Regulation of Myosin Synthesis by Thyroid Hormone: Relative Change in the α- and β-Myosin Heavy Chain mRNA Levels in Rabbit Heart†

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ABSTRACT: The expression of mRNAs for two cardiac myosins has been examined in the ventricles of hypo- and hyperthyroid rabbits by means of cloned cDNA sequences corresponding to the mRNAs of the α- and β-myosin heavy chains (HCs). The temporal change in the relative levels of the α- and β-HC mRNAs after 3,5,3′-triiodothyronine (T_3) treatment of hypothyroid rabbits was determined by nuclease S1 mapping. In the hypothyroid state, only HCβ-mRNA was expressed in the ventricles. The HCα-mRNA was first detectable 4 h after administration of T_3 (200 μg/kg) to hypothyroid animals. By 12 h, HCα-mRNA represented 20% of total myosin mRNA, increasing to 50% by 24 h and to about 90% by 72 h. The relationship between the relative mRNA levels and relative synthesis rates of the myosin HCs was evaluated in 5–6-week-old normal and thyrotoxic rabbits. Myosin synthesis rates were determined by labeling of protein in vivo with [^{3}H]leucine. The V1 (HCα) and V3 (HCβ) isomyosins were separated by affinity chromatography with monoclonal antibodies, and the HCs were isolated electrophoretically. In a normal euthyroid group of animals and in animals 12 and 24 h after administration of 200 μg of 3,5,3′,5′-tetraiodothyronine/kg, the relative mRNA levels and relative synthesis rates of the α- and β-HCs were not significantly different. Our results show that, first, thyroid hormone causes a rapid accumulation of HCα-mRNA and loss of HCβ-mRNA and, second, in normal and thyrotoxic rabbits, the relative synthesis rates of HCα and HCβ reflect the relative abundance of the α- and β-HC mRNAs. These data strongly suggest that thyroid hormone regulation of cardiac myosin synthesis occurs pretranslationally.

Two classes of myosin heavy chains (HCs),[1] referred to as HCα and HCβ, are expressed in the rabbit ventricle (Hoh et al., 1979). Both HCs are found during late gestation, the α-HC always being more abundant (Lompre et al., 1981; Chizzonite et al., 1982; Everett et al., 1983a). After birth, the ratio of these HCs changes, with the relative amount of HCα increasing at first and then declining again until in old rabbits HCβ becomes the predominant species. The developmental sequence of myosin HCs appearing in the heart may be modified by a variety of stimuli (Zak & Galhotra, 1983), thyroid hormone being particularly effective in this regard. Its administration results in rapid accumulation of HCα and elimination of HCβ (Hoh et al., 1978; Chizzonite et al., 1982; Martin et al., 1982). This redistribution of HCs has been shown to result from an increase in the synthesis rate of HCα and a decrease in that of HCβ (Everett et al., 1983a).

In this study, we have further explored the action of thyroid hormone on myosin HC expression in the myocardium. The relative levels of the cardiac α- and β-HC mRNAs were determined in euthyroid and thyrotoxic rabbits, and they were compared with the relative synthesis rates of the respective HCs. We analyzed myosin HC mRNA levels by nuclease S1 mapping, using a pair of cloned cDNA sequences corresponding to a divergent region of the mRNAs (Sinha et al., 1982). Our results show that thyroid hormone administration results in a dramatic change in the expression of myosin HC mRNA in the ventricles from the β to the α type and that the relative synthesis rates of HCα and HCβ correlate highly with the relative mRNA levels for these proteins in euthyroid and thyrotoxic rabbits.

