Regulation of Myosin Heavy Chain Genes in the Heart

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Myosin acts as the chemical-mechanical transducer of motion in heart and all forms of striated muscle by converting energy from the hydrolysis of ATP into the sliding of myofilaments. Each hexameric myosin molecule contains two myosin heavy chains (MHCs) and four myosin light chains (MLCs). For many years, distinctive myosin isoforms were recognized in skeletal muscle, e.g., slow-twitch (red) and fast-twitch (white) fibers, but cardiac muscle was considered to have only a single myosin type until isoform shifts were observed with alterations in thyroid status (reviewed in Reference 2). Under the nondenaturing electrophoretic conditions described by Hoh et al., myosin from ventricular muscle was found to contain three isoforms, V1, V2, and V3, in order of decreasing electrophoretic mobility and ATPase activity. These isoforms differ only in their MHC composition. The V1 form is composed of two α-MHCs, V2 has two β-MHCs, and V3 has one MHC of each type. Atrial muscle contains a different myosin isoform (V4n) composed of two α-MHCs and a separate set of MLCs. Although the ATPase activity of myosin is closely associated with the MHC component of the "head" region, the associated MLCs may be capable of modifying this activity.

A tremendous increase in our knowledge of the structure and function of cardiac myosin was brought about by the cloning and sequencing of the α- and β-MHC genes, including the complete cDNA sequences from human and rat. Introduction of chimeric plasmids containing 5' flanking sequences fused to the chloramphenicol acetyltransferase (CAT) gene made possible the study of cardiac MHC gene regulation in cultured heart cells. (The CAT gene is found only in bacterial cells; hence, its expression is a unique marker of the activity of the foreign plasmid.) Also, the long controversy regarding a possible etiological role for a defective form of myosin in heart failure has been put into new perspective by reports linking some cases of familial hypertrophic cardiomyopathy directly to missense mutations of the β-MHC gene.

In view of this new information, it would seem timely to review our current understanding of how the myosin isoform content of the heart is controlled. Because ventricular myosin isoforms contain the same complement of MLCs, the major questions to be addressed are related to control of MHC expression. How are the MHC genes initially switched on? What is the molecular basis for their control? Are the human cardiac MHC genes regulated by the same hormonal and physiological factors that have been identified in animal models? How is the expression of MHCs in the heart coordinated with that of other contractile proteins? More importantly, can insights gained from these basic molecular studies be used to design new strategies for the treatment of heart disease?

Study of cardiac MHC isoforms has assumed a special importance because changes in the proportion of these proteins have been found to be directly related to the level of mechanical performance in animal models, the high-ATPase activity V1 type being associated with faster speeds of contraction. Comparison of complete cDNA sequences for the rat α- and β-MHCs reveals that only a very small number of nonidentical amino acids (131 of 1,938) are able to account for the characteristic differences in ATPase activity and other biochemical properties.

Mammalian cardiac MHCs are encoded on closely homologous genes that are located on a separate chromosomal locus from the skeletal muscle MHCs. In human genome, the α- and β-genes are arranged in tandem on chromosome 14q12, separated by 4.5 kb pairs of intergenic sequence. Each of the genes contain 43 exons distributed over a distance of nearly 22 kb pairs. A similar arrangement of the cardiac MHC genes has been found in mouse, rat, rabbit, hamster, and other mammals, suggesting that they may have arisen from a transposition event followed by a gene duplication before the divergence of these species 70 million years ago.

Because the sequences 5' upstream of the transcription initiation site frequently contain important regulatory elements, analysis of sequences in this region can provide important clues about their control. In the case of the α- and β-MHC genes, the 5' flanking sequences show little resemblance, which is consistent with physiological data suggesting they are controlled differently. By contrast, these regions are highly conserved between species. Thus, rat and human cardiac MHC genes show remarkable homology for several hundred base pairs upstream from the transcription initiation site, suggesting they may be under similar control.
**MHC Genes in Development**

