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Characterization of Naturally Occurring Myosin Heavy Chain Antisense mRNA in Rat Heart

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Abstract Analysis of mRNA by Northern blot and reverse transcription–polymerase chain reaction demonstrated the expression of sense and considerable amounts of naturally occurring antisense mRNA for β-myosin heavy chain (MHC) and α-MHC in the neonatal rat heart: antisense MHC mRNA expression of α-MHC and β-MHC was approximately half of the corresponding sense MHC mRNA expression. Using a computational approach, we could identify a reverse Pol II promoter in the β-MHC gene. Both sense and antisense MHC mRNA demonstrated similar sizes of approximately 6,000 bp in the Northern blot. Alpha-MHC antisense mRNA consisted of approximately 3,700 bp of complementary exon sequences and β-MHC consisted of approximately 2,700 bp, suggesting a higher probability of α-MHC mRNA dimerization. Hence, sense mRNA transcripts and protein of α-MHC should exist at different relative levels in the neonatal state. In fact, the relative proportion of α-MHC was 52.0 ± 2.6% on the sense mRNA but only 36.3 ± 1.8% on the protein level. Because of its high abundance in the heart, we suggest that in the neonatal heart naturally occurring antisense mRNA may play a role in the regulation of MHC expression and, therefore, in the control of the energetical and contractile behaviour of the heart. J. Cell. Biochem. 70:110–120, 1998. © 1998 Wiley-Liss, Inc.

Key words: myosin heavy chains; rat heart; naturally occurring antisense mRNA

Two myosin heavy chain (MHC) isoforms, designated as α- and β-MHC, are expressed in the mammalian heart. They associate to form αα-homodimers, αβ-heterodimers, and ββ-homodimers. The α- and β-MHC isoforms arise from two different genes located in tandem on the same chromosome [Mahdavi et al., 1984]. Expression of both MHC gene changes during development and as an adaptation to different environmental demands [reviewed in Swynghedauw, 1986]. Work from several laboratories has demonstrated that in the rodent heart the MHC phenotype switches from the β-isoform to the α-MHC isoform during maturation [Lompré et al., 1979; Mahdavi et al., 1987]. This phenotypic conversion is associated with changes in the contractile and energetic behavior of heart myocytes. Expression of α-MHC leads to a higher maximal shortening velocity [Schwartz et al., 1981; Ebrecht et al., 1982] paralleled by a decreased economy of tension development [Alpert and Mulieri, 1982]. Furthermore, oxygen consumption of working hearts increases with α-MHC expression [Kissling et al., 1982]. Hence, MHC isoenzymes play a key role in cardiac contractility, and understanding its regulation expression would be beneficial.

In the rat heart, MHC expression is reported to be regulated on the transcriptional level [Lompré et al., 1984; Izumo et al., 1987] by interaction with enhancer and silencer elements in the promoter region of the genes. Thyroxine increases transcriptional activity of the α-MHC gene [Izumo and Mahdavi, 1988] and suppresses transcription of the β-MHC gene [Edwards et al., 1994]. Testosterone also enhances the expression of α-MHC mRNA [Morano et al., 1990]. Elevated levels of cAMP selectively promote α-MHC gene transcription with no effect on the β-MHC gene [Gupta et al., 1991]. This may be part of the pathway by which hemodynamic overload decreases α-MHC

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expression because adenylyl cyclase activity is low in hypertrophied and overloaded hearts, which is most likely due to overexpression of the inhibitory G-protein [Böhm et al., 1992]. The zinc-finger early gene Egr-1 protein, which is upregulated with α-adrenergic stimulation of heart cells [Iwaki et al., 1990], interacts with the promoter region of the β-MHC gene, thereby increasing its transcription rate [Kariya et al., 1994].

In contrast, posttranscriptional mechanisms that regulate MHC expression are still not well characterized. Naturally occurring antisense mRNA has the potential for posttranscriptional regulation of MHC expression. In eucaryotic cells, both DNA strands of a variety of genes are transcribed, leading to sense mRNA and naturally occuring antisense mRNA [reviewed in Greem et al., 1986; Simons, 1993]. The antisense mRNA is believed to form a duplex with sense mRNA, thereby deteriorating the expression of proteins. In *Xenopus* oocytes, antisense mRNA for basic fibroblast growth factor (bFGF) is expressed in 20-fold excess over the corresponding sense mRNA [Kimelmann and Kirschner, 1989], thus blocking bFGF expression. Antisense mRNA for bFGF has also been detected in human glial tumor cells [Murphy and Knee, 1994]. The existence of naturally occuring antisense mRNA for MHC in skeletal and cardiac muscle has been described [McCarthy et al., 1983; Heywood, 1986; Boheler et al., 1992; Luther et al., 1997], but their sizes and regulatory roles in the heart are still not understood.

In the present study, we characterized the expression of naturally occurring MHC antisense mRNA in the rat heart. Quantification demonstrated that antisense mRNA for both α-MHC and β-MHC was expressed in considerable amounts, i.e., half of the amount of the corresponding sense MHC-mRNA. These results led us to hypothesize that naturally occurring antisense mRNA plays a role in the posttranscriptional regulation of MHC expression in the heart.

