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# Analysis of Sense and Naturally Occurring Antisense Transcripts of Myosin Heavy Chain in the Human Myocardium

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**Abstract** Naturally occurring antisense RNA has the potential to form a duplex with its complementary sense mRNA, thereby regulating protein expression. Previously, we demonstrated considerable amounts of endogenous antisense RNA for both α- and β-myosin heavy chain (MHC) in rat heart suggesting a role in posttranscriptional MHC-regulation (Luther et al. [1997] J Mol Cell Cardiol 29(1):27–35). To evaluate whether antisense RNA is also involved in MHC regulation in human heart we analyzed ventricular myocardium transcripts in nonfailing hearts (n = 3) and hearts from patients undergoing heart transplantation (n = 5). Investigation of RNA by reverse transcription polymerase chain reaction (RT-PCR) detected an antisense RNA transcript for β-MHC but none for α-MHC. Northern blot analysis of normal and failing hearts detected sense mRNA for β-MHC, but not α-MHC suggesting no functionally relevant levels of α-MHC mRNA exist in the human ventricle. The results describe—for the first time—the existence of endogenous *polyadenylated* MHC antisense transcripts in the human heart. The potential effect of attenuating translation was shown in an in vitro translation assay using a synthetic antisense-oligonucleotide derived from the sequence of the naturally occurring antisense RNA. J. Cell. Biochem. 80:596–605, 2001. 

**Key words:** myosin heavy chain; antisense RNA; human heart

The myosin molecule of all muscle types is composed of two heavy chains (MHC) and four light chains. Heavy chains exist as two isoforms and their differential expression pattern determines the contractile properties of the muscle [Maughan, 1979]. In the rat, β-MHC is the abundant fetal form while α-MHC expression increases after birth and becomes the predominant form in adult animals [Lompré et al., 1979]. In human myocardium MHC expression does not change during ontogeny, with β-MHC being preferentially expressed in the ventricles and α-MHC in the atrium of fetuses or adults [Mercadier et al., 1983]. There have been recent reports showing significant amounts of α-MHC mRNA (up to 30% of total MHC) [Nakao et al., 1997] and α-MHC protein [Miyata et al., 2000] in the human heart ventricle, but a recent analysis by Western-blotting failed to detect appreciable amounts of α-MHC at the RNA- or protein-level in normal or hypertrophied myocardium [Ritter et al., 1999a].

A growing number of eukaryotic genes are now thought to be regulated at least in part by natural *cis*-encoded antisense RNA transcribed from the noncoding DNA strand of the gene [for review see Kumar and Carmichael, 1998; Vanhée-Brossollet and Vaquero, 1998]. It is becoming increasingly apparent that the actions of such endogenous antisense RNA molecules are analogous to those of synthetic oligomers with respect to modulating gene expression in prokaryotes as well as eukaryotes. By forming RNA duplexes with their complementary sense transcripts, they may regulate expression in the nucleus either at the level of transcription, processing or transport to the cytoplasm.

Abbreviations used: MHC, myosin heavy chain; RT-PCR, reverse transcription-polymerase chain reaction; PAGE, polyacrylamide gel electrophoresis; bp, base pair(s).

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However, it is thought that antisense regulation occurs more generally in the cytoplasm at the level of messenger stability and posttranscriptional control.

There are some reports about the regulatory role of naturally occurring antisense-RNA in contractile proteins. McCarthy et al. [1983] discovered RNA fragments which specifically quenched MHC translation by RNA–RNA hybridization in the avian skeletal muscle. However, in this case the antisense RNA, called translational control RNAs (tcRNAs), derived from a locus different from the sense RNA, and as such form short, imperfect duplexes with the mRNA [Kumar and Carmichael, 1998]. These RNA fragments were found to be expressed at a higher level in normal than in dystrophic chick pectoralis muscle, indicating a possible pathophysiological role. Boheler et al. [1992] reported complementary antisense β-MHC RNA in cardiac muscle but its physiological function remained undefined. In a recent study, we were able to detect antisense RNA in rat heart for both α- and β-MHC [Luther et al. 1997].

In order to investigate both the sense mRNA levels of MHC isoforms and the role of antisense RNA in MHC expression in human heart, we performed RT-PCR and Northern hybridization to detect RNA transcripts. Here, we report that only β-MHC antisense transcripts were detected in ventricular myocardium, with sense mRNA levels substantially higher than antisense RNA in both failing and nonfailing hearts. Part of this endogenous antisense RNA sequence was shown to be capable of attenuating translation in an in vitro assay.

## MATERIALS AND METHODS

### Heart Tissue

Myocardium from terminally failing human hearts (left ventricular myocardium) was obtained from patients after cardiectomy during cardiac transplantation (1 man, 4 women; age $34 \pm 18$ years; range, 11–57 years). The preoperative diagnosis was chronic heart failure in all patients. All patients had been classified as being in New York Heart Association class IV. Total heart RNA of normal myocardium (3 men, age $34.7 \pm 10.9$ years; range, 26–50 years, from persons without known heart disease), was obtained commercially from Invitrogen, Groningen.

