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Biochemistry

Cardiac α- and β-myosin heavy chain genes are organized in tandem

(gene organization/gene regulation/tissue-specific expression/DNA sequence/S1 mapping analysis)

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ABSTRACT **Two ventricular myosin heavy chains (MHCs), α and β, which exhibit different levels of ATPase activity, are differentially expressed during development, in response to thyroid hormone and in several pathological conditions. We have isolated and analyzed the structure of the genes coding for α- and β-MHC mRNAs in the rat. Detailed analysis of eight overlapping MHC genomic clones shows that the α- and β-MHC genes are organized in tandem and span 50 kilobases of the chromosome. The β-MHC gene, predominantly expressed in late fetal life, is located 4 kilobases upstream from the α-MHC gene, predominantly expressed in the adult. These two genes are very closely related at the nucleotide sequence level, suggesting that they have arisen by duplication of a common ancestor, yet their expression in the ventricular myocardium has been shown to be regulated in an antithetic fashion by thyroid hormone.**

In vertebrates the sarcomeric myosin heavy chains (MHCs) are encoded by a multigene family of 8–10 members (1–5). Each different sarcomeric MHC gene displays a pattern of expression that is tissue specific and developmentally regulated (3–8). The high degree of sequence conservation observed among the MHC genes, within a species (1, 6, 9), as well as throughout the evolutionary scale (1), suggests that these genes have arisen by duplication of a common ancestor. Moreover, in mouse (10, 11) and human (11), this gene family appears to be clustered onto a single chromosome, raising the possibility that the MHC genes are closely linked in the genome.

The modulation of the expression of two cardiac ventricular myosins, V1 and V3, has been documented in several mammalian species (12, 13). Myosin V1 contains two α-MHCs, whereas myosin V3, with a lower APTase activity, contains two β-MHCs. Both types of MHCs, α and β, are almost always expressed together in the normal animal, although their respective level varies during development (12, 13).

The ontogeny of the α- and β-ventricular MHCs can be entirely accounted for by changes in the level of their respective mRNAs (14). During heart development in the rat, the ventricular β-MHC mRNA, dominant in the late fetal life, is almost completely replaced after birth by α-MHC mRNA, which is dominant in the adult, leading to parallel changes in the MHC isozyme distribution (14). The ventricular MHC phenotype can also be directly modulated *in vivo* by hormonal and pathological stimuli (7, 12, 14, 15). Hypothyroid states in the rat result in a complete switch in the normal α/β MHC distribution (12, 14) by inducing the progressive disappearance of α-MHC mRNA and the appearance of β-MHC mRNA (14). Conversely, thyroxin replacement has the opposite effect, suggesting that the α- and β-MHC genes are regulated by thyroid hormone in an antithetic fashion. Interestingly, the expression of these genes is not limited to the ventricular cells since α-MHC mRNA is accumulated in the atrial myocardium, whereas β-MHC mRNA is present in slow-twitch skeletal muscle fibers (14).

We have isolated and characterized the α- and β-MHC genes, in an attempt to elucidate the possible relationship between the structure and organization of the cardiac MHC genes and the molecular basis for their developmentally, hormonally, and tissue-specific regulated expression. These genes are linked in the rat genome, 4 kilobase (kb) apart, and organized 5′ to 3′ according to their developmental expression.

MATERIALS AND METHODS

Isolation and Characterization of Cardiac MHC Gene Sequences. Rat genomic libraries constituted by partial *Eco*RI- or *Hae* III-digested spleen DNA cloned into λ Charon 4A were screened as described (4, 16). The DNA sequence analysis was performed by the base-specific chemical cleavage method (17) or by the dideoxy chain termination method (or both) (18). The DNA hybridization was performed as described (6, 19).