Between 7.5 and 8 days after fertilization in the mouse, the newly formed cardiac tube begins to express mRNAs encoding α-MHC, β-MHC, and atrial and ventricular MLC-1 together with skeletal and cardiac α-actins.\(^1\)\(^5\)\(^6\) As a distinct ventricular chamber forms, β-MHC mRNA becomes restricted to ventricular myocytes. This process is complete by 10 days. During this time, α-MHC levels decrease in ventricular muscle cells but continue to be expressed at high levels in atrial myocytes. α-MHC transcripts progressively decrease in ventricular myocytes until shortly after birth, when they increase rapidly, almost completely replacing β-MHC mRNAs by 7 days after birth. This developmental transition is most likely triggered by the surge in circulating levels of thyroid hormone that occurs shortly after birth.\(^1\)\(^9\) In humans, however, the V\(_3\) (ββ) isoform is predominantly expressed in the ventricle throughout life from fetus to adult, whereas the V\(_1\) (αα) form is expressed in the atrium.\(^1\)\(^8\)\(^9\)

The MHC genes are thought to be turned on initially as part of the genetic program responsible for myogenesis. In skeletal muscles, proteins encoded by four different but related genes, MyoD, myogenin, myf-5, and MRF4/Herculin/myf6, are known to be important. Each of these genes is sufficient to activate the myogenic program in skeletal muscle progenitor cells,\(^2\)\(^0\) but transcripts from these or homologous genes have not been detected in the heart. Forced expression of MyoD in the developing hearts of transgenic mice is associated with abnormal cardiac morphology and ultimately leads to death. At the molecular level, both cardiac-specific and skeletal muscle-specific genes are activated in affected hearts,\(^2\)\(^1\) suggesting that the myogenic programs in heart and skeletal muscle are able to exert their influences independently.

This evidence suggests that a separate “cardiac” regulatory system is responsible for myogenic development in the heart, but the key regulatory proteins involved are as yet unknown. Recently, activity from an α-MHC/CAT reporter plasmid has been found to be induced by cotransfection of egr-1, a gene that is transiently induced by a number of growth stimuli.\(^2\)\(^2\) Furthermore, transfection of cardiomyocytes with a plasmid expressing egr-1 stimulated endogenous α-MHC gene expression. These results indicate that the α-MHC gene is a target for the egr-1 gene product, but the relevance of this observation to normal development remains to be defined.

**Regulatory Factors**

A search that was begun more than 20 years ago for the mechanism responsible for stimulation of cardiac myosin ATPase by thyroid hormone led to the discovery that the hormone induces the high-activity cardiac MHC isoform (V\(_1\)) and suppresses expression of the low-activity (V\(_3\)) form in ventricular myocardium of rabbit and rat.\(^2\) The results of nuclear run-on experiments (an assay that measures the rate at which transcription of a particular gene is initiated) indicate that thyroid hormone control of cardiac MHC gene expression is exerted at the pretranslational level.\(^2\)\(^3\) Although thyroid hormone is clearly a major determinant of cardiac myosin isoform expression, several other factors have been identified that may play a modulatory role. The best-characterized of these factors is pressure-volume overload, which in rat models promotes β-MHC expression and decreases α-MHC mRNA levels.\(^2\)\(^4\) Imamura et al\(^2\)\(^5\) have shown that this switch is not associated with decreased serum thyroid hormone levels; nevertheless, the MHC pattern can be returned toward normal by doses of thyroxine (T\(_4\)) three to six times the physiological requirement.\(^2\)\(^6\)

Cardiac myosin isoenzyme patterns in the rat have been reported to change in response to gonadectomy in male and female rats.\(^2\)\(^7\) Streptozotocin-induced diabetes causes downregulation of the MHC isoforms that can be reversed either by insulin replacement or by treatment with thyroid hormone.\(^2\)\(^8\) Thyroid hormone also is able to reverse the downregulation that occurs in senescence.\(^2\)\(^9\) Upregulation has been reported in response to exercise and α1-adrenergic stimulation.\(^3\)\(^0\) Administration of a high-fructose diet by gavage increases production of α-MHCs as well as the expression of a number of other thyroid hormone–inducible enzymes and proteins.\(^3\)\(^1\) Recently, it has been shown that endothelin-1 binds to receptors on cardiomyocytes, causes hypertrophy, and increases the expression of both α- and β-MHC genes.\(^3\)\(^2\)