### RNA Preparation

Total RNA was prepared according to a modification of the protocol of Chomczynski and Sacchi [1987]. Briefly, small samples ($\approx 20$ mg) of minced ventricular tissue were dissolved in TRIZOL (Gibco-BRL) and homogenized. After centrifugation at $12000\,g$ for 20 min at 4°C, the aqueous phase was transferred into a fresh tube, 800 μl isopropanol was added, and the sample was centrifuged at $5000\,g$ for 10 min. The pellet was washed with 75% ethanol and the RNA dissolved in 20 μl water. The concentration was determined by UV absorption. The ratio of optical density at 260 and 280 nm was between 1.8 and 2.0 in all cases. Isolation of pure mRNA was performed with oligo(dT)-cellulose (QuickPrep Micro mRNA purification kit, Amersham).

### Reverse Transcription

Two micrograms of total RNA was used for reverse transcription into cDNARNA was denatured for 5 min at 70°C and then reverse-transcribed by incubation for 60 min at 42°C in the presence of 0.5 mM dNTP, 10 μM dithiothreitol, 25 pM of each primer, 1 unit MMLV superscript reverse transcriptase (Gibco-BRL), 1 U RNase H (Gibco-BRL) in 50 mM Tris–HCl, pH 8.3, 75 mM KCl, and 3 mM $MgCl_2$. The reaction was stopped by heating at 70°C for 10 min. For antisense RNA detection the forward primer was used in the reverse transcription reaction for first strand cDNA synthesis [cf. Marino et al., 1991]. To exclude amplification of contaminating genomic DNA, RNA samples were digested prior to reverse transcription by incubation with DNase (Gibco-BRL) at 37°C in a buffer containing 50 mM sodium acetate (pH 4.5) and 2 mM EDTA for 30 min. The samples were neutralized with 1 M NaOH to pH 7.2. DNase efficiency was checked using a DNA-specific primer for RT-PCR. In addition, equimolar amounts of prepared RNA were used instead of cDNA in the PCR cycling procedure. Up to 30 cycles produced no products under these conditions (not shown).

### Quantitative Polymerase Chain Reactions

Relative distributions of RNA isoforms were studied by the polymerase chain reaction (PCR). Oligonucleotide primers were designed to specifically amplify two distinct double-

**TABLE I. Sequence and Positions of Oligonucleotides Used in the RT-PCR and Northern Blot Hybridization**

| | Forward | Position | Access | Reverse | Position | Access |
|---|---|---|---|---|---|---|
| α-MHC RT-PCR | 5′ GGT GCC AAG CAA AAT GC | 5788–5807 | D00943 | 5′ GGC ACT CAT ATT TAT TAC AGG | 1650–1630 | M21664 |
| Northern | 5′ GCC GTG ACA TTG GTG CCA AGC AAA AAA TGC ACG ATG AGG AGT | 5777–5818 | D00943 | 5′ CAG GCA GAG TTT GGC ACT CAT ATT TAT TAC AGG TTG GCA AGA | 30654–30613 | Z20656.1 |
| β-MHC RT-PCR1 | 5′ CAT TGG CAC GAA GGG CTT GAA TG | 5864–5886 | NM_000257 | 5′ GGA AAA TTGC TTT TATT CTG C | 5979–5998 | NM_000257 |
| RT-PCR2 Northern | 5′ GCA GCT AGG AGA AGG AGG | 4628–4647 | NM_000257 | 5′ GGA AAA TTGC TTT TATT CTG C | 5979–5998 | NM_000257 |
| | 5′ CAA GAG CCG TGA CAT TGG CAC GAA GGG CTT GAA TGA GGA GTA G | 5852–5894 | NM_000257 | 5′ CTT CAA GGA AAA TTGC TTT TATT CTG CTT CCT CCC AAG GAG | 5969–6005 | NM_000257 |

stranded products from regions unique to the α- (84 bp) and β-MHC (134 bp) cDNAs. A second pair of isoform-specific primers for β-MHC amplifies a 958 bp fragment in the 5′ region of the cDNA (Table I; Fig. 1). Primers were prepared by Bio Tez, (Berlin, Germany) using an ABI-synthesizer (Perkin-Elmer) and purified by HPLC. The oligonucleotide positions in the cDNA sequences are indicated in Table I and Figure 1. For quantitative evaluation, cDNA products were amplified using 1.5 units TAQ-DNA polymerase (Gibco-BRL) in an assay mixture containing 1 μM of the respective oligonucleotide primers, 10 mM Tris–HCl, pH 8.3, 1.5 mM $MgCl_2$, and 20 μM dNTP. The mixture with a final volume of 50 μl was overlaid with mineral oil and amplified in a thermal cycler (Biometra). Denaturation was carried out at 94°C (1 min), followed by an annealing step at 54°C (1 min) and an exten-