MATERIAL AND METHODS

Animals

Male Wistar rats of different ages were used. Food and water were given ad libitum. Animals were killed by decapitation or cervical dislocation. The hearts were excised, blotted, and weighted, and the left ventricles were quickly frozen in liquid nitrogen. Tissue was stored for not longer than 2 weeks at -80°C.

RNA and Protein Preparation

Total RNA and protein were prepared from the same tissue extract according to the protocol of Chomczynski and Sacchi [1987]. In brief, small samples (≈50 mg) of minced ventricular tissue were dissolved in 800 µl of 4 M guanidinium thiocyanate, 25 mM sodium citrate, pH 7.0, 0.5% sarcosyl, and 0.1 M β-mercaptoethanol and homogenized. Eighty microliters of 2 M sodium acetate, pH 4.0, 800 µl water-saturated phenol (Roth), and 160 µl chloroform/isoamyl alcohol (25:1, v/v) were added. After centrifugation at 12,000*g* for 20 min at 4°C, the aqueous phase was transferred to a fresh tube, 800 µl isopropanol were added, and the sample was centrifuged at 5,000*g* for 10 min. The pellet was washed with 75% ethanol, and the RNA was dissolved in 20 µl water. The concentration was determined by ultraviolet absorption. The ratio of optical density at 260 nm and optical density at 280 nm was 1.8–2.0 in all cases. Integrity of RNA was determined by examining the 28S and 18S rRNA bands in ethidium-bromide-stained agarose gels using a 23S/16S rRNA from *Escherichia coli* (Boehringer-Mannheim, Mannheim, Germany) as standards. The protein present in the interphase of the phenol extract was washed with ethanol and destilled water and centrifuged (8,000*g*). Subsequently, samples were stored at -20°C for not longer than 2 weeks and used for Western blot analysis.

Quantification of MHC mRNA by Reverse Transcriptase–Polymerase Chain Reaction (RT-PCR)

Quantitative RT-PCR was performed as described elsewhere [Morano et al., 1990; Luther et al., 1997] except that each MHC mRNA isoform was reversely transcribed and amplified in different test tubes. The sequences of MHC cDNA were taken from McNally et al. [1989] for the α-isoform and from Kraft et al. [1989] for the β-isoform. Oligonucleotide primers were synthesized to match the amplification of two distinct double-stranded cDNA products specific for α-MHC (77 bp) and β-MHC (99 bp). Primers were prepared by Bio Tez (Berlin, Germany) by using an ABI synthesizer (Perkin-Elmer, Oak Brook, IL) and purified by high performan liquid chromatography. The sequences of the oligonucleotides were as follows: CAGAAAATGCACGATGAGGA (α-MHC) and GGCCTGAATGAAGAGTAGAT (β-MHC) as the

forward primers and GCATTCATATTTATT-GTGGG (α-MHC) and GTGTTTCTGCC TAAG-GTGCT (β-MHC) as the reverse primers. The position of the products in sequence (between 5834 and 5910 for α-MHC and between 5804 and 5902 for β-MHC) was chosen at the 3′ untranslated region [cf. Morano et al., 1990].

One microgram of total RNA was used for reverse transcription into cDNA. RNA was denaturated for 5 min at 70°C and then reversely transcribed by incubation for 60 min at 42°C in the presence of 0.5 mM dNTP, 0.01 mM dithiothreitol, 25 pmol of each primer, 1 U MMLV superscript reverse transcriptase (BRL-Life Technologies, Gaithersburg, MD), 75 mM KCl, and 3 mM $MgCl_2$, pH 8.3. The reaction was stopped by heating at 70°C for 10 min. Reverse primers for sense mRNA detection and forward primers for antisense mRNA detection were used in the reverse transcription reaction.

For quantitative evaluation, cDNA products were subsequently amplified for 20 cycles using 1.5 units TAQ-DNA polymerase (BRL-Life Technolgies) in an assay mixture containing 1 µM of respective oligonucleotide primers, 10 mM Tris-HCl, pH 8.3, 1.5 mM $MgCl_2$, and 0.2 mM dNTP. The mixture with a final volume of 50 µl was overlaid with mineral oil and amplified in a thermal cycler (Biometra, Göttingen, Germany). Denaturation was carried out at 94°C for 1 min, followed by an annealing step at 58°C for 1 min and an extension step at 72°C for 1.5 min. The corresponding PCR products were separated by an 8% polyacrylamide gel electrophoresis (PAGE) with subsequent ethidium bromide staining. Gels were photographed (Polaroid) and evaluated by film densitometry (ScanPack, Biometra, Göttingen, Germany). The relative amount of the isoforms was expressed as a percentage of the total sum of peak areas. Quantification of PCR products was performed within the linear range of cycle number versus the logarithm of accumulated PCR products as already described: a linear amplification rate could be observed in up to 25 PCR cycles [Luther et al., 1997].

Separate experiments were performed to exclude amplification of contaminating genomic DNA. Therefore, RNA was digested prior to reverse transcription by incubation with 40 units T2 RNAse (Gibco-BRL, Gaithersburg, MD) at 37°C at in a buffer containing 50 mM sodium acetate (pH 4.5) and 2 mM EDTA for 30 min. For RT-PCR, probes were neutralized with 1 M NaOH to pH 7.2. In addition, equimolar amounts of prepared RNA were used instead of cDNA in the PCR cycling procedure. In up to 30 cycles, products were not obtained by this arrangement (not shown).