S1 Nuclease Mapping Analysis. RNA·DNA hybridization was in 25 μl of 80% deionized formamide/10 mM Pipes buffered at pH 6.4/1 mM EDTA/0.05% $NaDodSO_4$ for 20 hr at 42°C. S1 nuclease digestion was in 300 μl for 1 hr at 25°C with 75–150 units of enzyme (New England Nuclear) in 300 mM NaCl/30 mM Na acetate pH 4.5/3 mM $ZnSO_4$. At the end of the reaction, samples were made 10 mM in EDTA, precipitated with ethanol, resuspended in 85% formamide, and run on 6% polyacrylamide/8.3 M urea gels (17).

RESULTS

Isolation of the Cardiac MHC Genomic Sequences. The structure and specificity of several MHC genomic clones has been described elsewhere (4). One of these clones isolated from a rat DNA library, named clone 287A1, contains sequences that hybridize preferentially to cardiac RNA (4) and to the 3′ untranslated gene-specific sequence of a cardiac MHC cDNA clone, pCMHC5 (6). Interestingly, sequences downstream from the putative 3′ end of the MHC gene in clone 287A1 were shown to hybridize also to cardiac MHC mRNA (4), suggesting that two cardiac MHC genes might be closely adjacent to each other. To further investigate this possibility and isolate the complete set of genes that code for the cardiac MHC mRNAs, we have isolated genomic fragments that contain 5′ and 3′ extensions of clone 287A1 by "walking" along the chromosome. By successive screenings of two different genomic libraries constituted by partial *Eco*RI- or *Hae* III-digested rat DNA, we have isolated seven different and overlapping MHC genomic clones. Characterization of these clones was carried out by RNA and DNA hybridization, DNA sequence analysis, and restriction mapping analysis. By these procedures the genomic clones were aligned into a continuous genomic fragment of 50 kb (Fig. 1).

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Abbreviations: MHC, myosin heavy chain; kb, kilobase(s); bp, base pair(s).

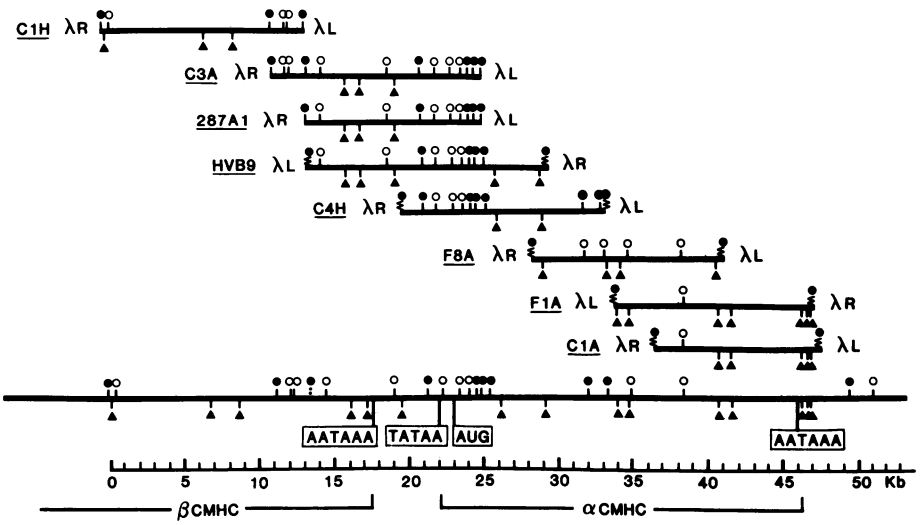

FIG. 1. Cardiac MHC gene cluster. Restriction endonuclease map of cardiac MHC genomic clones. The relative positions of the eight overlapping clones are given in the 5′ to 3′ orientation of the chromosomal map. Locations of the putative "TATAA" box, AUG start codon, and A-A-T-A-A-A polyadenylylation signals are indicated. Bottom scale is in kb. Restriction endonuclease sites: ▲, *Bam*HI; ●, *Eco*RI; ○, *Hin*dIII. ⋈●, *Eco*RI sites artificially generated during the cloning procedure; ···●, polymorphic *Eco*RI site in rat DNA (1).