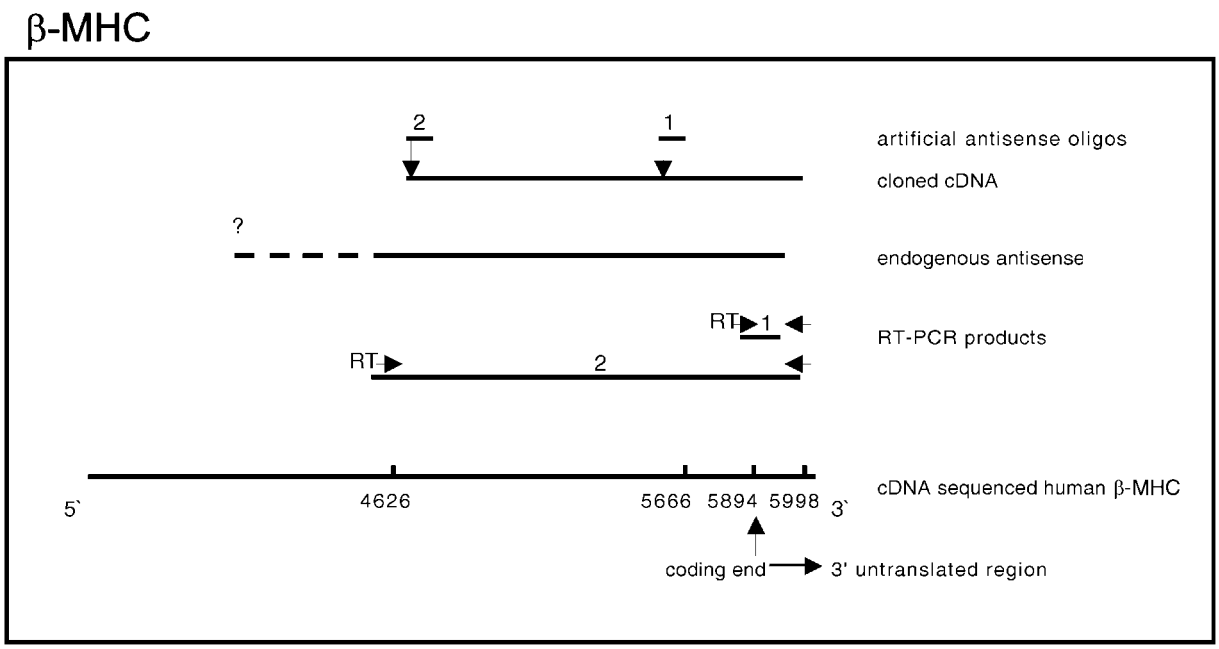


**Fig. 1.** Diagram of PCR-products, antisense-RNA, cloned cDNA and antisense-oligonucleotides in the β-MHC cDNA. Top: position of the antisense oligonucleotides (1 and 2, see Table II) used in the in vitro transcription/translation assays are shown in relation to the cDNA cloned in the pGEM3Z vector. Center: the endogenous antisense transcript is shown extending into an as yet undetermined 5′ region of the cDNA. Primers and RT-PCR products (1 and 2 see Table I) obtained in the quantitative assays are shown below. Bottom: the cDNA sequence of β-MHC indicating some nucleotide positions, the end of the protein coding sequence and 3′ untranslated region.

sion step at 72°C (1.5 min). Products were analyzed by 8% PAGE, stained with ethidium bromide, and evaluated by film densitometry. The relative amount of the isoforms was expressed as percentage of the total sum of the peak areas.

### Semiquantitative Evaluation

To allow semiquantitative evaluation PCR products were amplified in the presence of digoxigenin-11-dUTP (DIG-dUTP). Product accumulation was evaluated using electrophoretical separation, hybridization, and chemilumiscence detection. The latter was performed using a commercial luminescence detection kit CSPD (Boehringer). The chemiluminescent signal of the PCR products was evaluated after 12, 15, 18, 21, 24, 27, and 30 cycles. The logarithm of the optical density was plotted versus cycle number and analyzed by linear regression. Plots fit well into a linear function. The amplification efficiencies calculated for sense RNA [1.7, cf. Ritter et al., 1999b] could be assumed for naturally occurring antisense RNA since identical double stranded cDNA products result in both cases. PCR-products were checked by DNA sequencing of both strands (ABI 373 DNA sequencing system, Perkin-Elmer), using ABI PRISM Dye Terminator Cycle Sequencing Kit and restriction enzyme analysis.

### 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. RNA was transferred to nylon membranes and cross-linked by ultraviolet radiation. Prehybridization was performed for 1 h at 68°C in a buffer 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).

