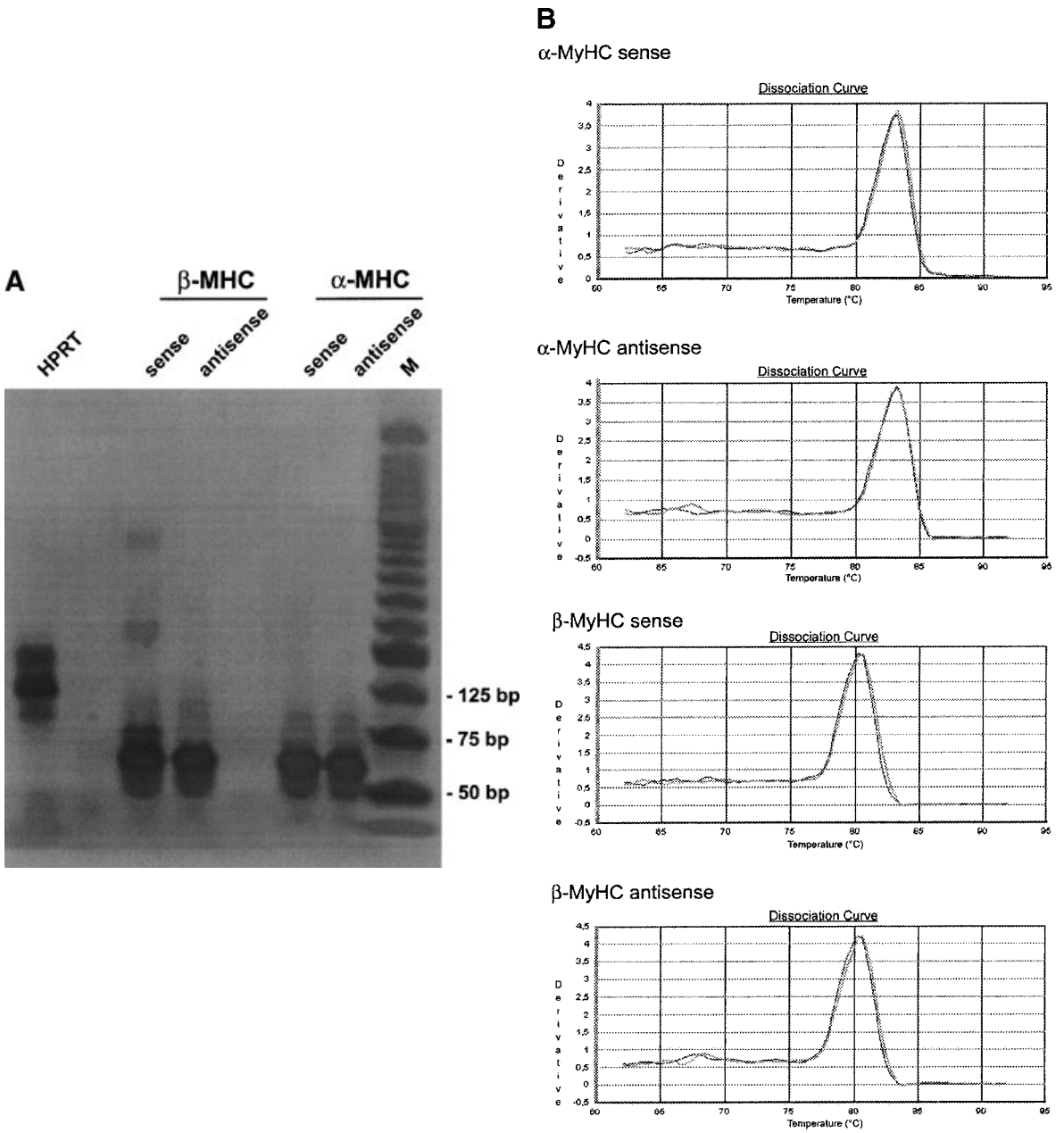

Fig. 1. Analysis of real-time PCR products after 40 cycles indicates absence of contaminating products (**A**) gel electrophoresis (polyacrylamide gel, 5%, silver staining) with the expected size of PCR products: sense and antisense of α-MyHC (61 bp), and β-MyHC (60 bp)). M indicates the molecular weight marker. **B**: Display of melting curves during PCR by monitoring the fluorescence of dsDNA dyes as the temperature passes through the product denaturation temperature.

use of primer in RT, which are specific for orientation and for the isogene. PCR products were checked by gel electrophoresis in order to exclude additional, non-specific fragments that could interfere with the quantification using SYBR Green dye. Additionally, we analyzed the melting curves for the same reason. The data of product analysis with a close match of sense and antisense is explainable with perfect complementarity of both transcript. It means that the antisense transcript do not comprise intron sequences since primer were located in different exons. Due to the identical parameters of PCR products, relative quantification of antisense and sense occurred under ideal conditions.