Experimental Procedures

Treatment of Animals. The temporal change in myosin mRNA levels after T_3 treatment was determined in adult male New Zealand white rabbits fed a diet containing 0.15% propylthiouracil (Teklad Diets, Madison, WI) for between 8 and 11 weeks. The rabbits consumed an average of 86 ± 6 (±SE, n = 9) g of food/day and thus received, on the average, 129 mg of propylthiouracil/day. The average body weight remained constant at 4.1 ± 0.1 (±SE, n = 9) kg while the animals were on the diet. The total serum T_3 concentration fell from 1.6 ± 0.2 (±SE, n = 6) to 0.9 ± 0.1 ng/mL 8 weeks after the beginning of the propylthiouracil diet. The total serum T_4 concentration fell from 4.2 ± 0.4 to 1.9 ± 0.1 g/100 mL over the same period. No further decline in either T_3 or T_4 levels was observed after 11 weeks on the diet. Serum thyroid hormone concentrations were measured with a standard clinical RIA kit (Clinical Assays, Cambridge, MA).

The relationship between the relative mRNA levels and relative synthesis rates of the myosin HCs was determined in 5–6-week-old New Zealand white rabbits maintained on a normal laboratory diet. Some animals were given thyroxine (200 μg kg^{-1} day^{-1} intramuscularly as a fine precipitate in pH 7.0 phosphate-buffered saline. At sacrifice, a sample from the apex of the heart was taken for native gel electrophoresis of myosin (Clark et al., 1982). Each heart was then cut in half by a transverse section through the aortic trunk and both ventricles. The atria were discarded. Half of each heart was used for RNA extraction (Towle et al., 1980) and the other

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[1] Abbreviations: T_3, 3,5,3′-triiodothyronine; T_4, 3,5,3′,5′-tetraiodothyronine; HC, heavy chain.

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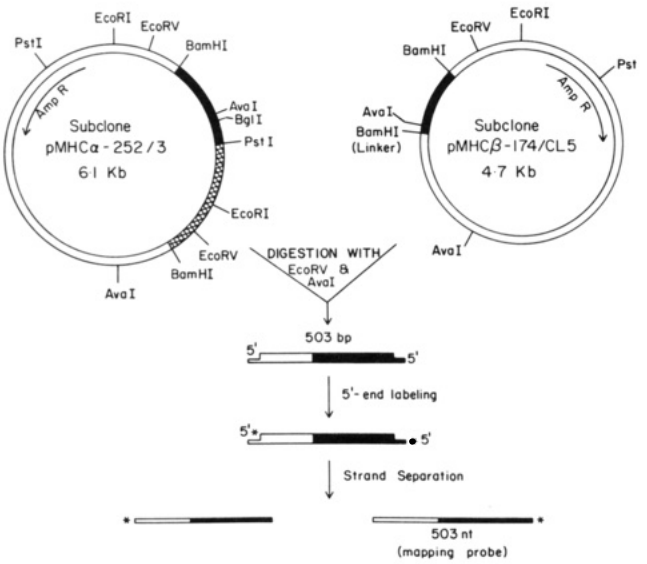

FIGURE 1: Preparation of nuclease S1 mapping probes. Subclone pMHCα-252/3 was constructed by insertion of a 1.8 kilobase pair *Bam*HI restriction fragment from pMHCα-252 (Sinha et al., 1982) into the *Bam*HI site of pBR322. This fragment extends from the unique *Bam*HI site of the vector pBR322 to the middle *Bam*HI site of the insert of pMHCα-252. The shaded segment represents myosin-coding sequences, and the cross-hatched segment represents plasmid sequences from the original clone. The plasmid was digested with *Eco*RV and *Ava*I to yield the 503-nucleotide α-cDNA probe. For construction of subclone pMHCβ-174/CL5, a 3.75 kilobase *Bgl*I fragment was first isolated from pMHCβ-174 (Sinha et al., 1982). This fragment extends from the *Bgl*I site at position 928 in pBR322 to the unique *Bgl*I site of the insert of pMHCβ-174. The *Bgl*I sites were made blunt ended with T4 DNA polymerase, and a *Bam*HI synthetic linker was added with T_4 ligase. The preparation was digested with *Bam*HI, and a 350 base pair BamHI fragment was isolated and inserted into the *Bam*HI site of pBR322 for cloning. This plasmid was also digested with *Eco*RV and *Ava*I to yield the 503-nucleotide β probe.

for myosin isolation (Everett et al., 1983).

Determination of the Protein Synthesis Rate. The fractional synthesis rates of HCα and HCβ were determined by [^{3}H]-leucine incorporation as has been previously described (Everett et al., 1983a).