For hybridization oligonucleotides were used with a sequence directed against the 3′ end of the cDNA. Labeling of sense and antisense oligonucleotide probes was performed using digoxygenin-dUTP with comparable efficiency. Hybridization was carried out in the prehybridization solution at 45°C overnight. Hybrids were detected by incubation with an antidigoxygenin/alkaline phosphatase conjugate and subsequent color reaction of the substrate 5-bromo-4-chlor-3-indolyl-phosphate/nitro blue-tetrazloium chloride.

**TABLE II. Sequence and Positions of Oligonucleotides Used for In Vitro Transcription/Translation of Cardiac β-MHC According to Jaenicke et al. [1990], M58018**

| | Antisense | Position | Scrambled |
|---|---|---|---|
| 1 | 5′ TTC CGC TGG AAC CCA ACT GC | 4647–4628 | 5′ CTG CTT CCG TAA CCC AA GGC |
| 2 | 5′ GTT TTT CCT GTC CTC CTC CGT CTG GTA GGT GAG CTC CTT GAT | 5666–5624 | 5′ GAT TTG TCT TCG CCG TTC TTG ACG TCC GGT CCG TTT GTA CCT |

### In Vitro Translation

Total RNA from human cardiac tissue was reverse transcribed into DNA according to standard protocols and amplified with primers containing *Kpn*1/*Hind*III restriction sites (forward: CTA TCG GTA CCT CTA CCA TGG AGC AGT TGG GTT CCA GC, reverse: GAT GGC AAG CTT TCT GGA AAA TTG CTT TAT TCT GC). The PCR product (human cardiac β-MHC 4626–5998, according to X54163), including ATG initiation codon and stop codon, was cloned into the *Kpn*1/*Hind*III sites of the vector pGEM3Z (Promega) and sequenced in both directions.

In vitro transcription/translation experiments were performed with circular plasmid DNA (0.8 μg) in 25 μl reactions using the TNT T7 Quick Coupled Transcription/Translation System (T7TNT, Promega) containing [$^{35}$S]-labeled methionine/cysteine (10 μCi). Oligonucleotides (Table II) were added in a final concentration from 0 to 8 μM. After incubation for 90 min at 30°C the translation products were separated by SDS-PAGE. The gels were dried and the radioactive bands were visualized by autoradiography and quantified by densitometry of the signals. The X-ray films were exposured for 20 h.

### Statistics

Values are expressed as means ± SD (n = 3–5, unless otherwise stated). Significance analysis was performed with Student's *t*-test (RT-PCR), one-way ANOVA and Bonferroni Post-hoc Test for selected pairs (in vitro-translations-assay).

## RESULTS

### Detection and Quantification of Sense and Antisense RNA

Sense or antisense RNA detection was performed by reverse transcription using primers (with reverse or forward orientation, respectively) at different position in the cDNA followed by PCR. Amplification of an antisense signal with isoform-specific primers directed against the respective cDNA 3′ ends (see Materials and Methods and Table I) was successful for β-MHC (between 5998 and 5864; Fig. 1, RT-PCR product 1) but not α-MHC (not shown). Using isoform-specific primers directed to positions closer to the 5′ end, an other antisense product for β-MHC was identified between nt 4628 and 5998 of the cDNA, which was analyzed by sequence-specific restriction by Sac I (Fig. 3). Similar oligonucleotides with an α-MHC specific sequence again did not generate any products, indicating that there is no antisense sequence in this region of the α-MHC cDNA either.

Each amplified antisense β-MHC PCR-product did not differ in size from the corresponding sense PCR-products, so that contaminating, nonspliced genomic DNA as a source can be unequivocally excluded. Moreover, the antisense detection signal was not significantly different for all β-MHC antisense products, which suggested they all derived from one contiguous antisense transcript. In further RT-PCR experiments using purified mRNA isolated with oligo(dT)-cellulose as opposed to total RNA preparations we obtained the same amounts of amplified antisense products (not shown), suggesting, in addition, that this endogenous transcript is polyadenylated.

For relative quantification of antisense and sense MHC RNA, PCR was performed in the presence of DIG labeled nucleotides and the products were analyzed after increasing numbers of cycles. Twelve PCR cycles (Table I) were enough to yield a product signal from sense-β-MHC after PAGE and chemiluminescence detection. To obtain a similar signal from