Characterization of Naturally Occurring Antisense mRNA of MHC

To characterize the size of naturally occurring antisense mRNA for α- and β-MHC, sets of oligonucleotide primers were chosen to obtain overlapping cDNA products (Table I). The RT-PCR procedure was performed according to the protocol described above (cf. quantitative RT-PCR) except that the extension step in the PCR was increased to 2 min, 30 PCR cycles were applied, and products were analyzed by 1% agarose containing ethidium bromide in 50 mM Tris-acetate, pH 8.3, 87 mM borate, and 2 mM EDTA (TBE buffer). DNA sequencing of both strands was carried out on an ABI 373 DNA sequencing system (Perkin-Elmer) by using ABI PRISM Dye Terminator Cycle Sequencing Kit. Ten to twenty nanograms of agarose gel-purified PCR fragment and 3.2 pmol primer were used in a total volume of 20 µl. The sequencing reactions were performed on an GeneAmp PCR System 2400 (Perkin-Elmer) and run on a 5.5% gel (32 cm "well to read" distance) at 32 W for 11 h, theoretically producing about 450 bases.

In addition to RT-PCR, sense and antisense MHC mRNA were investigated by Northern blot hybridization. Total RNA (10 µg/lane) was electrophoretically separated by 1% agarose gels containing 2.2 M formaldehyde, 20 mM N-morpholino propane-sulphuric acid, pH 7.0, 5 mM sodium acetate, and 1 mM EDTA. The RNA was then transferred to nylon membranes (3 mA/cm^2, 1 h) and cross-linked by ultraviolet irradiation. The membranes were prehybridized for 1 h at 68°C in a solution containing 5× standard saline citrate, 1% of blocking reagent (DIG oligonucleotide tailing kit, Boehringer-Mannheim), 0.1% N-lauroylsarcosine, and 0.02% sodium dodecyl sulphate (SDS). Sense and antisense α-MHC mRNA were detected by hybridization with the specific 20-base oligodnucleotide probes described for RT-PCR by using the 3′ reverse and forward primers for detection of sense and antisense mRNA, respectively. Furthermore, Northern Blot hybridization was also performed with 40 base pairs of oligonucleotides consisting of the above-mentioned 20 bp of oligonucleotides enlarged by 20 oligonucleotides [CAGAAAATGCACGATGAGGAATAACCT-

TABLE I. Position and Size of Overlapping Polymerase Chain Reaction Products for Characterization of Naturally Occurring Antisense mRNA of α-MHC and β-MHC[a]

cDNA	Designation	Position in sense sequence	Size (bp)
α-MHC	A1	5910–5306	605
	A2	5365–4882	484
	A3	4944–4567	378
	A4	4671–4151	521
	A5	4219–3093	1,127
	A6	3137–2201	957
	A7	2256–1260	997
	A8	1312–646	667
	A9	702–18	685
β-MHC	B1	5902–5278	625
	B2	5340–3971	1,370
	B3	4071–2968	1,104
	B4	3029–1934	1,096
	B5	2011–1229	782
	B6	1286–568	719
	B7	641–16	625

[a]Sequence adopted from McCarthy et al. [1983] and McNally et al. [1989]. MHC, myosin heavy chain.

GTCCAGCAGAAAG (α-MHC) and GGCCTGAATGAAGAGTAGATCTTGTGCTACCCAACCCTAA (β-MHC) as the forward primers and GCATTCATATTTATTGTGGGATAGCAACAGCGAAACTCTT (α-MHC) and GTGTTTCTGCCTAAGGTGCTGTTTCAAAGGCTCCAGGTCT (β-MHC) as the reverse primers]. Oligonucleotides were 3′ labeled with digoxygenin-dUTP with a commercially available kit (Boehringer-Mannheim). The hybridization reaction was carried out in the prehybridization solution at 42°C for 18 h. MHC mRNA/oligo nucletide hybrids were visualized by the anti-digoxygenin/alkaline phosphatase conjugate and subsequent color reaction with 5-bromo-4-chlor-3-indolyl-phosphate/nitro blue-tetrazloium chloride at room temperature for 2 h (Boehringer-Mannheim). Labeling efficiencies of the oligonucleotides were found to be comparable.

Quantification of MHC by Western Blot Analysis

Cell culture samples or washed protein samples obtained from the RNA preparation procedure were homogenized in SDS sample buffer (5% SDS, 50 mM Tris-HCl, pH 7.5, 250 mM sucrose, 75 mM urea, 60 mM β-mercaptoethanol), boiled for 2 min, and cleared by centrifugation. Aliquots from the supernatant were subjected to SDS-PAGE. Briefly, we used a 4% stacking gel and a 5% separation gel, both containing 25% glycerol. The gels were run (30-mA constant current) at 15°C for 4 h. Proteins were electrophoretically transferred from SDS gels to nitrocellulose (45 μm; Hybond-C, Amersham, Braunschweig, Germany) in a buffer containing 25 mM Tris, 192 mM glycin, 0.1% SDS, and 20% (v/v) methanol (2 h, 0.8 mA/cm^2) by using the Bio-Rad Mini-Protean II system (Cambridge, MA) and were processed further for immunostaining as described elsewhere [Calovini et al., 1995]. Briefly, the nitrocellulose was blocked with 3% ovalbumin overnight at 4°C and subsequently incubated with a monoclonal antibody (mAb) raised against α-MHC (mAb 12 F8; 90 min at room temperature) in a dilution of 1:5,000 and the secondary peroxidase-conjugated antibody in a dilution of 1:2,000 (anti-mouse IgG; Sigma, St. Louis, MO) for 1 h at room temperature. Proteins were visualized with the enhanced chemoluminescence reaction kit (ECL, Amersham) and X-ray film (X-Omat, Kodak, Rochester, NY). The signals were scanned densitrometrically with an Epson GT 8000 (ScanPack, Biometra).