All eight overlapping genomic clones contain MHC coding sequences, as determined by RNA blot (data not shown), DNA blot, and sequence analyses (see below), and together define a 50 kb segment of the genome.

The 50-kb Genomic Sequence Defined by the Overlapping Genomic Clones Contains the Cardiac α- and β-MHC Genes Organized in Tandem. A minimum of two MHC genes is expressed in the rat ventricles (6). These genes code for two closely related MHC mRNAs that have 95% homology in the portion represented by their respective cDNA clones, pCMHC5 and pCMHC21, but are completely different at their 3′ end termini (6). These MHC cDNA clones, previously identified as adult cardiac specific (6), were subsequently shown to represent β-MHC mRNA (pCMHC5) and α-MHC mRNA (pCMHC21) (14).

All fragments in the genomic DNA previously shown to hybridize to these two cardiac MHC cDNA clones (6) are present in the MHC genomic clones shown above. This fact suggests that the genes corresponding to the α- and β-MHC mRNAs are represented in the 50-kb chromosomal sequence defined by these clones. However, due to the high degree of sequence homology between the two cDNA clones, only their 3′ end gene-specific sequences could be used to identify the respective gene (6, 9). The gene-specific sequence in pCMHC5 hybridizes to the 2.3-kb *Bam*HI fragment contained in clones C3A, 287A1, HVB9, and C4H and the gene-specific sequence in pCMHC21 hybridizes to the 4.7-kb *Bam*HI fragment contained in clones F1A and C1A (data not shown). These two fragments were isolated, further digested with other restriction enzymes, and hybridized again with the MHC cDNA probes, to generate segments of convenient size for DNA sequence analysis.

As shown in Fig. 2*a*, the 620-base-pair (bp) *Bam*HI–*Pst* I fragment mapping at coordinates 17.3–17.9 of Fig. 1 contains, in a separate exon, the codons for the five carboxyl-terminal amino acids and the complete untranslated 3′ end of the β-MHC mRNA represented by pCMHC5. The nucleotide sequence of the 405-bp *Pst* I–*Bam*HI fragment (coordinates 45.9–46.3) shown in Fig. 2*b* contains, in a separate exon, the codons for the seven carboxyl-terminal amino acids and the complete untranslated 3′ end of the α-MHC mRNA represented by pCMHC21. The two sequences, which are separated by ≈29 kb, delineate the last exon of two different cardiac MHC genes, respectively. The fact that the highly divergent, gene-specific sequences at the 3′ end of the two cardiac MHC mRNAs are precisely separated by an intron from the conserved sequences situated upstream uncovers an interesting feature of the structure of their genes. Thus, α- and β-MHC genes are linked in the genome. The β-MHC gene, predominantly expressed in the late fetal life, is located upstream from the α-MHC gene, which is predominantly expressed in the adult.

The Cardiac α- and β-MHC Genes Are 4 kb Apart. To determine the distance separating the two MHC genes, the position of the 5′ end of the α-MHC gene was localized. The 3.4-kb *Eco*RI genomic fragment downstream from the β-MHC gene (coordinates 21.2–24.6 in Fig. 1), which hybridizes to cardiac MHC mRNA, was subjected to sequence analysis (Fig. 3). Comparison of the open reading frames to

a

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GGATCCCTCAAGGTCACACAAGGGTCTCCACCTACCAGGTGCCCTAGTCTCAACTTCAGTTTCCATGTGC   70
TGTCCCTACAATGTTGGCCTCCCCAGAGCTAATTTTGACTTGGTTTTATTTCAAAAGGGCCTGAATGAAG  140
                                                    G  L  N  E  E
AGTAGATCTTGTGCTACCCAACCCTAAGGATGCCTGTGAAGCCCTGAGACCTGGAGCCTTTGAAACAGCA  210
  END
CCTTAGGCAGAAACACAATAAAGCCAATTTTCCTTCAAGCCAAAATCCTGTCTCTAGACTCTTCTTCACT  280
GACCTCGGTCCCTGGGGCTCTAGGGTGGGGAGGTGGGACTTGAAAGAAGGAGGGAAGTGGCAAGGCCACT  350
CCCCAGGACTCTGTGAAGTTCTGAGCCTTCCCAGTACAGTGCTGGCTCATAGATCCTCCTCCAGCCAAAC  420
ACAGCAAGAAGTGATGCTTCCTTTGTGACTTCCCCAGGCCCAGTGCCTGTCCGGTTGAAACAGGATTTAG  490
AGAAGCCTCTAAACTCACCTGGAGCTCTGAGCCCATCTACCTCACACCTACATGCCGGTTCTAATCAGTA  560
TCCTACACTGATAATACGCAGAGCTGACCACGGGAGAAGGCCTGGAGAGTAGGAACTGGCCAGAGACTTT  630
TCAGTCTGCAG
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b