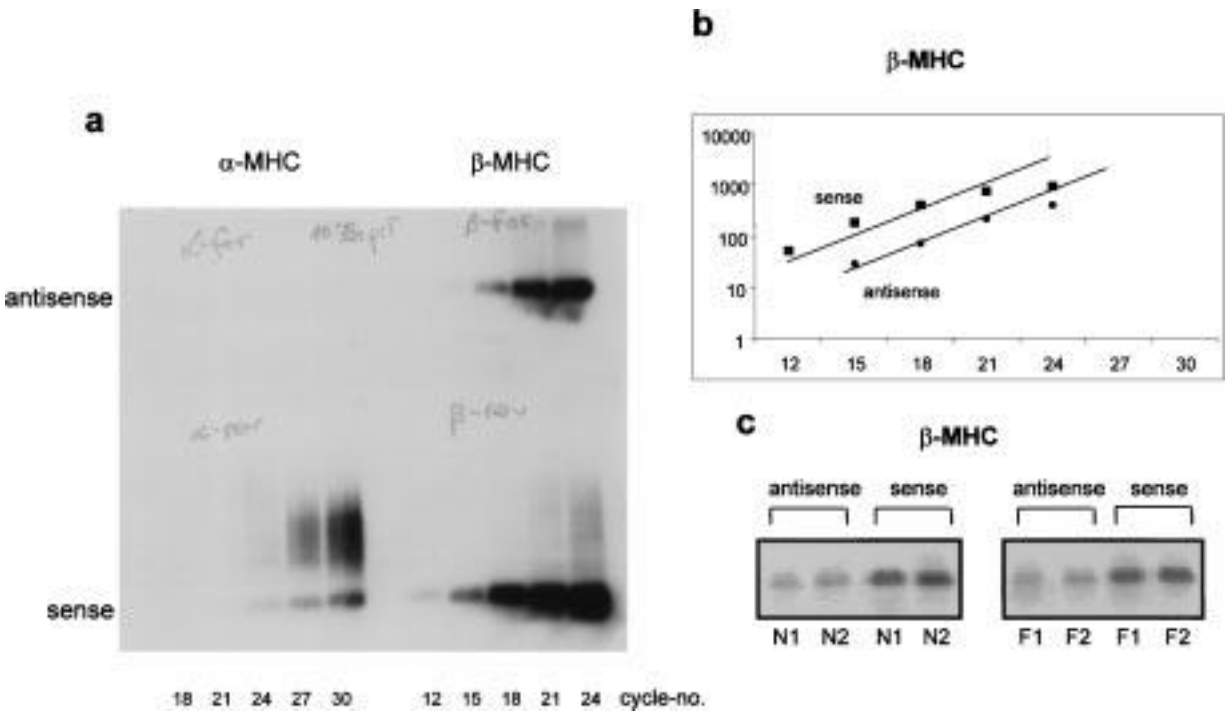


**Fig. 2.** (**A**) Chemiluminescent signals (CSPD, Boehringer, Germany) of sense and antisense RNA for α- and β-MHC after increasing number of PCR cycles. All products (top: antisense products, bottom: sense products) were analyzed on a single gel to minimize differences of detection. Antisense β-MHC signal was detected after 15 cycles, while the sense signal was obtained within 12 cycles. No signal for antisense α-MHC was found even after 30 cycles. (**B**) Signal of the β-MHC PCR products were evaluated after 12, 15, 18, 21, 24, 27, and 30 cycles. The logarithms of the optical density were plotted versus cycle number and analyzed by linear regression. Plots fit well into a linear function. (**C**) β-MHC-PCR-products (sense and antisense) of normal (N) and failing hearts (F) (two examples each) with no significant difference in signal intensities.

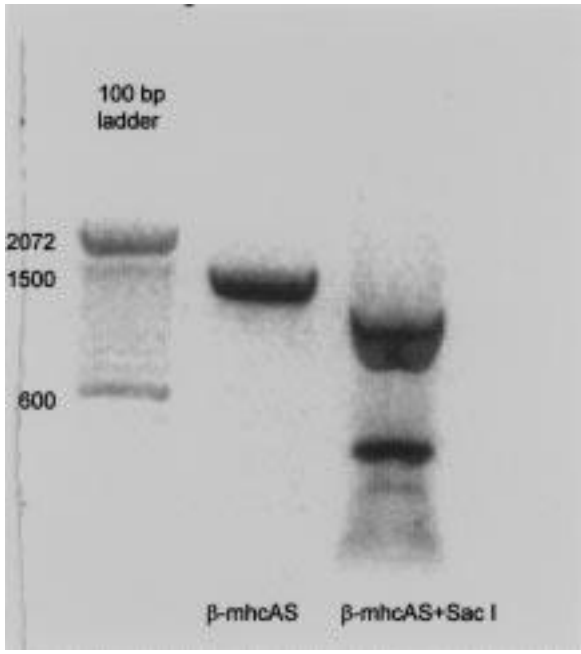


**Fig. 3.** Analysis of long β-MHC antisense transcript. RT-PCR produced an antisense RNA fragment for β-MHC using primers with positions 5979–5998 (reverse) and 4628–4647 (forward) with the expected size of 1371 bp (β-MHCAS). Molecular weight marker is a 100 bp DNA ladder (Gibco-BRL). In the second lane restriction of the PCR product by Sac I produced fragments with the expected size of 1008 and 363 bp (restriction site 5630 GAGCT′ C 5636).