Total myosin synthesis rates, R_s (Zak et al., 1979), in each heart (milligrams of HC synthesized per gram of heart per day) were also calculated from the product of the fractional synthesis rate (day^{-1}) and the pool size (milligrams per gram of heart) of each HC. The total pool of myosin heavy chain in the ventricles averaged 22 mg/g of tissue, determined by an isotope dilution protocol previously described (Everett et al., 1983b). The relative contribution of HCα and HCβ to this total pool was determined by the relative ventricular content of isomyosins V1 (HCα) and V3 (HCβ). The isomyosins were separated by native gel electrophoresis, and their relative amounts were determined by densitometric scanning of the Coomassie blue stained gels (Everett et al., 1983a).

Preparation of Nuclease S1 Mapping Probes. Two cardiac myosin HC cDNA clones, pMHCα-252 and pMHCβ-174 (Sinha et al., 1982), were subcloned to obtain two 503-nucleotide cDNA probes consisting of 313 myosin coding sequences (from nucleotides 6 to 318 in the above reference) and 190 plasmid sequences (see Figure 1). These additional plasmid sequences allowed us to distinguish between protection of the probe sequences hybridized with mRNA and incomplete digestion of the probe itself.

The 503 base pair *Eco*RV/*Ava*I restriction fragments from subclones pMHCα-252/3 and pMHCβ-174/CL5 (Figure 1) were 5′ end labeled with [γ-^{32}P]ATP (5000 Ci/mmol, Amersham) and T4 polynucleotide kinase (P-L Biochemicals), and the strands were separated (Maxam & Gilbert, 1980). The more slowly migrating band containing sequences complementary to myosin heavy chain mRNA was eluted and ethanol precipitated. The specific radioactivity of each probe was about 1×10^7 cpm/μg.

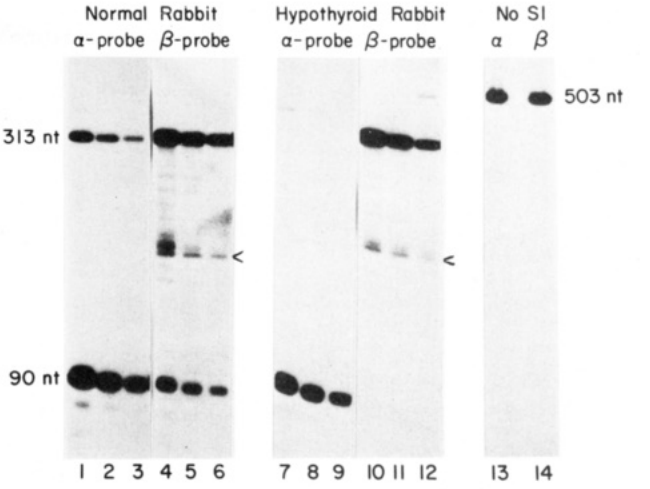

FIGURE 2: Nuclease S1 mapping of cardiac muscle RNAs with the α- and β-cDNA probes. Mapping was conducted with a range of nuclease S1 concentrations (2000, 3000, and 4000 units) as described under Experimental Procedures. The products were denatured and fractionated on a 5% polyacrylamide/urea sequencing gel. Shown are autoradiographic results for gels after hybridization of both probes with RNA from normal and hypothyroid (propylthiouracil treated) rabbits (see Experimental Procedures). The lanes marked "No S1" show the mobility of the undigested probes. The arrows indicate an intermediate-sized DNA fragment with a mobility of about 160 nucleotides.

Nuclease S1 Mapping. Between 15 and 20 ng (1.5×10^5 cpm) of each cDNA probe was hybridized at 68 °C for 18 h with 50 μg of total ventricular RNA. The amount of cDNA was in excess of myosin mRNA. Digestion was carried out with a range of nuclease D1 (Boehringer/Mannheim) concentrations (2000–4000 units/155-μL reaction volume) at 37 °C for 4 h (Orkin & Goff, 1981). The digested products were separated on a 5% polyacrylamide sequencing gel (Orkin & Goff, 1981), and their positions were located by autoradiography. Sections of the gel containing the labeled cDNAs were excised, and the radioactivity was measured by liquid scintillation counting.