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CTGCAGACACCTATAACCTCCGAATCCACTCTCCCACTCCAAACGGCCCCAGATCTGTGTTCTAAACTAC   70
AAGCACCACCATCATGGGACTTCAAGACTCCTGAGGTTTAACTCCTCCCTTTCCAAGGGCATCTTCCTAG  140
AGCCACTGAAATCCCTAGAGATTCCTCCCCAGACTATCTTCCCCGTTGGCCTCACACCTTTTATTTCTTT  210
CTCAGCAGAAAATGCACGATGAGGAATAACCTGTCCAGCAGAAAGAGCCTCGCTGTTGCTATCCCACAAT  280
    Q  K  M  H  D  E  E  END
AAATATGAATGCTTGACTTTGCCTGTACCCTCTGTCCTTCCCATTAAATTATCTATCTGGGGTGGGAATT  350
TTGGTGGCCCAGGAGGAAGGTGGCCACAGCAGAGACTGTCAGAGCTGTAGGATCC
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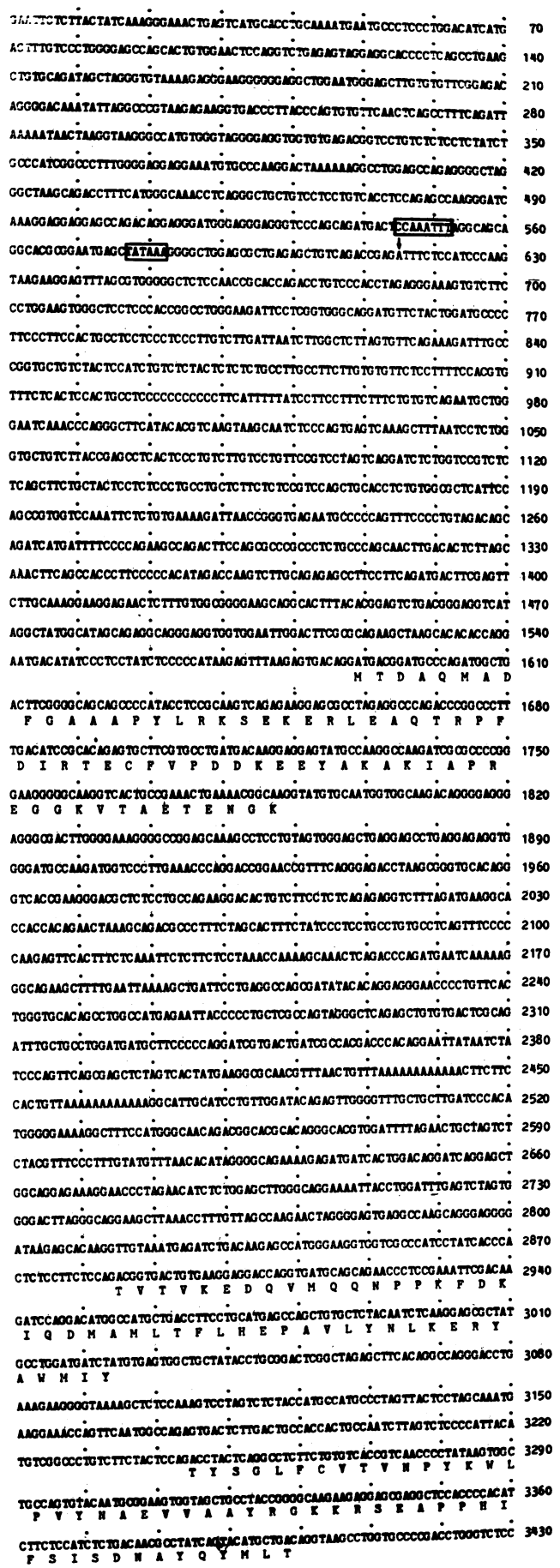
FIG. 2. Nucleotide sequence of the 3′ end termini of the cardiac α- and β-MHC genes. (*a*) The 640-bp *Bam*HI–*Pst* I fragment in clone 287A1. The sequence of the last exon of the β-MHC gene is underlined. (*b*) The 405-bp *Pst* I–*Bam*HI fragment in clone F1A. The sequence of the last exon of the α-MHC gene is underlined. The two different polyadenylylation sites (6) are indicated by an arrow.