Using real-time PCR revealed some differences compared to results obtained with conventional RT-PCR: investigating neonatal rat

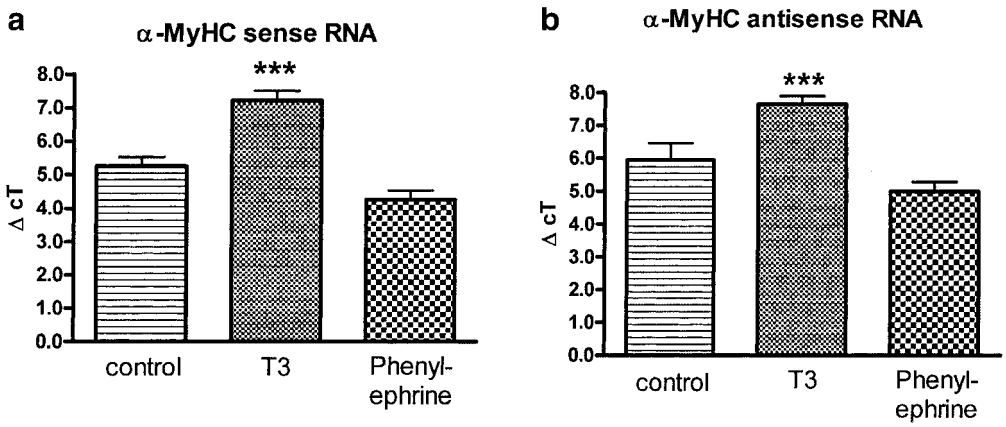

Fig. 2. Level of sense (**a**) and antisense (**b**) α-MyHC in neonatal cadiomyocytes cultivated in a serum-free medium (control), in presence of trijodidthyronin (T3), or phenylephrine (phe). Ct-values were obtained by real-time-PCR and normalized against the external control of a housekeeping gene (*HPRT*). The resulting ΔCt-value is the difference of cycle-number between specific product and housekeeping gene. Values are based on data from n = 6 and expressed as means ± SEM. Significance was tested by ANOVA including Tukey's multiple comparison test. $^{***}P < 0.001$.

heart cells, which were cultivated for 4 days in a serum-free medium showed in controls a sense α/β-MyHC relation of 5.8 to 1 which is around three times higher than detected by conventional RT-PCR [Luther et al., 1997a]. Another difference was the significantly elevated level of antisense compared to sense level with 1.6 to 1 for α-MyHC and 1.9 to 1 for β-MyHC compared to 0.5 to 1 (α- and β-antisense) in our prior investigation [Luther et al., 1998]. Quantification of MyHC antisense RNA by RT-PCR is intricate. For example, a RT-PCR protocol which included coamplification of both isogene antisense transcripts was seen to cause an artificial underestimation of β-MyHC antisense [Luther et al., 1998].

To elucidate antisense regulation isogene specific MyHC inductors were chosen which are well characterized. This design comprises positive control for both α- and β-MyHC detection since the results of sense MyHC upon stimulation were in the expected range: T3 increased α-MyHC and decreased β-MyHC significantly, phenylephrine increased β-MyHC and did not affect α-MyHC. Quantification of antisense transcripts revealed a coordinated regulation: increased sense was accompanied by an increased corresponding antisense level. This coregulation was not expected since—according to a downregulating function of antisense—one would expect an inverse regulation.