Statistics

Values are expressed as means ± S.E.M. Significance analysis was performed with Student's t-test.

RESULTS

Quantification of MHC Sense and Antisense mRNA

To quantify MHC mRNA of sense and antisense MHC mRNA, we applied the Northern blot hybridization technique (Fig. 1) and quantitative RT-PCR (Fig. 2) with mRNA prepared from neonatal rat hearts (4 days postpartum). To detect low levels of expressed antisense MHC mRNA, we performed long-term hybridization, i.e., 18 h at 42°C. In the Northern blot analysis, sense and antisense α- and β-MHC mRNA had similar sizes located as single bands above 28S rRNA (4,800 bp; Fig. 1), which corresponds to about 6,000 bp of sense and antisense MHC.

Densitometrical analysis and quantification of the Northern blot signals demonstrated the antisense messages to be around 50% of the corresponding sense messages (antisense α-MHC mRNA was 53 ± 4% of sense the β-MHC mRNA signal; antisense β-MHC was 45 ± 6% of the sense β-MHC signal; mean ± S.E.M. of six determinations each; $P < 0.001$). Northern blot

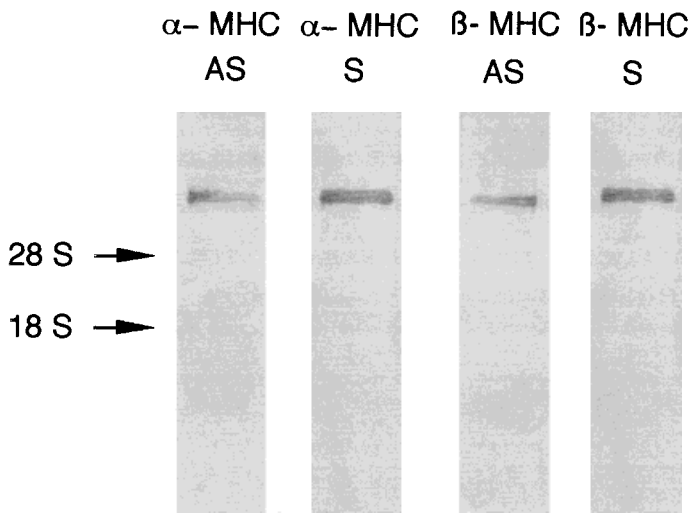

Fig. 1. Northern blot analysis of α-myosin heavy chain (MHC) and β-MHC sense (S) and antisense (AS) mRNA. Ten micrograms of total cardiac RNA of neonatal rats were hybridized with the corresponding DIG-labeled 3′ forward or reverse oligonucleotide (40 nt) probes (forward primers for antisense, reverse primers for sense MHC mRNA isoforms). Migration of 28S and 18S RNA is indicated.

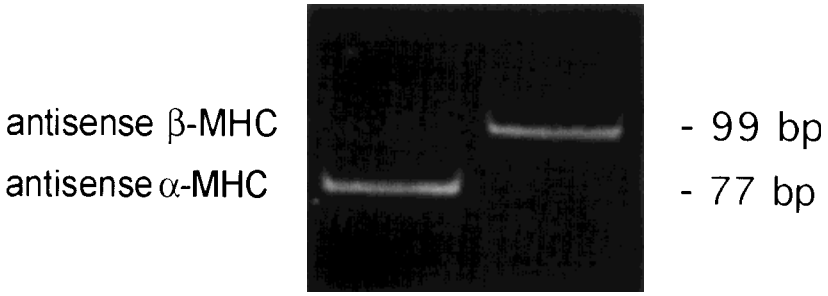

Fig. 2. Quantification of naturally occurring antisense MHC mRNA in the neonatal rat heart. Photograph shows an ethidium bromide-stained polyacrylmide gel with PCR products derived from α-MHC (left) and β-MHC (right) antisense mRNA (20 cycles). The cDNAs were obtained by using forward primers of both α- and β-MHC in the reverse transcription reaction.

signals of sense α-MHC and sense β-MHC mRNA demonstrated equal amounts (sense β-MHC was 95 ± 3% of the sense α-MHC signal; mean ± S.E.M. of six determinations; Fig. 1). This is in line with our previous study using RT-PCR to quantify MHC mRNA [Luther et al., 1997].

As demonstrated by quantitative RT-PCR, cDNA products amplified with antisense mRNA reverse transcription demonstrated the predicted size, i.e., 99 bp for β-MHC and 77 bp for α-MHC (Fig. 3). Equal amounts of α- and β-MHC antisense mRNA isoforms were expressed in the neonatal state, namely 47 ± 3% α-MHC antisense mRNA of whole MHC antisense mRNA (Fig. 2).