the available sequences for rabbit skeletal MHC (20) and nematode *Caenorhabditis elegans unc*-54 MHC (21) revealed that this fragment contains, in three separate exons, the amino-terminal sequence of α-MHC—namely, the codons for amino acids 1–67, 68–115, and 116–167. It merits mention that the location of two of the first three introns is conserved in the MHC genes of species as distant in evolution as rat and *C. elegans*.

The fact that the only C-C-A-A-A-T-T-T and T-A-T-A-A-A sequences (22) are found 1032 bases and 1006 bases, respectively, upstream from the AUG start codon (Fig. 3) suggests that the 5′ untranslated sequence of this MHC mRNA is interrupted by intron(s). For this reason, S1 mapping experiments were performed to analyze the 5′ end of the α-MHC mRNA. Ventricular and atrial mRNAs from a normal adult animal, where the α-MHC gene is expressed, were used, whereas ventricular mRNA from a thyroidectomized animal, where β- and not α-MHC is expressed (14), was used as negative control. The RNAs where hybridized to a 712-bp *Hin*dIII–*Ava* I genomic fragment, which contains the sequence coding for the first 53 amino acids of α-MHC and 553 additional bases extending in the 5′ direction of the gene (coordinates 22.5–23.2). As shown in the last lane of Fig. 4, no S1-protected fragment is observed with RNA from the thyroidectomized rat, indicating sequence divergence between the 5′ ends of α- and β-MHC mRNAs, at least at the labeling site of the hybridization probe. Two S1-protected fragments are generated by hybridization of the genomic probe with ventricular and atrial MHC mRNAs from normal rats (Fig. 4). The two S1 cleavage points can be localized in the nucleotide sequence of the DNA probe, a few bases away from the AUG start codon and within two adjacent and directly repeated T-A-A-G-A-G-T sequences.

It is unlikely that the 5′ end of the sequence protected from S1 nuclease corresponds to the cap site of the α-MHC mRNA, because it is located too close (≈12 bases) to the AUG start codon and too distant (≈1000 bases) from the C-C-A-A-A-T-T-T and T-A-T-A-A-A promoter sequences (22). Therefore, these results strongly suggest that the untranslated 5′ region of the α-MHC mRNA is interrupted by intron(s), as predicted by the sequence (see above). In the *C. elegans unc*54 MHC gene (21) the only putative promoter sequences are ≈400 bp upstream from the AUG start codon, thereby suggesting an interrupted untranslated 5′ end as well.

The origin of two S1-protected fragments in the α-MHC DNA·RNA hybrids is unclear. As shown, their ratio does not change with increasing concentrations of S1 nuclease. A possible interpretation is that an alternative splicing pathway in the untranslated 5′ end might be used in both ventricular and atrial cells. Alternatively, the shortest S1-protected fragment could be generated by tertiary structure in the RNA·DNA hybrid produced as a consequence of the sequence repeat in this region (23). Despite these unsolved questions, the experiments shown above provide information on the length of a sarcomeric MHC gene in vertebrates and demonstrate that the α-MHC gene that codes for a 6.2-kb mRNA is 24.5 kb long. This MHC gene is located 4 kb downstream from the β-MHC gene.