Regulation of MHC expression in rabbit ventricle, which contains a predominance (>90%) of β-MHCs, provides an interesting contrast to studies in rat heart. Pressure overload increases β-MHC synthesis, lowering α-MHC content by dilution rather than by inhibition of synthesis.\(^3\)\(^3\) On the other hand, as in the rat, treatment with T\(_4\) switches rabbit ventricular myosin from the V\(_3\)(ββ) to the V\(_1\)(αα) isoform.\(^3\)\(^4\) Thyroid hormone has been shown to regulate MHC expression in cardiac myocytes maintained in defined medium, ruling out the requirement for other hormones or regulating factors as intermediates.\(^3\)\(^5\)\(^3\)\(^6\) Efforts to demonstrate a role for catecholamines in the control of MHC expression in cultured cells have produced conflicting results. Norepinephrine was found to increase total MHC production in cultured fetal rat heart cells through the α1-adrenergic receptor.\(^3\)\(^7\) The level of α-MHC protein was unaffected, and the increase in MHC content could be accounted for entirely by a selective increase in the β-MHC isoform. By contrast, activation of adenyl cyclase has been reported to elevate α-MHC mRNA.\(^3\)\(^8\) These results are contrary to an earlier study by Gustafson et al\(^3\)\(^9\) and suggest that the outcome of experiments involving hormone interactions may depend on the tissue culture conditions.

Because of the phenomenon of receptor desensitization, it is not possible to equate the effects of brief treatment with adrenergic agonists in cultured cardiomyocytes with the effects of sympathetic stimulation in the intact animal. Efforts to induce α-MHC expression by injection of hypothyroid rats with doses of isoproterenol sufficient to increase intracellular cAMP have been unsuccessful.\(^3\)\(^1\) Continuous infusion of norepinephrine in euthyroid rats results in only transient increases in α- and β-MHCs.\(^3\)\(^9\)

**Expression of MHC Genes in Humans and Large Mammals**

β-MHC is the major MHC form expressed in human ventricular myocardium, but a few fibers also contain α-MHCs.\(^4\)\(^0\)\(^4\)\(^1\) Marked changes in human atrial MHC
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These other observations are interesting in two respects. First, the α-MHC gene is T4 inducible in subhuman primates, but the response is small compared with the induction observed in rat and rabbit. Second, despite wide differences in the effect of T4 on α-MHC expression between calf and dog, the hemodynamic responses in these species are very similar. For these reasons, a switch in MHC isoforms is unlikely to be the major underlying mechanism responsible for the inotropic action of thyroid hormone. Recently, attention has focused on the possibility of an alternative mechanism involving increased uptake and release of intracellular Ca2+. T4 is known to stimulate Ca2+ uptake by sarcoplasmic reticulum and coordinately control the expression of Na/K-ATPase and several proteins involved in intracellular calcium handling (e.g., Ca2+-ATPase of the sarcoplasmic reticulum, phospholamban, ryanodine receptor, and calcineurin, etc.).

The results shown in Figure 1 also indicate that administration of T4 to calf and baboon caused a partial switch in atrial myosin MHC content from the α to the β form. This shift has not been reported in small rodents treated with T4 but is similar to that observed in human atria subjected to pressure–volume overload and illustrates the interplay between thyroid hormone and mechanical factors in the regulation of MHC expression.

General Features of Transcriptional Control

Cell-specific expression of many genes is regulated at the transcriptional level by the interaction of activator proteins with specific DNA sequences organized in modular arrays often located within the 5' flanking region of the transcribed genes. Transcription factors and RNA polymerase II bind to promoter elements such as the CAAT and TATA sequences, which are located within 100–150 base pairs of the start site to initiate a "basal" level of transcription (Figure 2). Additional activator proteins bind to sequence elements located farther upstream to create an "induced" level of expression. According to this idea, activator proteins bind to DNA and interact with proteins in the tran-

**Figure 1. Effects of treatment with thyroxine on the electrophoretic pattern of myosin heavy chains in baboon, calf, and dog.** Animals were treated with 1.0–1.5 mg thyroxine per kilogram body wt for 4–6 weeks. Myosin was prepared as described, and the heavy chains were separated by sodium dodecyl sulfate gradient gel electrophoresis. In each row, lane A is mixture of hypothyroid and euthyroid ventricular myosins from rat as a separation standard; lane B is euthyroid ventricular myosin; lane C is thyrotoxic ventricular myosin; lane D is euthyroid atrial myosin; lane E is thyrotoxic atrial myosin.
Genes initially by transfection of cultured cells. Studies of transcriptional control of MHC fusion genes by transfection into cultured cells

A number of positive and negative elements have been localized upstream from the basal promoter region of cellular and viral genes, and some of their binding factors have been characterized. Specific DNA sequences are responsible for hormonal regulation as well as limitation of expression to specific tissues and developmental stages. Enhancers (defined as stimulating the promoter irrespective of their orientation and distance from the start point) have been found at a distance of several thousand kilobases upstream or downstream from the transcription initiation site, including introns of the gene.