Characterization of Naturally Occurring Antisense MHC mRNA

We probed complementary exon sequences of both α- and β-MHC antisense mRNA by using synthetic oligonucleotides (Table I) and RT-PCR. These oligonucleotides were constructed on the basis of the sense mRNA sequences [Kraft et al., 1989; McNally et al., 1989].

Not all oligonucleotide pairs caused the generation of cDNA in RT-PCR: we observed an extension of antisense α-MHC mRNA from position 2201 to 5910 (Fig. 3A). However, no antisense cDNA for α-MHC could be generated by using oligonucleotides from position 18 to 2201. The whole exon sequences of antisense message for α-MHC had a length of at least 3,700

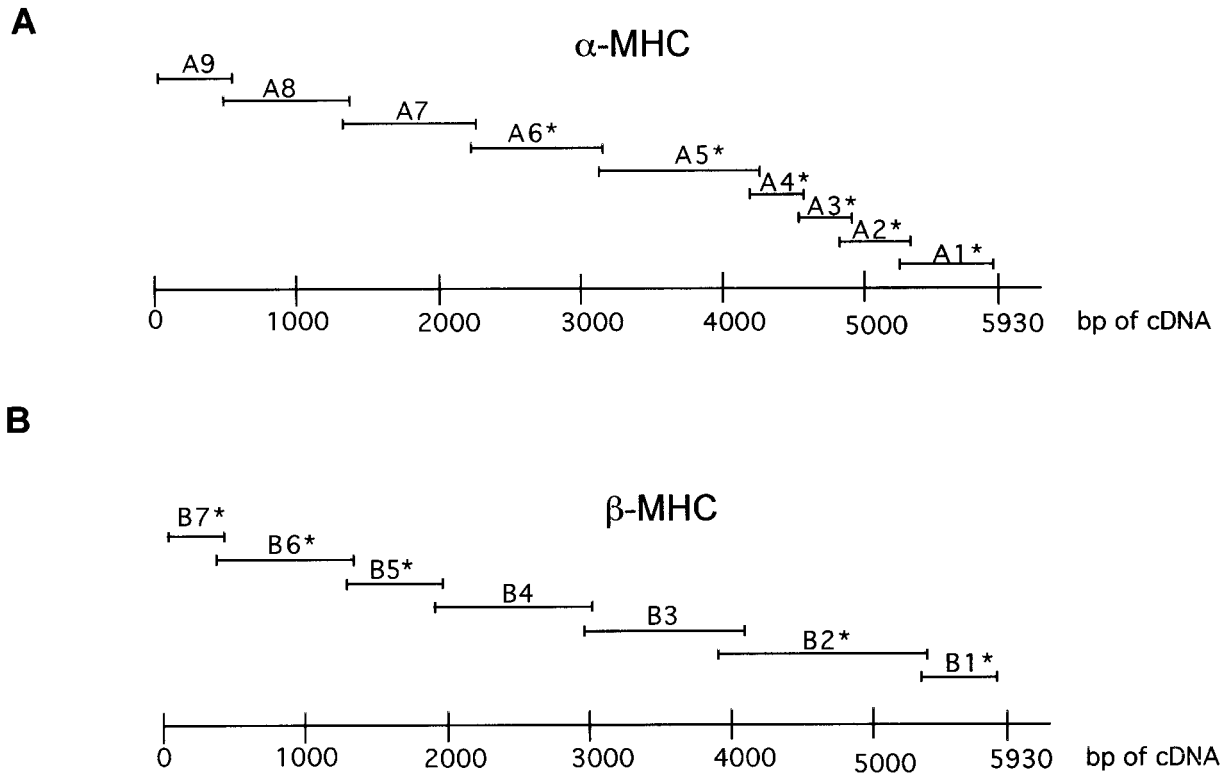

Fig. 3. Schematic representation of the analysis of antisense MHC mRNA by RT-PCR. Forward primers were used in the RT reaction specifically to detect MHC antisense mRNA. They were constructed on the basis of the published sense mRNA sequence for α-MHC (**A**) and β-MHC (**B**). Those primer pairs that detected antisense MHC mRNA, i.e. that caused the generation of a cDNA product in RT-PCR, are labeled with an asterisk.

bp. The β-MHC antisense mRNA consisted of approximately 2,700 bp of exon information at the 5′ end (approximately 2,000 bp) and at the 3′ end (approximately 630 bp). Antisense transcripts of β-MHC were detectable by using oligonucleotides from position 16 to 2011 and from 5278 to 5902 (Fig. 3B). Between positions 623 and 5278, no antisense cDNA for β-MHC could be generated by RT-PCR.

Immunodetection of α-MHC

We analyzed the isoenzyme pattern of MHC in fetal, neonatal, and adult rat hearts. We used the protein fraction obtained from the RNA preparation and employed the specific immunodetection of the α-MHC isoform by the mAb 12F8. Samples from human heart and from rat heart of different developmental stages were probed with the anti-α-MHC antibody mAb 12F8 (Fig. 4). For this series of experiments, one gel was stained for protein with Coomassie blue and the identical gel was processed for immunodetection of MHC with mAb 12F8. In normal human heart, which predominantly expresses the β-MHC isoform [Swynghedauw, 1986], no immunoreaction was detected under our experimental conditions. In rat hearts, however, a 200-kDa protein was stained by the antibody. This band clearly comigrated with the protein band of the MHC. Immunorecognition of the MHC by mAb 12F8 increased during fetal and neonatal development, producing the strongest signal at day 28 of postnatal life (Fig. 4). These data clearly demonstrate a specific immunorecognition of the α-MHC isoform by the mAb 12F8. At present, there is no antibody exhibiting a similar specificity against the β-MHC isoform.