The Cardiac α- and β-MHC Genes Have Highly Conserved Sequences. The striking sequence conservation in the light meromyosin coding portion between the cardiac genes (6), and among the sarcomeric genes (1), led us to obtain infor-

FIG. 3. Nucleotide sequence of the 5′ end and adjacent sequence of the α-MHC gene: the 3.4-kb *Eco*RI fragment in clone 287A1. Boxes indicate C-C-A-A-A-T-T-T and T-A-T-A-A-A sequences. Arrow indicates putative cap site of the mRNA. The deduced amino acid sequence is given in the one-letter code, below the first nucleotide of the corresponding codon.

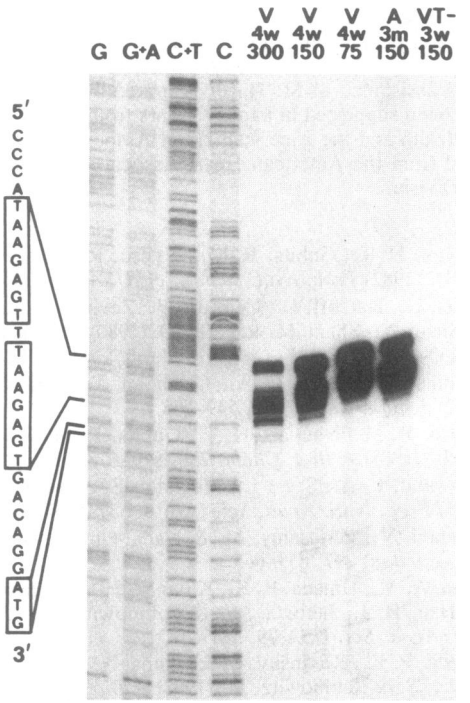


FIG. 4. S1 mapping of the 5' end of the cardiac MHC mRNAs. The 712-bp *Hin*dIII–*Ava* I fragment in clone 287A1, labeled at the 5' end with [γ-^{32}P]ATP, was hybridized, in DNA excess, to 30 μg of cardiac RNAs, extracted from the left ventricle of a 4-week-old normal rat (V4w), from the atria of a 3-month-old normal rat (A3m), and from the left ventricle taken 3 weeks after thyroidectomy (VT-3w). S1 nuclease concentrations used were 75, 150, or 300 units, as indicated on top of the corresponding lane. S1-resistant fragments were run adjacent to sequencing reactions of the same DNA fragment (17). For complete nucleotide sequence of this fragment, see Fig. 3, nucleotides 1034–1746.