Studies of Transcriptional Control of MHC Fusion Genes by Transfection Into Cultured Cells

Molecular control of cardiac MHC genes was studied initially by transfection of MHC/CAT fusion plasmids into primary cultures of rat heart cells. In the constructs used for these studies, about 2 kb pairs of 5' flanking sequences of the α-MHC gene was fused to the CAT coding sequences. Because DNA replication occurs only to a limited extent in primary heart cell cultures, plasmids bearing the promoter sequences are not stably incorporated into genomic DNA in experiments of this type but rather enter the nucleus and become associated with regulatory proteins. Assays of this type, referred to as transient assays, are rapid, generally being complete within 2–3 days, and are not influenced by the site of integration of the chimeric gene in the DNA of the host. Analysis of the activity of the constructs permits an identification to be made of the DNA sequence elements responsible for regulation of transcription.

To date, the most detailed analyses of promoter elements within the cardiac MHC genes have been carried out using transient assays in cultures of heart or skeletal muscle cells. Despite their tandem arrangement in genomic DNA, cardiac MHC/CAT fusion genes are regulated independently. In each of these genes, there is a basal promoter with TATA and CAAT sequences and a complex series of upstream regulatory elements. As might be anticipated from the divergence of sequences in the promoters of the α- and β-MHC genes, the DNA elements that control their transcription are quite different.

Analysis of the α-MHC Gene Promoter in Cultured Heart Cells

When transfected into fetal rat heart cells, functional activity of human α-MHC/CAT fusion plasmids is very low in the absence of T3 (Figure 3). Deletion of sequences upstream from position −78 in the human gene (or position −86 in the homologous rat gene) results in a marked loss of overall activity, as might be anticipated, because these constructs extend only a short distance 5' upstream from canonical CAAT and TATA sequences and thus represent the activity of the basal promoter. Chimeric plasmids pHaMHC-120 and pHaMHC-159 show higher levels of expression both in the presence and absence of T3, suggesting that one or

FIGURE 2. A general model of transcriptional control. The activity of the basal promoter is stimulated by an interaction between proteins of the transcription initiation complex (open circles) and an activating protein (filled circle) bound to an upstream DNA element. CAAT and TATA sequences in the basal promoter serve as recognition signals for binding of initiation factors and RNA polymerase II. The arrow indicates the direction in which transcription proceeds.

FIGURE 3. Bar graph of functional activity of human α-myosin heavy chain/chloramphenicol acetyltransferase fusion plasmids containing deletions in the 5' flanking sequences after transfection into primary cardiomyocytes prepared from 18–19-day (gestational age) fetal rats. All constructs contained a common 3' HindIII site located +421 base pairs 3' downstream from the transcription initiation site. Experiments were carried out in the presence (○) or absence (■) of 10−8 M T3 (thyroxine) in the culture medium. Cotransfection with pRSV/βgal was used as control for transfection efficiency. Values represent the mean for five or six experiments using duplicate plates.
more thyroid hormone response elements (TREs) are located in the additional sequences contained in these clones. In longer constructs, there is a remarkable rise and fall in T3-inducible activity, indicating the presence of upstream positive and negative elements.

Addition of T3 to cultures containing human or rat α-MHC/CAT fusion plasmids stimulates CAT activity with an EC50=5×10−8. This is consistent with the generally accepted mechanism of T3 activation of genes by binding to high-affinity nuclear T3 receptors (TRs), which are known to be products of the c-erbA proto-oncogenes (see References 49 and 50 for reviews).

**Characterization of the α-MHC TRE**

Initial studies of the DNA sequences involved in TR binding in the α-MHC gene54 suggested that the α-MHC TRE might resemble the palindromic model binding site proposed by Glass et al.52 More recently, determination of the contact points between the receptor proteins and the DNA sequences by DNase I “footprinting” and methylation interference analysis indicates that within the proximal promoter, TRs interact within two octameric imperfect direct repeats (TRE1) containing the consensus sequence 5′-(T/A)GGAGG(T/A)-3′.53 An identical sequence is present in a similar location in the rat gene. There is a second, weaker element (TRE2) located downstream of TRE1 in the human gene that has no counterpart in the rat α-MHC promoter and may not be functionally significant. Importantly, the binding affinity of TRs for the TREs in the human α-MHC gene seems to correlate with their functional activity. The affinity for TRE1 is approximately 20 times higher than TRE2 and is similar to the high-affinity TRE located on the growth hormone gene.54