To quantify the α-MHC isoform, we elaborated a calibration curve by using a 28-day-old rat heart sample as the standard for α-MHC (Fig. 5). Increasing amounts of this standard were analyzed for immunoreactivity and protein in parallel. The intensity of immunostaining (optical density, ECL) was plotted against the signal for protein staining (optical density, protein). Figure 5 shows that (1) the detection limit of α-MHC was very low and (2) there was

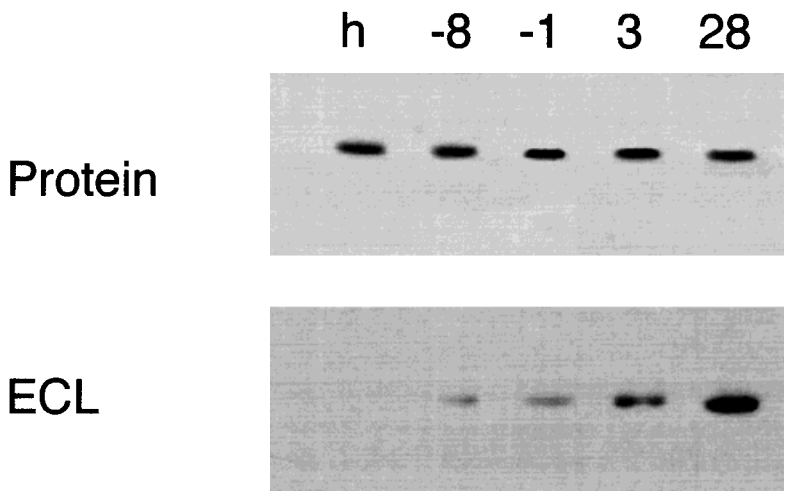

Fig. 4. Immunorecognition of MHC isoforms in human and rat heart by the monoclonal antibody (mAb) 12F8. Samples from human heart (h) and from rat heart at different days of development were separated by SDS-PAGE. One gel was stained for protein by Coomassie blue (**top**) and the respective gel was used for immunodetection of MHC with the mAb 12F8 and the enhanced chemoluminescence (ECL) detection kit (**bottom**). Samples were analyzed from rat hearts 8 days (-8) and 1 day (-1) before birth and at days 1, 2, and 28 of postnatal development.

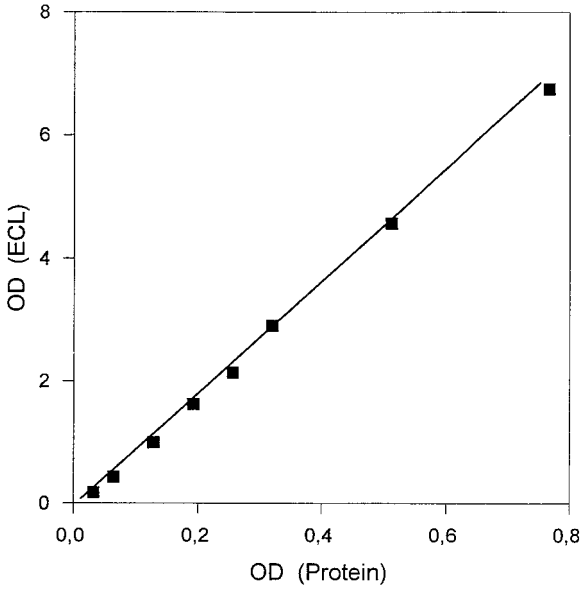

Fig. 5. Calibration of the α-MHC immunoreactivity signal. Increasing amounts of cardiac MHC obtained from a 28-day-old rat were processed for immunostaining by using the mAb 12F8. Densitometric evaluation of the immunostained and the Coomassie-stained MHC band resulted in relative values for optical densities (OD) of the ECL signal and protein signal, respectively.

a linear correlation between immunostaining and protein staining within a wide range of α-MHC concentration.

The α-MHC isoform expression was determined in samples containing the α- and β-isoforms by plotting the immunoreactivity signal against the MHC protein signal. To normalize the values, the 28-day-old rat heart sample was run at each gel and was identically processed for immunostaining. At a given optical density of MHC protein, the immunorecognition of the MHC band by mAb 12F8 was lower in samples from fetal and young rat hearts than was immunorecognition of the α-MHC standard. This relation was used to calculate the percentage of the α-MHC expression (Fig. 6). In fetal rat hearts, low levels of α-MHC were observed, amounting to 8% and 20% at days 8 and 1 before birth, respectively. There was a steady increase in the α-MHC isoform during postnatal development: α-MHC expression rose to 33% at parturition and remained about constant up to 4 days postpartum (37%). Subsequently, a further increase in α-MHC expression was observed reaching 98% α-MHC protein at 3 weeks of postnatal maturation.