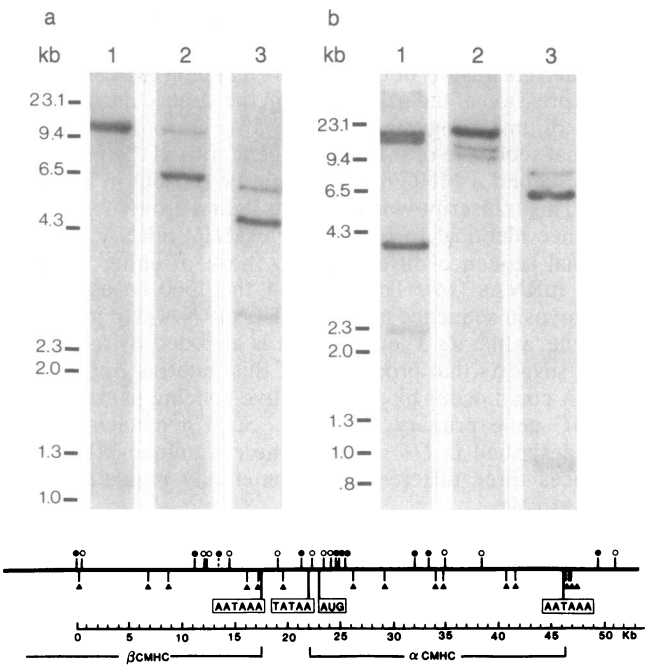


FIG. 5. Identification of cardiac MHC sequences in genomic rat DNA. Hybridization of the 3.3-kb *Eco*RI fragment (*a*) and of the 7.8-kb *Eco*RI fragment (*b*) of clone F8A to rat DNA digested with *Hin*dIII (lane 1), *Eco*RI (lane 2), and *Bam*HI (lane 3). Hybridization washes were in 15 mM sodium chloride/1.5 mM sodium citrate/0.1% $NaDodSO_4$ at 55°C.

mation on the extent of sequence homology between the two cardiac and other sarcomeric MHC genomic sequences. The 3.5-kb and 7.8-kb *Eco*RI fragments of the genomic clone F8A, which covers half of the α-MHC gene (see Fig. 1), were hybridized to restriction digests of rat DNA. As shown in Fig. 5, both probes generate a pattern of hybridization that demonstrates that the strongly hybridizing bands can be mapped in the homologous α-MHC gene, whereas the weaker hybridizing bands can be mapped in the adjacent β-MHC gene.

In Fig. 5*a*, the 3.5-kb probe hybridizes in the α-MHC gene to a 11.0-kb *Hin*dIII fragment (coordinates 24–35), a 6.5-kb *Eco*RI fragment (coordinates 25.5–32.0), and two contiguous *Bam*HI fragments of 3.0 and 4.8 kb (coordinates 26.2–34.0). The hybridization to bands that can be mapped in the β-MHC gene corresponds to a 11-kb *Eco*RI fragment (coordinates 0–11.0) and a 6.4-kb BamHI fragment (coordinates 0.2–6.6). The 11.7-kb *Hin*dIII fragment in the β-MHC gene (coordinates 0.5–12.2) is not visualized on these blots because it comigrates with the fragment of the approximate same size corresponding to the α-MHC gene.

In Fig. 5*b*, the 7.8-kb probe hybridizes in the α-MHC gene to three contiguous *Hin*dIII fragments of 11.0, 3.5, and 12.5 kb (coordinates 24.0–51.0), a 16-kb *Eco*RI fragment (coordinates 33.3–49.3), and four contiguous *Bam*HI fragments of 4.8, 0.8, 6.0, and 0.9 kb (coordinates 29.2–41.7). The 7.8-kb probe cross-hybridizes in the β-MHC gene to two *Hin*dIII fragments of 11.7 kb (not separated in this single digest from the 11.0-kb fragment of the α-MHC gene) and 2.3 kb (coordinates 0.5–14.5), respectively, two *Eco*RI fragments of 11 and 10.2 kb (coordinates 0–21.2; note that the *Eco*RI site at coordinate 13.5 is polymorphic in rat DNA; see ref. 1), and two *Bam*HI fragments of 1.8 kb (not visible in short exposure of the autoradiogram) and 7.5 kb (coordinates 6.8–16.1).

The sequence conservation throughout the length of the two genes was confirmed by using, as hybridization probes, other genomic segments of the α-MHC gene and genomic segments of the β-MHC gene (data not shown). From the relative intensity of hybridization between homologous and heterologous sequences, the average sequence conservation in the two cardiac MHC genes can be estimated to lie between 20% and 30%. Assuming that the intervening sequences that represent 75% of the length of these genes are nonhomolgous, the conservation between coding sequences of the α- and β-MHC genes could be as high as 90–95%. Such a high level of sequence homology was previously observed in the light meromyosin coding portion of the α- and β-MHC mRNAs (6). The absence of hybridization, at high stringency, to MHC genomic fragments other than the cardiac MHC genes, demonstrates that the degree of homology between these two cardiac MHC genes is much higher than between cardiac and skeletal MHC sequences. It is therefore very likely that the α- and β-MHC genes have originated by duplication after the divergence of the ancestral cardiac gene from the skeletal sequences. This conclusion is supported by the close linkage of the two cardiac MHC genes and by the existence of several sarcomeric MHC genes in invertebrates (1, 21), before the appearance of the heart in the phylogenetic ladder.