Further analysis of the region within the α-MHC TR footprints by site-specific mutagenesis suggests that there is a core binding motif consisting of hexameric imperfect direct repeats with the consensus sequence AGG(T/A)G(C)/A separated by four base pairs.54 This motif is similar to the consensus recognition sequences for retinoic acid and vitamin D receptors, except that the “half-sites” for these receptors are separated by three and five base pairs, respectively. Binding of all three receptor types is enhanced by auxiliary proteins contained in nuclear extracts, one of which has been identified as RXRβ, a retinoic acid receptor homologue.56 Although incapable of high-affinity binding of retinoic acid, RXRβ acts as a coregulator by forming stable heterodimers with retinoic acid, thyroid hormone, and vitamin D receptors, which enhance binding to their cognate DNA response elements.

The distinct yet sometimes overlapping patterns of activation of MHC genes and other genes controlled by thyroid hormone and steroids thus seem to involve at least three components: 1) the orientation and spacing of similar hormone receptor binding motifs, 2) similarities in receptor structure in some cases permitting formation of functional heterodimers, and 3) the existence of common coregulators. Additional interactions between thyroid hormone and steroid hormones at both the cellular and molecular levels are likely to be important in the overall pattern of MHC regulation in the heart and skeletal muscles.

**Interactions of α-MHC TRE With Upstream Elements**

The human and rat α-MHC genes also are regulated by positive and negative regulatory elements located within 1 kb pairs of 5′ flanking sequence upstream of the TRE.57,58 The positive elements amplify the transcriptional activity in the presence of T3, >10-fold over the level of activity produced by the TRE acting on the basal promoter. These data suggest a minimum model for T3 regulation consisting of a basal promoter followed 5′ upstream by a TRE and more distal upstream elements acting to modify the activity induced by the hormone. By analogy with the general model of transcription (Figure 2), the TRE-TR-T3 ternary complex may interact with one or more upstream elements, looping out intervening DNA sequences to form a complex that strongly activates transcription. The possibility that the upstream sequences examined in this study might contain additional TREs was ruled out by experiments showing that when ligated to a heterologous promoter, these sequences were unresponsive to T3.50

**Direct Injection of the α-MHC Gene**

MHC/CAT fusion plasmids injected directly into the heart are taken up by cardiomyocytes and express the marker CAT gene for at least several weeks.60,61 The plasmids are not stably incorporated into genomic DNA, however. This technique is relatively inexpensive and permits mapping of the elements that regulate gene expression in response to complex stimuli, such as pressure-volume overload, exercise, etc., that cannot be modeled in vitro. There are several limitations to this approach. First, only a very small number of cells along the tract of the needle are transfected successfully. Second, the foreign gene is not stably incorporated into chromosomal DNA and, therefore, is not expressed indefinitely. Third, only a single construct usually can be tested in each heart. Given the variability in uptake of injected plasmids, comparison of the activities of large numbers of constructs is laborious.

Direct injection has been used to evaluate the role of upstream DNA elements in mediating hormonal responses of the α-MHC gene. Kitis et al62 and Ojamaa and Klein63 observed that the activity of a rat α-MHC fusion plasmid containing 613 base pairs of upstream sequence increased in response to T3 when injected into the ventricles of both in euthyroid and hypothyroid animals. The level of expression of the fusion plasmid in hypothyroid animals in both studies seemed high in comparison to that of the endogenous α-MHC gene, suggesting that additional elements may be needed for control of activity in vivo.

**Transgenic Experiments With the α-MHC Gene**

In an effort to determine whether the molecular mechanism proposed for the control of the α-MHC gene in cell culture applies in vivo, several strains of transgenic mice have been prepared using α-MHC/CAT constructs.64,65 Fusion genes containing as much as 5.5 kb pairs of sequence upstream from the mouse α-MHC transcription initiation site were fused to the bacterial CAT coding sequence. In situ hybridization analyses confirmed that expression of a transgene containing 600 base pairs of 5′ flanking sequence mimics the activity of
the endogenous gene in cardiac tissue during development and in response to changes in thyroid status. Specifically, atrial levels of CAT activity are relatively unaffected in the hypothyroid state, but no enzymatic activity could be detected in transgenic ventricle. Interestingly, when 5.5 kb pairs of upstream sequence were included in the reporter plasmid, mutation of sequences within the proximally located putative TRE did not cause a loss of activity in euthyroid transgenic animals. This mutation appeared to interfere with downregulation when hypothyroidism was induced by treatment with propylthiouracil, however.