To compare developmental changes in MHC isoform expression on the mRNA and protein levels, data of α-MHC mRNA expression are also shown in Figure 6, which we replotted from a previous work [Luther et al., 1997]. In general, isoform transition from β-MHC to α-MHC occurred during heart development on both the mRNA and protein levels. No significant differences were observed between mRNA and protein expression during fetal life when β-MHC was the predominant isoform. The same holds true for rat hearts older than 2 weeks when α-MHC is preferentially expressed. In the

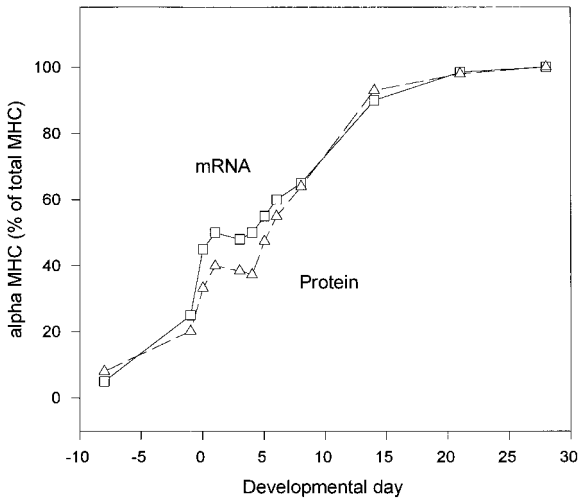

Fig. 6. Parallel determination of α-MHC expression on mRNA and protein levels during rat heart development. The same tissue specimens were analyzed for the relative amounts of α-MHC mRNA and protein. Data of mRNA were replotted from Luther et al. [1997, Fig. 3]. Data of α-MHC protein were obtained by comparing the immunoreactivity signals with signals of rat cardiac MHC at day 28 of postnatal life (cf. Fig. 6). Values are means of three determinations for both α-MHC mRNA and α-MHC protein from one tissue specimen.

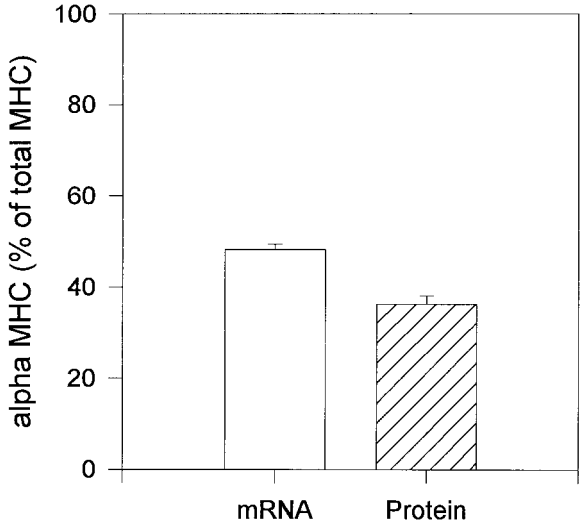

Fig. 7. Comparison of α-MHC expression on mRNA and protein levels in neonatal rat hearts. Data are means ± S.E.M. of mRNA and protein levels from seven independent experiments using neonatal rat hearts 4 days after birth ($*P < 0.05$). Protein and mRNA analysis were derived from the same tissue extract.

neonatal state, however, a noncoordinated expression was apparent: the rise in α-MHC expression was more pronounced at the mRNA level than at the protein level.

In comparing MHC protein and mRNA (quantitative RT-PCR) of cardiac tissue from rats 4 days postpartum, we observed a significantly different ($P < 0.05$) α-MHC expression on the sense mRNA and protein level, 52.0 ± 2.6% and 36.3 ± 1.8% (7 animals), respectively (Fig. 7). In these experiments, protein and mRNA were analyzed from the same tissue extracts.

Computational Analysis of Antisense Promoter

By using the genomic sequence for β-MHC of the syrian hamster and a computational approach (Gene Recognition and Analysis Internet II), we could identify a reverse Pol II promoter between 33,365 bp and 33,575 bp of the reported sequence (CAAT: 33,575 bp; TATA: 33,481 bp; CAP signal: 33,439 bp; ATG: 33,365 bp [sequence from Wang et al., 1995]). A corresponding reverse promoter for the α-MHC gene of the hamster could not be identified with the available sequence obtained from Wang et al. [1994].

DISCUSSION

In the present study, we characterized and quantified sense and naturally occurring antisense mRNA of α-MHC and β-MHC in the neonatal rat heart. Quantititative RT-PCR was performed with the same isoform-specific primers used for sense mRNA quantification. The only difference was that the forward primers were used for reverse transcription of antisense mRNA and the reverse primers were used for sense mRNA reverse transcription [Marino et al., 1991; Luther et al., 1997]. Conditions for quantitative RT-PCR were established previously [Luther et al., 1997], i.e., quantification within the linear range of the relationship between cycle number and logarithm of cDNA product accumulation. A prerequisite for detection of antisense mRNA using forward primers is that these primers do not bind to the sense mRNA: it is theoretically possible that a forward primer binds downstream of the selected position, thus producing a cDNA that could subsequently be amplified properly in the PCR. To minimize this risk, we selected the 3′-untranslated part of the MHC mRNA for RT-PCR analysis. To exclude further sense mRNA detection with forward primers, we used a computational approach (Primer from the GCG packet) to search for possible complementary regions in the cDNA sequences of both rat MHC isoforms. Because there were virtually no

complementary sequences in the MHC sense mRNA with the forward primers used in the present study, we suggest that our forward primers used to quantify antisense mRNA selectively detected the corresponding antisense MHC mRNA rather than sense mRNA.