Our results are in contrast to a report of Haddad et al. [2003], who investigated myocardium of adult rats, which were exposed to β-MyHC stimulating conditions (induction of hypothyroidism or diabetes). Antisense β-MyHC

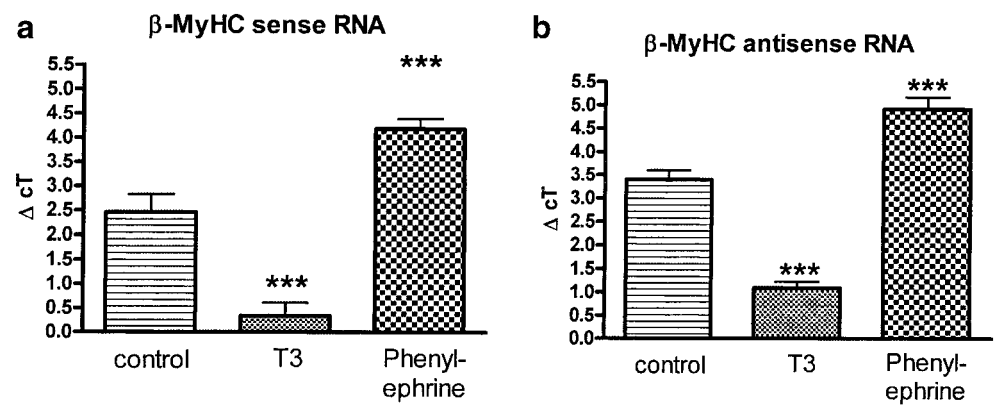

Fig. 3. Level of sense (**a**) and antisense (**b**) β-MyHC in neonatal cadiomyocytes cultivated in a serum-free medium (control), in presence of trijodidthyronin (T3) or phenylephrine. (For further details see legend of Figure 2).

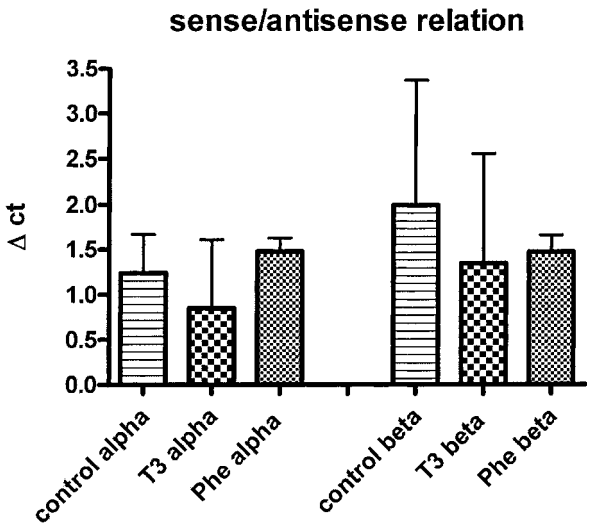

Fig. 4. Antisense/sense relation for α- and β-MyHC. Relative level of antisense/sense RNA determined by subtraction of mean Ct-value of sense from referred antisense product. A higher value indicates a lower relative antisense level and vice versa.

was shown to decrease under these conditions. A possible explanation is the different mode of stimulation. Hypothyroidism and diabetes are assured conditions of β-MyHC induction but the exact mechanism is unknown. It is not clear, whether under this conditions a defined promoter interaction is involved (what is described for phenylephrine) or β-MyHC transcription is a result of a more complex process. According to different mechanisms of sense induction antisense transcription may be regulated in a different manner.

An other explanation are differences due to the ontogenesis with different antisense regulation in neonatal and adult rat. In the neonatal heart, the MyHC phenotype switches from the β-isoform into the α-MyHC isoform. This antithetical shift is thought to be driven not only by thyroid hormones but additional mechanisms, which may be activated during this period. However, Haddad et al. describe a bidirectional promoter which was hypothized to be located in the intergene region of the two isogenes. The result of this promoter/gene architecture should be an enhanced MyHC isoform switch in physiological and pathophysiological situations with increased β-MyHC antisense transcription upon α-MyHC sense stimulation. Our results of antisense transcription under T3 (which was not investigated by Haddad et al.) revealed an induction of α-MyHC antisense and a decrease of β-MyHC antisense. This result is hardly compatible with a significant impact of the postulated bidirectional promoter. However, the relative α-MyHC sense/antisense level was slightly decreased. A conclusive interpretation of the results is difficult but it is reasonable to state that (1) the results of antisense levels are not explicable by a regulation model with one promoter: beside the described bidirectional promoter in the intergenic region, whose activity might reflect the relative β-MyHC antisense increase, one has to postulate another mechanism of transcription regulation. A sequence analysis identified a potential reverse polymerase II promoter for β-MyHC [Luther et al., 1998], which may be a factor of regulation to explain the parallel increase of absolute level of sense and antisense β-MyHC. However, a corresponding element for α-MyHC has as yet not been detected, but α-MyHC antisense levels are changing in same manner. (2) The distinct change in MyHC isoform expression from fetal to neonatal and adult rat hearts is the result of an altered activity of different regulation mechanism, so that MyHC antisense regulation may be different in neonatal and adult rat heart. (3) MyHC antisense level detected by RT-PCR (conventional or real-time) in a whole RNA preparation may not reflect the functional activity of the transcripts. Since the quantity of sense and antisense was equal it is hardly conceivable that all detected antisense transcripts form duplex with the corresponding sense transcript to inhibit translation. One would expect no changes in protein expression under thyroxin and phenylephrine, but in fact they do arise.