The transgene results probably can be reconciled with the "loop" model proposed earlier by assuming that when the TRE is mutated, upstream elements are able to directly activate transcription without the participation of TR-T complex. Both the results obtained in transgenic animals and in tissue culture indicate that more than one upstream element is able to activate transcription from the \( \alpha \)-MHC promoter. The reason for this redundancy is unclear. Possibly, some of these elements may be activated selectively during development or under various physiological conditions.

**Analysis of the \( \beta \)-MHC Gene Promoter by Transfection Into Cultured Heart Cells**

Functional assays of a series of human and rat \( \beta \)-MHC/CAT fusion plasmids containing progressive deletions of the 5' flanking sequence revealed a strong positive element that is required for high-level expression of the \( \beta \)-MHC promoter in fetal rat heart cells (Figure 4). This element is located in the proximal region of the promoter between positions -298/-273 upstream from the transcription initiation site. Multiple copies of the element are active when inserted into a heterologous promoter, although they do not act as a classical enhancer there, showing some orientation dependency.

The sequences in the region of this element are shown in Figure 5. A strong DNase I footprint (positions -278/-296) coincides with sequences that were shown to be required for strong expression in heart cell cultures. The pattern of protein contact points identified by dimethyl sulfate interference suggests that the protein activator of this element may bind as a dimer to two hexameric imperfect direct repeats with the consensus sequence 5'-\( (C/G)TG(T/A)G-3' \). The pattern also might be produced by the binding of two unrelated proteins. This motif does not correspond to protein binding sites previously reported, but the surrounding sequences contain several motifs known to be important in activation of cellular and viral genes.

![Diagram](attachment:diagram.png)

**Figure 5.** Recognition sequences for cellular and viral transcriptional factors that are contained within the proximally located strong positive element of the human \( \beta \)-myosin heavy chain gene. Sequences that bind transcriptional factors M-CAT, TEF-1, and AP-4 are boxed. The AP-5 binding site, which overlaps the AP-4 site and extends two base pairs upstream, has been omitted for clarity. The region that is protected from DNase I digestion is underlined. *Protein interactions as demonstrated by methylation interference analysis.
**Similarity of the Positive Element With Viral and Cellular Enhancers**

Of particular interest is an almost perfect copy (six of seven base pairs) of the M-CAT element (5'-CATTCCCT-3') that is required for muscle-specific expression of troponin-T gene in mammalian skeletal muscle. The protein that binds to the strong positive element is competitively inhibited strongly by the M-CAT sequences, suggesting that it may be closely related to the M-CAT factor. The importance of this interaction was shown by the fact that deletion of the M-CAT sequences diminished β-MHC promoter strength by ≈98%. The strong positive element also contains extensive sequence homology with several functional elements (enhancers) within the SV 40 enhancer that are involved in activation of viral transcripts. One sequence (5'-GTGGATGT-3') is identical to the GT-IIC enhancer of SV 40 (Figure 5). The M-CAT-like motif is embedded within the opposite strand of this sequence. The GT-IIC enhancer interacts with transcription enhancer factor-1 (TEF-1), which recently was cloned from human liver. TEF-1 transcipts also have been identified in heart tissue, raising the possibility that TEF-1 and the M-CAT factor may be the same or related proteins.

The M-CAT domain is conserved in the human, rat, and rabbit β-MHC genes. Thus, it is possible that TEF-1 or other proteins that bind to the M-CAT/GT-IIC motif are involved in activation of the β-MHC gene and represent part of a mechanism for coordinated expression of contractile protein genes in the heart that is independent of MyoD and related proteins.

Overlapping the TEF-1 binding site in the β-MHC promoter are two other motifs that interact with SV 40 transcriptional factors AP-4 and AP-5. The functional significance of these sequence homologies is unclear.