Results

Analysis of Myosin mRNAs by Nuclease S1 Mapping. We examined the cardiac myosin mRNA composition by nuclease S1 mapping using 5′-labeled single-stranded cDNA probes encoding mRNA sequences for the α- and β-myosin HCs. The probes were 503 nucleotides in length; 313 of the nucleotides were myosin coding sequences, and the remaining 190 were plasmid sequences at the 3′ end (see Figure 1). Both probes were complementary to the same region of the α- and β-mRNAs (Sinha et al., 1982), and the sequences were identical from the 5′ end to the 90th nucleotide, where a tetranucleotide mismatch occurs.

Results of nuclease S1 protection experiments with RNA from normal and propylthiouracil-treated (hypothyroid) adult rabbits are shown in Figure 2. Each RNA sample was hybridized with each probe and then treated with increasing concentrations of S1 to ensure that digestion was complete and that the ratio of radioactivity determined in the 313- and 90-nucleotide fragments was constant. In a rabbit fed a normal diet, both the α- and β-mRNAs were expressed, with

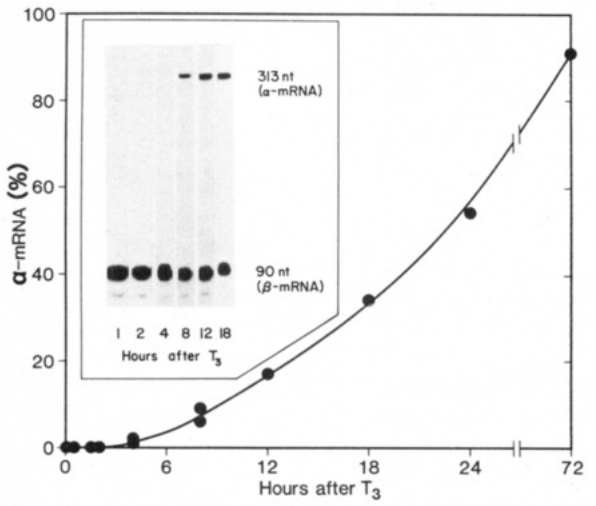

FIGURE 3: Temporal change in the relative amounts of the α- and β-myosin mRNAs in the ventricles of hypothyroid rabbits injected with T_3. The relative amounts of the two mRNAs were determined from the radioactivity in the 313- and 90-nucleotide DNA fragments after nuclease S1 mapping with both cDNA probes as described under Experimental Procedures. The amount of HCα-mRNA is expressed as the percent of total myosin mRNA (HCα + HCβ). The results with both probes were similar and were therefore averaged for calculation of relative mRNA levels. The inset shows autoradiographic results after S1 mapping with the α probe at the highest nuclease S1 concentration (4000 units) employed.

the α type making up 13% of the total myosin mRNA (lanes 1–6, Figure 2). The presence of a small amount of α-mRNA is indicated by the band at 313 nucleotides in lanes 1–3 when the α probe was used, and at 90 nucleotides (lanes 4–6) when the β probe was used. Similar results have been obtained with other normal adult rabbits (Sinha et al., 1982). In a propylthiouracil-treated rabbit, only the β-mRNA was expressed, since only a single band at 90 nucleotides (lanes 7–9) and at 313 nucleotides (lanes 10–12) was seen after S1 mapping with the α and β probes, respectively. Lanes 13 and 14 represent the full-length probes (503 nucleotides) which were not treated with nuclease S1.