To characterize the molecular size of sense and antisense MHC mRNA, we applied the Northern blot hybridization technique with mRNA prepared from neonatal rat hearts. In the Northern blot, sense and antisense α- and β-MHC mRNA demonstrated similar sizes located as single bands at about 6,000 bp, which could be predicted from the MHC cDNA sequences [cf. Kraft et al., 1989; McNally et al., 1989]. Similar sizes of sense and antisense MHC mRNA, however, provide no information about those parts of the antisense mRNA molecules that could hybridize with the corresponding sense mRNA. Those parts could be deduced from the fact that the splicing signals on the exon/intron boundaries of the sense and antisense DNA strands must be located at different regions. Thus, in contrast to sense mRNA, the corresponding antisense message may contain both complementary intron and exon information. However, only those parts of the antisense mRNA molecules containing complementary exon sequences could hybridize with the sense mRNA and could be of functional importance. Therefore, we probed complementary exon sequences of both α- and β-MHC antisense mRNA by using synthetic oligonucleotides and qualitative RT-PCR on the basis of the corresponding sense mRNA [Kraft et al., 1989; McNally et al., 1989]. The whole exon sequences of antisense message for α-MHC had a length of at least 3,700 bp, and the β-MHC antisense mRNA had a length of about 2,700 bp. The results are in accordance with those by Boheler et al. [1992] who reported on the detection of antisense mRNA of β-MHC but not α-MHC using probes raised against the 5′ end of MHC mRNAs. Because only about half of the antisense MHC mRNA species consisted of complementary exon information but had approximately similar sizes in the Northern blot analysis, we suggest that both antisense MHC mRNA species contain considerable amounts of sequences that could not hybridize with the corresponding sense MHC mRNA.

By using the Northern blot hybridization technique, we demonstrated considerable amounts of both antisense α-MHC and β-MHC mRNA in the neonatal heart, which constituted approximately 50% of the amount of the corresponding sense MHC-mRNA isoform. This result is in accordance with that of a previous study in which we demonstrated that antisense α-MHC mRNA was half the amount of the corresponding sense mRNA species in the neonatal rat heart when using RT-PCR for quantification [Luther et al., 1997]. In addition, we have reported on approximately equal amounts of α- and β-MHC antisense mRNA isoforms in the neonatal state as assessed by quantitative RT-PCR. This result is in contrast to the result of a previous study [Luther et al., 1997] in which we reported different amounts of α- and β-MHC antisense mRNA. In the previous study, simultaneous reverse transcription of both antisense MHC mRNA isoforms was performed in the same test tube. We found that this procedure led to an artifactual underestimation of antisense (but not sense) β-MHC mRNA. There was an approximately twofold excess of sense mRNA versus antisense transcripts for α-MHC [Luther et al., 1997] and equal proportions of antisense mRNA for α-MHC and β-MHC (present study); an approximately twofold excess of sense mRNA MHC isoforms over the corresponding antisense mRNA MHC species in the neonatal state could be derived. Because of its high expression level in the rat heart, antisense mRNA may play a significant role in the expression regulation of MHC in the neonatal state.

If antisense mRNA could form pairs with sense mRNA, the amount of free (translatable) sense mRNA should be quenched. Because the antisense mRNA of α-MHC contained 3,700 bp, which could hybridize with the corresponding sense mRNA, whereas the antisense β-MHC mRNA contained only 2,200 bp of complementary exon sequences, the probability of quenching the α-MHC sense signal by dimerization may be higher. To verify this hypothesis, we analyzed the isoenzyme pattern of MHC in fetal, neonatal, and adult rat hearts. We used the protein fraction obtained from the RNA preparation and employed the specific immunodetection of the α-MHC isoform by the mAb 12F8. Immunorecognition of the MHC by mAb 12F8 increased during fetal and neonatal development, producing the strongest signal at day 28 of postnatal life. There was a steady increase in the α-MHC isoform during postnatal development. To compare developmental changes in MHC isoform expression on the mRNA and

protein levels, data of α-MHC mRNA expression observed from a previous study were used [Luther et al., 1997]. In the neonatal state, a noncoordinated expression of protein and mRNA was apparent. Obviously, the rise in α-MHC expression was more pronounced at the mRNA level than at the protein level. This rise could be due to the fact that in the growing heart mRNA transcription rate is higher than the rate of protein translation. In addition, the noncoordinated expression of α-MHC mRNA and protein may be due to the existence of corresponding, naturally occurring antisense mRNA in rat heart.

Expression of naturally occurring antisense mRNA requires a promoter in the 3′ end of a gene serving as a signal for interaction with polymerase II. Using the genomic sequences for α- and β-MHC of the Syrian hamster [Wang et al., 1994, 1995] and a computational approach (Gene Recognition and Analysis Internet II) to identify promoter regions [Uberbacher and Mural, 1991; Xu et al., 1994], we could identify a reverse Pol II promoter in the β-MHC gene. The failure to detect a corresponding reverse promoter for the α-MHC gene of the hamster does not necessarily indicate the absence of a reverse promoter for the α-MHC gene. Rather, the gene may be located beyond the already published sequence.

In conclusion, naturally occurring antisense seems to play an important role in the regulation of cardiac MHC expression and thus in the determination of cardiac energetics and contractility.

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