Because of the functional importance of MyoD and related proteins to the expression of contractile proteins in skeletal muscle, it is noteworthy that the strong positive element of the human β-MHC gene also contains an E-box motif (5'-CANNTG-3'), which is identical to the cognate recognition site for the transcriptional factors, Pan-1 and Pan-2. These proteins have recognition sites in the promoter regions of pancreatic exocrine genes. The skeletal muscle MLC 1/3 gene and creatine kinase gene contain similar E-box motifs that function as muscle-specific transcriptional enhancer elements. In the human β-MHC gene, elimination of the E-box sequences by mutation or deletion reduces activity of the strong positive element but not to the extent that might be anticipated if expression were highly dependent on an E-box binding protein.

The β-MHC gene also is expressed in slow-twitch skeletal muscle fibers. Results of transient assays of β-MHC promoters from rat and rabbit in skeletal muscle assay systems suggest that a broader enhancer-like domain is required for activity in skeletal muscle cell cultures than in heart cells. In addition to the 22-base pair strong positive element, several additional sites were identified, including a C-rich sequence resembling an SP-1 binding site, an AP-2 site, and an element referred to as βe3, which contains a sequence similar to the NFe binding site in the rat embryonic MHC promoter. The C-rich element and the NFe-like sequence are required for expression of the β-MHC gene in heart.

**Identification of a Repressor Element**

Functional assays of the human β-MHC gene in rat heart cells also indicate that a repressor element is present immediately 5' upstream to the strong positive element. The location of the repressor adjacent to the strong positive element and its ability to suppress the activity of heterologous enhancers suggest that it may play a role in modulating the level of β-MHC expression.

An oligonucleotide corresponding to the sequence of the repressor element was used to screen a heart expression library, resulting in the identification of a cDNA encoding a possible repressor protein. Interestingly, the protein is an isoform of cellular nucleic acid binding protein (CNBP), which was originally identified in liver and shown to bind to the promoter regions of several enzymes involved in cholesterol biosynthesis. A second clone identical to the previously reported CNBP clone also was isolated from the heart library. Cotransfection of the two versions of CNBP into cardiomyocytes together with a β-MHC/CAT reporter construct revealed that the form originally identified in liver strongly represses activity, whereas the version first found in heart slightly stimulates activity, perhaps by completing with the endogenous protein(s) to relieve repression.

**Characterization of the β-MHC TRE**

The mechanism responsible for inhibition of β-MHC expression by T3 was assessed by transfection of a series of human β-MHC/CAT fusion constructs into fetal rat heart cells in the absence or presence of 10^{-8} M T3. Addition of T3 to the plates caused a 40–60% reduction in CAT activity in all of the clones except the shortest one containing the basal promoter; the low unstimulated activity of this clone makes these measurements difficult to evaluate. Similar results were obtained with a series of constructs containing progressive deletions of the rat β-MHC 5' flanking sequences. Taken together, these results suggest that a negative TRE may be present in the basal promoter region of both the genes. Although additional TR binding sites were identified by gel mobility shift assays (which involve binding of 125I-labeled nuclear extracts to DNA sequences) and DNase I footprinting with bacterially expressed TRs, these sites could be deleted or mutated without affecting T3 regulation.

A TRE located within the basal promoter has been described in negatively regulated genes encoding the α-subunit of thyroid stimulating hormone (TSH) and rat growth hormone. TREs located in this region may interfere with initiation of transcription; hence, their effects are most clearly discernible when the activity of the basal promoter is enhanced by transcriptional activators.

Interestingly, one of the DNase I footprints adjacent to the TATA box in the β-MHC promoter contains the sequence, 5'-GGGTTGG-3', which is similar to the single TRE half-site (5'-AGGTGAG-3') found in the basal promoter of the negatively regulated gene encoding the α-subunit of TSH. Another similar sequence has been identified in the silencer element of the lysozyme
Another mRNA

Direct Injection of the β-MHC Gene

Edwards et al.8 evaluated the activity of the proximally located strong positive element and repressor by direct injection into rat ventricle. The pattern of activity was the same as found with these constructs in cultured fetal rat heart cells.

Transgenic Experiments With the β-MHC Promoter

The ability of the murine β-MHC promoter to drive CAT expression has been tested in transgenic mice.83 Constructs contained approximately 7.0, 2.5, and 0.6 kb pairs of β-MHC S' flanking sequence fused to the CAT gene and were generally incorporated as head-to-tail concatamers. All constructs exhibited muscle-specific expression in transgenic animals, but the activity of the longest clone was severalfold greater than the two shorter constructs. CAT activity of the longer constructs usually was not downregulated in adult ventricular myocardium. Furthermore, ectopic expression was observed in muscles that do not contain an abundance of slow fibers.