antisense transcripts (product 1 depicted in Fig 1) of β-MHC a minimum of 15 PCR cycles were necessary in human ventricular tissue (Fig. 2). The amount of cDNA products synthesized and number of PCR cycles can be used to estimate the relative abundance of the transcripts providing the rate of amplification of both forms is identical. Using the efficiency factor of 1.7, i.e. the rate of cycle-dependent product generation as derived for the identical products amplified here we calculated that sense transcripts are in about 6.5-fold excess of their antisense counterparts. For α-MHC sense RNA detection a minimum of 24 cycles were necessary meaning about 40-fold less RNA is present in the initial samples [Ritter et al., 1999a,1999b]. No antisense RNA for α-MHC was detected after 30 cycles under these conditions. Results were identical for both normal and failing myocardium (Fig. 2).

Northern hybridization of total RNA from normal and failing human myocardium all showed the same results, successfully detecting sense β-MHC RNA (not shown) with a specific signal of the expected size of around 6000 bp. However, no signal was obtained for antisense β-MHC or sense/antisense α-MHC RNA transcripts, confirming the PCR results above where α-MHC sense RNA seems to be present at relatively low levels.

### In Vitro Transcription/Translation

Potential effects of endogenous antisense transcripts on MHC gene expression in vivo were investigated using an in vitro transcription/translation assay in the presence of oligonucleotides corresponding to two different positions within the antisense transcript (Fig. 1). In each case the effect was compared to that of a mismatched antisense oligonucleotide (scrambled, Table II). An antisense oligonucleotide directed against position 5666–5625 (Table II; Fig. 1) was shown to attenuate specific translation of β-MHC to 75% (±14.8%) of control levels, as determined by autoradiography of a protein PAGE analysis; but this was not significantly different to the effect of the scrambled oligonucleotide (81.3%; ±10.8 of control protein synthesis). Incubation with an antisense oligonucleotide closer to the 5′ end (4647–4628: Table II; Fig 1) revealed a significant and specific effect, reducing translated protein amounts to 69% (±1%) of the untreated control, while the equivalent scrambled oligonucleotide only reduced levels to 90% (±6%) (Fig. 4A). The specific inhibitory effect of the antisense oligonucleotide 4647–4628 was determined by adding different concentrations ranging from 0 to 8 μM to the transcription/translation assay. A maximum effect was determined at 6 μM with no additional effect with increasing concentrations (Fig. 5).

## DISCUSSION

In a previous study, considerable amounts of α- and β-MHC antisense RNA were detected in rat myocardium [Luther et al., 1997, 1998]. An important role in posttranscriptional regulation was hypothesized to explain the striking noncoordinated expression of α-MHC mRNA and protein in the neonatal rat heart [Luther et al., 1998]. The aim of the present study was to address the role of endogenous MHC antisense RNA in human myocardium, and by association, to question whether there is evidence for noncoordinated expression of MHC in human myocardium. Indeed, significantly higher amounts of α-MHC mRNA than reflected at

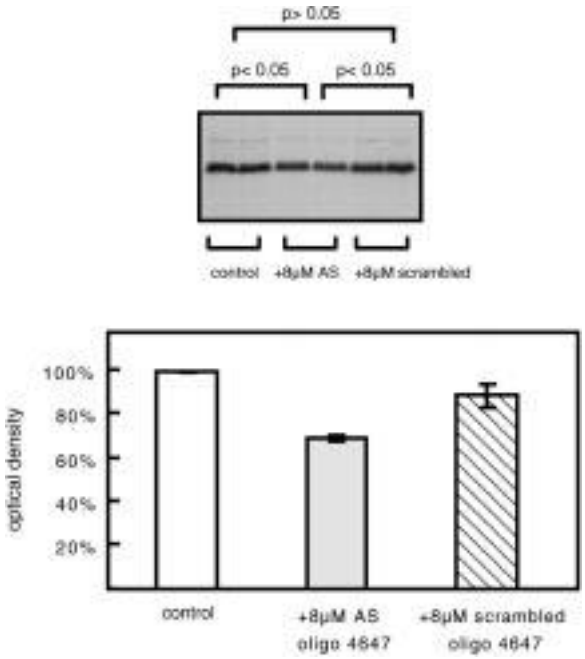


**Fig. 4.** Inhibition of in vitro translation by antisense oligonucleotides. Circular plasmid DNA (pGEM3Z, Promega) with cloned cDNA from human cardiac β-MHC sequences 4626–5998 (Fig. 1) provided the template for the TNT T7 Quick Coupled Transcription/Translation System (Promega) containing [$^{35}$S]-labeled methionine/ cysteine (10 μCi). Products were separated by SDS-PAGE and the radioactive bands were visualized by autoradiography. Top: the effect of an antisense oligonucleotide (AS) directed against position 4647–4628 compared to the control with no added oligonucleotide and a scrambled oligonucleotide on β-MHC translation. Bottom: data from densitometry measurements of the bands show as a bar chart. Each value is based on three experiments. The percentage effect of inhibition refers to the control which was set at 100%. Values are means ± SD one-way ANOVA with Bonferroni Post-hoc test, level of significance indicated.