DISCUSSION

We have isolated two MHC genes that are organized in tandem in a 50-kb continuous segment of the rat genome. These genes code for the cardiac α- and β-MHC mRNAs because they contain the sequences of these MHC transcripts, including, delineated by the last exon, their gene-specific 3' terminal end.

Although the possibility that other MHC genes might be expressed in the ventricles cannot be excluded, the DNA hybridization analysis using MHC genomic probes (Fig. 5)

and MHC cDNA probes (6) strongly suggest that these genes represent the only cardiac α- and β-MHC sequences in the genome. The results obtained by S1 mapping experiments on the expression of the MHC genes in the ventricular and atrial myocardium, using probes at the 5′ (Fig. 4) or the 3′ (14) terminal sequence of the α-MHC gene, raised the possibility that a second α-MHC mRNA might be present in these tissues. This transcript would have its light meromyosin coding sequence identical to that of the α-MHC mRNA and a 3′ terminal sequence different from those of either α- or β-MHC mRNAs (14). The fact that the 1500 bases of light meromyosin sequence in the α-MHC mRNA map into a single gene, while its 3′ end portion is encoded by a separate exon, suggests that production of this putative α-like MHC mRNA could occur by an alternative splicing pattern of the α-MHC gene primary transcript. Such a mechanism has been described in *Drosophila*, where a unique MHC gene produces three different developmentally regulated MHC mRNAs (24).

The β-MHC gene, predominantly expressed in the ventricle of fetal hearts (14), is located ≈4 kb upstream from the α-MHC gene, predominantly expressed in the ventricle of adult hearts (14). The close linkage of this gene arrangement and the observation that the sarcomeric MHC genes in mouse (10, 11) and human (11) have been mapped onto a single chromosome raises the possibility that embryonic, newborn, and adult skeletal muscle MHC genes (9) might also be organized in a head to tail fashion and in the order of their developmental expression.

The tight clustering of the cardiac MHC genes is reminiscent of other developmentally regulated gene families such as the β-globin family (22) and the immunoglobulin gene families (25). A few striking features, however, set the cardiac MHC genes apart. First, in the case of the globin and immunoglobulin genes, the developmental pathway is unidirectional. Once a gene has been switched off in order to turn on the next gene in the pathway, the expression of the former gene is irretrievably lost. The gene switched off will no longer be able to be expressed in the same terminally differentiated cell. This is clearly not the case for the cardiac MHC genes. The β-MHC gene is switched off during normal development and the α-MHC gene is switched off in response to thyroid hormone withdrawal (14). Both genes, however, can be turned back on, in the absence of DNA replication and cell division, either "spontaneously" as is the case for the β-MHC gene in older animals or experimentally in response to thyroid hormone depletion/replacement and different mechanical stimuli (14). Second, the globin and immunoglobin genes have a narrow tissue specificity of expression. Their expression is restricted to erythrocyte precursor cells and B cells, respectively. On the contrary, the α- and β-MHC genes are expressed in other muscle tissues, such as atrial muscle for the α-MHC gene and skeletal slow-twitch muscle for the β-MHC gene (14).

The fact that the α- and β-MHC genes display an opposite pattern of expression in response to thyroid hormone (14) makes these genes a very attractive model in which to analyze the basic mechanisms involved in their control. Significant evidence has accumulated to suggest that regulatory 5′ flanking sequences are responsible for the temporal and spacial expression of the gene(s) under their control. The responsive potential of enhancer elements in several hormone-inducible genes has been recently proven (26–28). The isolation and characterization of the cardiac MHC gene sequences described in this study make it possible to determine the existence of such enhancer elements in the cardiac α- and β-MHC genes as well as offer a good model for the study of the molecular mechanisms of thyroid hormone action.

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