These results are consistent with earlier data indicating there are important elements in the proximal sequences that are required for muscle-specific expression and suggest that an additional strong positive element may be located upstream. The putative distal element has not been localized or otherwise characterized, however. Since the long constructs used in the transgenic studies were incorporated in 10–30 copies, it is possible that their higher CAT activity may represent the summation of activity from multiple low-activity elements. Also, depending on their site of incorporation, some of the constructs could have fallen under the control of strong promoters in adjacent sequences. In any case, additional experiments are needed to more precisely locate the putative upstream strong positive element and to define its interactions with the proximal sequences.

Another mRNA Associated With the β-MHC Gene

Unexpectedly, in nuclear run-on assays, the sense probe corresponding to the S' region of the β-MHC gene gave an antisense mRNA.83 Antisense transcripts have been identified for several proto-oncogenes and growth factors,85 but this would be the first instance of an antisense mRNA to a contractile protein gene. In some instances, an identifiable protein is encoded by the antisense message, but it is unclear whether the complementary RNA or the encoded protein plays a role in regulating the level of the sense transcript.

Potential Therapy for Heart Disease

A change in cardiac myosin isoforms may have therapeutic applications in certain clinical situations. A T3-induced switch from the V2 to the V1 isoform (or stimulation of other genes under T3 control, e.g., Na/K-ATPase, Ca++-ATPase of the sarcoplasmic reticulum, etc.) might improve performance of the failing heart. In particular, induction of the α-MHC gene might be beneficial in cases of familial hypertrophic cardiomyopathy in which there is a defective β-MHC gene. Since the human α-MHC gene may be relatively resistant to the effects of thyroid hormone, there probably are limitations to the degree of induction that can be achieved by this means without causing significant side effects. Presently, no other agents capable of inducing α-MHC expression are known. On the other hand, induction of the V1 isoform may not be directly linked to the cardiotonic actions of thyroid hormone, leaving open the possibility that T3 analogues with selective cardiotonic properties might be useful in the management of heart failure.87

Gene transfer represents another potential means of increasing α-MHC expression in the heart. Because of the very small number of cells that can be transfected, direct injection offers little immediate promise. With the development of techniques capable of stably transfecting a larger number of cells by use of viral vectors to transport the gene,86 it may become possible to replace the low-activity V2 myosin isoform with the high-activity V1 form.

Also, it may be possible to obtain information about the pathogenesis of hypertrophic cardiomyopathy by using the technique of homologous recombination to target insertion of a mutated MHC gene into genomic DNA and inactivate the one normally expressed. The same methodology could be used to evaluate the effects of myosin isoform switching on cardiac performance without altering thyroid status. Thus, in the future, molecular genetic approaches are likely to provide further insights into the role of MHC genes in physiological adaptations and clinical disorders.

Summary

Advances in our knowledge of the regulation of cardiac myosin isoforms made possible by molecular cloning of the α- and β-MHCs genes are reviewed. Expression of these genes in heart does not seem to require MyoD or related proteins of the skeletal muscle myogenic program. Cardiac MHC genes are under the control of T3, which stimulates transcription of the α-MHC gene and inhibits β-MHC mRNA production both in vivo and in cultured heart cells. The responsiveness of the genes to T3 varies in different mammals, however. The genes are most responsive in rat and rabbit, intermediate in sensitivity in calf and subhuman primate (baboon), and very resistant in the dog. The human α-MHC gene is T3-inducible in ventricle, but the degree of response has not been quantified. Introduction of chimeric plasmids containing S' flanking sequences of cardiac MHC genes fused to the CAT gene into cultured heart cells and transgenic animals has permitted identification of regulatory elements. Although the genes are closely linked in genomic DNA, they are controlled independently. The element within the α-MHC promoter responsible for induction by T3 is located approximately 160 base pairs from the transcription initiation site. Additional transcriptional activators located S' upstream amplify the response to T3, probably by looping out intervening DNA sequences. The proximal region of the β-MHC gene contains important regulatory elements, including those required for repression by T3, muscle-specific expression, α-MHC-
independent positive element, and a hormone-independent repressor. Transgenic experiments suggest that one or more additional positive elements located farther 5′ upstream may be required for full expression of the β-MHC gene. Further applications of molecular genetic techniques should permit greater understanding of the role of cardiac MHC genes in physiological adaptations and in hypertrophic cardiomyopathy.

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