In human ventricle with nearly exclusively β-MyHC protein, antisense pattern was shown to be coregulated as well: a high abundance of β-MyHC sense corresponded to an antisense expression of exclusively β-MyHC, α-MyHC antisense was not detected: the expression of sense and antisense was not changed in failing myocardium [Ritter et al., 1999; Luther et al., 2001]. Although a number of examples describe cases with inverse relationship [Kimelman and Kirschner, 1989], there are examples of sense and antisense coexpression [Shi et al., 2000; Podlowski et al., 2002], also for coordinated regulation [Armstrong and Krystal, 1992].

The effect of natural MyHC-antisense seems to be different compared to that of artificial antisense: an inhibition of β-MyHC translation by synthetic antisense oligonucleotide was clearly demonstrated by an in vitro-assay. The

issue was shown to be dependent on concentration and on the position of oligonucleotide in the cDNA [Luther et al., 2001]. Therefore it is reasonable, to assess that the interaction between sense RNA with long natural antisense is more complex than with a small oligonucleotide. Hence, one could hypothize that duplex formation reflects functional activity in a better way than natural antisense level itself. In this context, duplex formation would not be a random event as a result of stoichimetric amounts of sense and antisense transcript but a regulated process, that takes place in a cell compartment [Krystal et al., 1990]. Investigating antisense of troponin I, another core protein of heart muscle function, it was possible to demonstrate a small fraction of transcripts to be in the duplex state [Podlowski et al., 2002].

Again further studies will be needed to determine control elements responsible for antisense transcription modulation by endogenous and exogenous signals. The role of RNA-binding proteins and the differentiation of cell specific antisense transcription have to be in the focus. Taken together, we were able to show that enhanced transcription of α/β-MyHC sense RNA is paralleled by an increased level of the respective isoform specific antisense RNA in neonatal cardiomyocytes. The coregulation of sense and antisense RNA challenge the hypothesis of a translation inhibiting function of natural MyHC antisense transcripts.

ACKNOWLEDGMENTS

We thank Anke Stach for excellent technical assistance.

REFERENCES

Armstrong BC, Krystal GW. 1992. Isolation and characterization of complementary DNA for *N-cym*, a gene encoded by the DNA strand opposite to *N-myc*. Cell Growth Differ 3:385–390.

Boheler KR, Chassagne C, Martin X, Wisnewsky C, Schwartz K. 1992. Cardiac expressions of alpha- and beta-myosin heavy chains and sarcomeric alpha-actins are regulated through transcriptional mechanisms. Results from nuclear run-on assays in isolated rat cardiac nuclei. J Biol Chem 267:12979–12985.

Bustin SA. 2000. Absolute quantification of mRNA using real-time reverse transcription polymerase chain reaction assays. J Mol Endocrinol 25:169–193.

Chomczynski P, Sacchi N. 1987. Single-step method of RNA isolation by acid guanidinium thiocyanate–phenol–chloroform extraction. Anal Biochem 162:156–159.

Edwards JG, Bahl JJ, Flink IL, Cheng S, Morkin E. 1994. Thyroid hormone influences beta myosin heavy chain (beta MHC) expression. Biochem Biophys Res Commun 199:1482–1488.