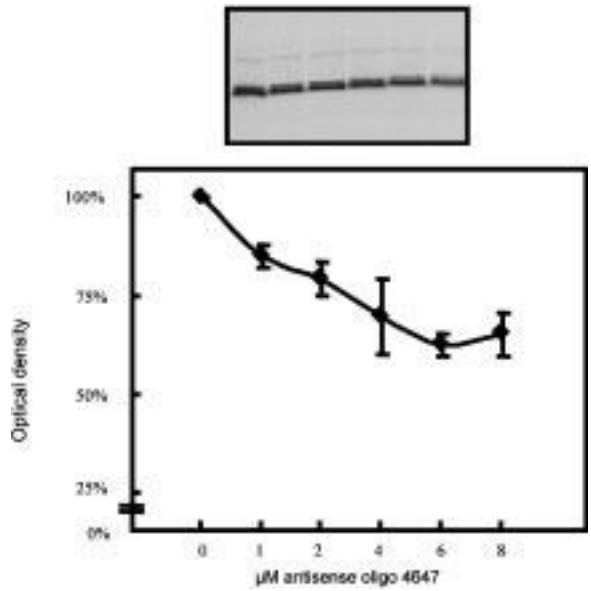


**Fig. 5.** Concentration dependency of antisense oligonucleotide translation inhibition. The specific inhibitory effect of the antisense nucleotide directed against position 4647–4628 of β-MHC on translation of the cloned β-MHC cDNA was measured as in Figure 4. Top: autoradiograph of SDS-PAGE proteins produced in the presence of increasing concentrations of oligonucleoide. Bottom: plot of the densitometry data. Each data point is based on three experiments. Values are means ± SD.

the protein level were recently reported in non-failing myocardium [Miyata, 2000] suggesting posttranslational mechanisms may be involved. However, these data are not substantiated by our previous results and those presented here analyzing MHC-isoform distribution: the sense-transcripts of MHC in non-failing myocardium were found to consist almost exclusively of the β-isoform. We calculated about a 40-fold excess of β-MHC over the sense α-MHC which was in accordance with the inability to detect sense mRNA in Northern blots and α-MHC protein in Western blots [Ritter et al., 1999a].

In view of the controversial data regarding coordination of mRNA and protein levels in human myocardium we performed MHC antisense RNA detection. There was no difference in antisense β-MHC mRNA levels between failing hearts and controls, the ratio of antisense to sense β-MHC mRNA being equal in both sets of samples. To minimize the risk of amplifying sense sequences arising from the sense primer binding in the opposite orientation to a sequence in the sense RNA downstream from its intended hybridization sequence, we initially selected the 3′-untranslated part of the MHC mRNA for RT-PCR analysis. Although, if the primer bound well enough to this "secondary" sequence to produce reverse transcripts, one might expect it to do the same in the subsequent PCR, thus producing a DNA product of an unexpected size, or maybe a mixture of products, neither of which were observed in our experiments. The 3′-end analysis was successful for β-MHC, producing an amplified antisense product identical in size to the reciprocal sense, reverse primer RT-PCR product, but was unsuccessful for α-MHC (Fig. 2). An additional RT-PCR using a sense primer further upstream in the β-MHC coding sequence also successfully amplified antisense sequences displaying an extension of antisense β-MHC mRNA from position 4628 to 5998. These

results indicate the existence of a contiguous antisense transcript of at least 1371 bp. Even though attempts with specific primers directed against the 5′ end of the cDNA failed to generate specific PCR products, a longer extension of the antisense transcript cannot be excluded so far. Possible contamination of RNA with unspliced genomic DNA could be unequivocally excluded since the size of the antisense β-MHC PCR-products generated was identical to the sense PCR-products. Concerning the origin of antisense transcripts at least three mechanisms can be considered: antisense could be transcribed from either the opposite strand of the gene [Tommasi and Pfeifer, 1999] or from a pseudogene [Korneev et al., 1999], or alternatively, derived from transcription of the sense transcript by RNA dependent RNA polymerase in the cytoplasm. This was suggested for antisense globin RNA and the M40 subunit of the insect V-ATPase [Volloch et al., 1996; Merzendorfer et al., 1997], since—identical to our finding—antisense transcripts are complementary to the spliced sense RNA.