At low S1 concentration, a number of intermediate-sized bands were seen after hybridization of the β probe with RNA from normal and hypothyroid rabbits. These bands do not represent incomplete S1 digestion of hybrids formed with α-mRNA, because they are present in hypothyroid rabbits which contain only the β-mRNA. The minor band at about 165 nucleotides (arrows, Figure 2) persists at high concentrations of S1. In contrast, no intermediate-sized bands are observed with the α probe at any concentration of S1 employed. The band indicated by the arrows in Figure 2 suggests the possibility of an additional myosin HC-mRNA sequence present in low abundance that is partly homologous to the β probe. Alternately, the band may be generated by selective S1 nuclease digestion of the HCβ-mRNA/cDNA hybrid at an AT-rich region. We also observed that the amount of radioactivity in the 313-nucleotide band when the β probe was used was less than that expected on the basis of the results with the α probe. Consequently, a small, but significant, difference in the relative amounts of the α- and β-mRNAs was determined with the two probes. For example, in a group of seven young rabbits that had similar amounts of the mRNAs, the amount of β-mRNA averaged 10 ± 2 (±SE) percentage points less ($p < 0.001$, paired t test) with the β probe than with the α probe. The finding may be accounted for by the existence of a low abundance β-type mRNA that is not entirely homologous with the β probe, thus reducing the amount of radioactivity in the 313-nucleotide band.

Table I: Relationship between Synthesis Rates and mRNA Levels of α- and β-Myosin Heavy Chains[a]

	R_s (mg g^{-1} day^{-1})			relative mRNA level
	HCα	HCβ	HCα/(HCα + HCβ)	HCα/(HCα + HCβ)
untreated				
rabbit 1	1.1	1.0	0.52	0.55
rabbit 2	3.6	0.9	0.80	0.69
rabbit 3	3.5	2.0	0.64	0.70
			0.65 ± 0.08[b]	0.65 ± 0.05[b]
0.5-day T_4				
rabbit 4	2.0	1.5	0.57	0.58
rabbit 5	2.4	1.7	0.59	0.60
rabbit 6	2.1	2.0	0.50	0.44
rabbit 7	1.9	2.5	0.43	0.43
			0.52 ± 0.04[b]	0.51 ± 0.05[b]
1-day T_4				
rabbit 8	5.5	0.4	0.93	0.88
rabbit 9	6.8	0.6	0.92	0.87
rabbit 10	5.1	0.7	0.88	0.91
			0.91 ± 0.01[b]	0.89 ± 0.01[b]

[a] The synthesis rates (R_s values) are a product of the fractional synthesis rate (day^{-1}) and the pool size (milligrams per gram of heart) of each HC. The fractional synthesis rates were determined by the specific radioactivities of leucine in the heavy chains and precursor (plasma) after a 1-h infusion of [^{3}H]leucine. The pool sizes of HCα and HCβ were determined indirectly from the percent V1 (or V3) × 22 mg, where 22 mg is the average total myosin HC content per gram wet weight of ventricle. The relative mRNA levels for HCα and HCβ were determined as described in the legend to Figure 3. Rabbits received a single injection of thyroxine 12 or 24 h prior to [^{3}H]leucine infusion. Average total serum thyroxine levels were 4 times the normal range of 3–6 μg/100 mL at 12 h after thyroxine treatment. [b] Mean ± SE.

Effect of T_3 on Myosin HC mRNA. The change in the relative amounts of the α- and β-HC mRNAs after T_3 administration to propylthiouracil-treated rabbits is illustrated in Figure 3, which shows results derived only with the highest nuclease S1 concentration. After 1–2 h of hormone treatment, only the β-type myosin mRNA was detected (band at 90 nucleotides, inset of Figure 3), as was observed with the propylthiouracil-treated rabbit shown in Figure 2. The α-mRNA was first seen after 4 h of T_3 treatment (band at 313 nucleotides) and thereafter showed an increased abundance with time (inset, Figure 3). A more precise measure of the relative changes in the α- and β-mRNA levels was determined by the measurement of the relative radioactivity in the 313- and 90-nucleotide DNA fragments by liquid scintillation counting. The amount of α-mRNA first seen at 4 h represented 1–2% of the total mRNA. The proportion of α-mRNA increased to 20% by 12 h, 50% by 24 h, and about 90% by 72 h (Figure 3). The high specific radioactivity of the probes made it possible for 1% of either mRNA to be readily determined.