Haddad F, Bodell PW, Qin AX, Giger JM, Baldwin KM. 2003 Role of antisense RNA in coordinating cardiac myosin heavy chain gene switching. J Biol Chem 278: 37132–37138.

Iwaki K, Sukahatme VP, Shubjeita HE, Chien KR. 1990. Alpha- and beta-adrenergic stimulation induces distinct patterns of immediate early gene expression in neonatal rat myocardial cells. *fos/jun* expression is associated with sarcomere assembly; Egr-1 induction is primarily an alpha 1-mediated response J Biol Chem 265:13809–13817.

Izumo S, Mahdavi V. 1988. Thyroid hormone receptor alpha isoforms generated by alternative splicing differentially activate myosin HC gene transcription. Nature 334:539–542.

Kariya K, Karns LR, Simpson PC. 1994. An enhancer core element mediates stimulation of the rat beta-myosin heavy chain promoter by an alpha 1-adrenergic agonist and activated beta-protein kinase C in hypertrophy of cardiac myocytes. J Biol Chem 269:3775–3782.

Kimelman D, Kirschner MW. 1989. An antisense mRNA directs the covalent modification of the trascript encoding fibroblast growth factor in *Xenopus* oocytes. Cell 59:687–696.

Knee R, Murphy P. 1997. Regulation of gene expression by natural antisense RNA transcripts. Neurochem Int 31: 379–392.

Krystal GW, Armstrong BC, Battey JF. 1990. *N-myc* mRNA forms an RNA–RNA duplex with endogenous antisense transcripts. Mol Cell Biol 10:4180–4191.

Luther HP, Morwinski R, Wallukat G, Haase H, Morano I. 1997a. Expression of sense and naturally occurring antisense mRNA of myosin heavy chain in rat heart tissue and cultivated cardiomyocytes. J. Mol Cell Cardiol 29:27–35.

Luther HP, Hille S, Haase H, Morano I. 1997b. The influence of mechanical activity, adrenergic stimulation, and calcium on the expression of myosin heavy chains in cultivated neonatal cardiomyocytes. J Cell Biochem 64: 458–465.

Luther HP, Haase H, Hohaus A, Beckmann G, Reich J, Morano I. 1998. Characterization of naturally occurring myosin heavy chain antisense mRNA in rat heart. J Cell Biochem 70:110–120.

Luther HP, Podlowski S, Hetzer R, Baumann G. 2001. Analysis of sense and naturally occurring antisense transcripts of myosin heavy chain in the human myocardium. J Cell Biochem 80:596–605.

Morrison T, Weis J, Wittwer C. 1998. Quantification of low-copy transcripts by continuous SYBR Green I monitoring during amplification. Biotechniques 24:954–958.

Podlowski S, Bramlage P, Baumann G, Morano I, Luther HP. 2002. Cardiac troponin I sense-antisense RNA duplexes in the myocardium. J Cell Biochem 85:198–207.

Ririe K, Rasmussen R, Wittwer C. 1997. Product differentiation by analysis of DNA melting curves during the polymerase chain reaction. Anal Biochem 245:154–160.

Ritter O, Luther HP, Haase H, Baltas LG, Baumann G, Schulte HD, Morano I. 1999. Expression of atrial myosin

light chains but not alpha-myosin heavy chains is correlated in vivo with increased ventricular function in patients with hypertrophic obstructive cardiomyopathy. J Mol Med 77:677–685.

Røsok Ø, Sioud M. 2004. Systematic identification of sense-antisense transcripts in mammalian cells. Nat Biotechnol 22:104–108.

Shi M, Yan X, Ryan DH, Harris R. 2000. Identification of urocortin mRNA antisense transcripts in rat tissue. Brain Res Bull 3:317–324.

Waspe LE, Ordahl CP, Simpson PC. 1990. The cardiac beta-myosin heavy chain isogene is induced selectively in alpha 1-adrenergic receptor-stimulated hypertrophy of cultured rat heart myocytes. J Clin Invest 85:1206–1214.

Yelin R, Dahary D, Sorek R, Levanon EY, Goldstein O, Shoshan A, Diber A, Biton S, Tamir Y, Khosravi R, Nemzer S, Pinner E, Walach S, Bernstein J, Savitsky K, Rotman G. 2003. Widespread occurrence of antisense transcription in the human genome. Nat Biotechnol 21: 379–386.