We report for the first time the existence of a polyadenylated antisense β-MHC transcript in tissue of the human heart, since identical amounts of amplified product were detected in RNA samples purified with oligo(dT)-cellulose compared to total RNA preparations (not shown). We assume that the poly (A) tail is probably localized at the 3′ end of the antisense transcript, which was shown by Merzendorfer et al. for the antisense M40 subunit of the insect V-ATPase [Merzendorfer et al., 1997]. Furthermore, there is no evidence that modification of antisense transcipts is different from those of sense transcripts, which are exclusively polyadenylated at the 3′ end. In vivo polyadenylation is associated with transport of RNA transcripts from the nucleus to the cytoplasm as well as increased stability [Bernstein and Ross, 1989]. However, in a study by Bechler [1997] in vitro transcribed antisense RNA was shown to have no additional effects on translation if it is polyadenylated, although there were two-fold differences if it was microinjected into the cytoplasm compared to the nucleus—thus transport and stability probably both play a role in endogenous systems.

The amount of antisense mRNA for β-MHC was quite low, as demonstrated by semiquantitative evaluation using labeled nucleotides. The failure of Northern hybridization to detect a signal for antisense RNA is likely due to the low amounts, since sense α-MHC RNA was also not detectable under these conditions. Another explanation would be reduced hybridization capability of antisense transcript. It is commonly believed that most duplexes will become degraded by RNAses either specific for dsRNA or specific for single stranded RNA but activated by dsRNA [Vanhée-Brossollet and Vaquero, 1998]. Since at least a partial duplex-formation between sense and antisense transcripts can be assumed modification of double-stranded RNA is conceivable. By changing adenosine to inosine [Bass and Weintraub, 1987], which behaves as guanosine, the RNA sequence is changed and hybridization may be affected.

These results are distinct from those obtained in rat hearts, which revealed considerable amounts of both α- and β-MHC mRNA antisense transcripts with RT-PCR and detectable signals by Northern blotting [Luther et al., 1997, 1998]. However, they confirm our RT-PCR results showing considerably less α-MHC than β-MHC sense mRNA in human ventricular myocardium.

In an attempt to study the potential role of antisense RNA for MHC regulation in vivo, the specific effect of oligonucleotides selected with reference to the endogenous antisense transcript was analyzed by an in vitro translation assay. These experiments detected a significant and specific attenuation of translation by one of the sequences selected which, in addition, was shown to be concentration-dependent. The antisense oligonucleotide against the 5′ end of the cloned β-MHC exerted a better inhibitory effect than the antisense against the 3′ end of the coding sequence suggesting that binding of the translation machinery was more effectively prevented by oligo 1. Similar results were obtained by Chen et al. screening the potential of different antisense oligonucleotids for inhibition delta antigen synthesis in vitro [Chen et al., 1997].

The proof of a specific effect is important, since it was shown that antisense oligonucleotides cannot always modify translation, even when present in excess [Crooke and Bennett, 1996]. The analogous investigation involving the respective scrambled oligonucleotide, which resulted in considerably less reduction of translation, is a crucial control since a range of nonspecific effects of antisense oligonucleotides

have been described [Branch, 1996]. The demonstrated concentration dependence of the attenuating effect on translation by antisense nucleotides suggests that also in vivo a particular transcription level of endogenous antisense RNA is probably necessary to affect protein expression. Therefore, an accurate quantification of the sense/antisense ratio is necessary to address any questions concerning the potency of expressed antisense RNA in vivo. Here, we determined a ratio of about 6.5 sense to antisense β-MHC transcripts, based on the number of PCR cycles required to obtain detectable products and previous calculations determining the amplification rate of these fragments [Luther et al., 1997; Ritter et al., 1999b]. In rat heart the ratios of antisense transcripts were appreciably higher, with both α- and β-MHC sense mRNA only about two-fold more abundant than their antisense counterparts [Luther et al., 1998]. The high abundance of naturally occurring antisense RNA in the neonatal rat heart implicates it as playing a role in the disproportionate, noncoordinated expression of α-MHC mRNA and protein in the growing heart [Luther et al., 1998]. In a recent study antisense RNA for the human atrial essential myosin light chain (ALC-1) gene was detected in hypertrophied myocardium [Ritter et al., 1999a]. The higher the ALC-1 RNA antisense/sense ratio, the lower the ALC-1 protein expression, suggesting that ALC-1 translation is disturbed by high levels of antisense RNA expression in the hypertrophied, but not in the normal, ventricle. Since we did not find noncoordinated MHC RNA and protein expression patterns in normal and failing human myocardium a major role for intrinsic antisense transcripts in posttranscriptional regulation seems to be unlikely under these conditions.

Conversely, our results finding relatively low amounts of antisense β-MHC and the absence of antisense α-MHC transcripts are not incompatible with such coordinated MHC expression, since they do not exclude the possibility that under different circumstances elevated antisense expression may contribute to posttranslational regulation.

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