Although no α-mRNA was detectable for 4 h, the total serum T_3 concentration was twice the normal range of 1–2 ng/mL at 1 h, and it continued to rise to about 25 times the normal level by 24 h after administration of T_3. The total serum T_4 concentration was unchanged after T_3 was given.

Relationship between Protein Synthesis Rate and mRNA Levels. The relative HC mRNA levels were compared with myosin synthesis rates (R_s). The unit for R_s is milligrams of HC synthesized per gram of heart per day and represents synthesis normalized for the size of HCs pool.

In each untreated rabbit, the R_s of the HCs varied, in one animal (number 3) by a factor of 3 (Table I). Despite this

difference, however, the relative R_s and relative mRNA levels for the HCs in the untreated group were not significantly different ($p < 0.05$, t test). Similarly, in the 0.5-day T_4 group, the relative R_s and mRNA levels were almost identical. In these animals, no significant effect of thyroid hormone on HC synthesis was observed. One day after T_4 treatment, the R_s of HC averaged about twice that of normal animals, whereas the R_s dropped to only 10% of all myosin synthesized per unit mass of tissue. A correlation coefficient (r^2) of 0.92 was obtained between the relative R_s and relative mRNA levels of the HCs of all 10 rabbits (last two columns, Table I).

Discussion

We have studied the change in the expression of myosin mRNA in ventricles of normal and hypo- and hyperthyroid rabbits. The mRNA was analyzed by nuclease S1 mapping with cloned cDNA probes coding for α- and β-myosin HC mRNAs.

To determine the time for appearance of the α-type mRNA after administration of T_3, the normal expression of α-mRNA was suppressed by treatment of adult rabbits with a diet containing propylthiouracil. Although this diet produced only a modest decrease (40–50%) in the total serum T_3 (and T_4) concentration, it nevertheless reduced the α-mRNA to undetectable levels (<1% of total HC mRNA). After a single injection of T_3 (200 μg/kg), a short lag period (<4 h) was observed before the α-mRNA was first detectable. The α-mRNA then continued to accumulate in the ventricles, reaching 90% of the total myosin mRNA by 3 days. Although the procedure employed could only evaluate relative changes in the amounts of the α- and β-myosin mRNAs, it is likely that T_3 resulted in both an absolute increase in α-mRNA content and a decrease in β-mRNA content in the cells. Evidence for this conclusion is provided by the increase in the synthesis rate of HCα and the concomitant decrease in that of HCβ after administration of the hormone.

Other cardiac myosin mRNA transcripts that are homologous to either of the two cDNA sequences would not be discriminated in these experiments and would be included in the two major classes, α and β. Our nuclease S1 mapping with the α-cDNA probe provides no evidence for the existence of a second α-type mRNA. On the other hand, with the β probe, an intermediate-sized band at about 165 nucleotides was found to persist after digestion with high concentrations of nuclease S1 (Figure 2). The band could not represent a partial digest of the probe hybridized with the α-mRNA because it was present in hypothyroid rabbits, which express only the β-mRNA.

This band could either correspond to a second type of mRNA or be a result of selective cleavage by S1 nuclease of the mRNA/cDNA hybrid at the region high in A and T. The first possibility is supported by microheterogeneity in the amino acid sequence of peptides derived from bovine ventricular myosin (Flink & Morkin, 1979).

In order to elucidate the control of myosin HC expression, we have compared, as a first step, the levels of mRNAs with the synthesis rates of corresponding HCs. Our data have shown that the relative amounts of synthesized HCα and HCβ correlate highly with the relative concentration of their respective mRNAs, in both euthyroid and thyrotoxic rabbits. These data indicate that the regulation of cardiac myosin synthesis occurs at the pretranslational level. This conclusion is consistent with the data from Hoh & Egerton (1979), who reported that the increased incorporation of labeled amino acids into isomyosin V1 in vitro, observed after administration of T_3 to rats, could be prevented by pretreatment with actinomycin D. Whether the action of the hormone can be attributed to a changed rate of transcription or to a change in mRNA processing still remains to be elucidated.

Registry No. T_3, 6893-02-3; T_4, 51-48-9.

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