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Expression of slow skeletal myosin heavy chain 2 gene in Purkinje fiber cells in chick heart

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Abstract

In avian, there are three slow skeletal myosin heavy chain (MHC) isoforms, slow skeletal MHC 1, 2, and 3. While slow skeletal MHC 3 has been characterized, slow skeletal MHC 1 and 2 are not yet fully studied. To determine the complete sequence of slow skeletal MHC 2, we isolated six overlapping cDNA clones, each encoding a portion of chick slow skeletal MHC 2, using the reverse transcription polymerase chain reaction (RT-PCR). The entire slow skeletal MHC 2 cDNA consisted of 5927 nucleotides including a 104 bp 3'-untranslated region and encoded 1941 amino acids. Using one of the cDNA clones, we made a probe for in situ hybridization. We also used immunohistochemistry to localize slow skeletal MHC 2 in skeletal and cardiac tissues. These studies showed that in addition to its expected expression in the adult chicken slow skeletal muscle, slow skeletal MHC 2 was expressed in the subendocardial cluster of cells and around the blood vessels within the ventricle of late embryos and adults. This isoform was not expressed in the myocardium throughout the life of the chicken. Based on morphological criteria as well as rich desmin expression, we concluded that the subendocardial cluster of cells were Purkinje cells. Although the physiological significance of the slow skeletal MHC expression remains elusive at this time, this MHC isoform may be used as a specific marker for Purkinje cells.

Keywords: Developing chick heart; Slow skeletal myosin heavy chain 2; Conduction system; Purkinje myocytes

1. Introduction

The structural and functional diversity of the striated muscle is reflected by the presence of a variety of myosin isoforms that are encoded by a family of myosin heavy chain (MHC) genes (Schiaffino and Reggiani, 1996). Myosin is composed of two heavy chains and four light chains. The type of MHC expressed in a muscle cell defines the specific type of muscle fiber, significantly affects its contractile properties (i.e. its ATPase activity), and serves as an excellent marker for differentiated cardiac and skeletal muscle (Masaki and Yoshizaki 1974; Moore et al., 1992; Nguyen et al., 1982, Nudel et al., 1980; Reiser et al., 1988; Robbins et al., 1986). The expression of each MHC isoform is regulated in the tissue-specific and developmental stage-specific manners (Bader et al., 1982; Bandman et al., 1982; Evans et al., 1988; Lompre et al., 1984).

Heart contraction is coordinated by conduction of electrical excitation through specialized tissues of the cardiac conduction system. Formation of the cardiac conduction system

Abbreviations: ALD, anterior latissimus dorsi; ED, embryonic day; MHC, myosin heavy chain; RT-PCR, reverse transcription polymerase chain reaction; SSMHC, slow skeletal myosin heavy chain.

☆ Sequence data from this article have been deposited with DDBJ/EMBL/Gene Bank nucleotide sequence databases under Accession No. AB057661.

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is a critical step during normal heart development. However, relatively little is known about the molecular mechanisms of differentiation of the cardiac conduction system. Chick and quail embryos and neonates have been used as model systems for studying the heart organogenesis in vertebrates. In adult chicken heart, different MHC isoforms have been identified at the protein level in the atria, ventricles, and conduction system (de Jong et al., 1988; Evans et al., 1988; Gonzalez-Sanchez and Bader, 1984, 1985; Zhang et al., 1986). We have recently reported that the chick neonatal fast skeletal MHC is expressed in the heart conduction cells during the late embryonic to newly hatched stages but not in adults (Machida et al., 2000a). Immunohistochemical evidence has suggested that slow skeletal MHCs are present in the developing and adult conduction system (Gonzalez-Sanchez and Bader, 1985; de Groot et al., 1987; Sanders et al., 1986). In the avian slow skeletal muscle, there are three different slow skeletal MHC isoforms, slow skeletal MHC 1, slow skeletal MHC 2, and slow skeletal MHC 3 (also called atrial MHC) (Page et al., 1992). It is not known, however, which of these isoforms is(are) expressed in the conduction system.

To better understand the molecular mechanisms responsible for the chamber-specific expression of MHCs during development, we have isolated and characterized cDNAs corresponding to different MHCs (Machida et al., 2000a, 2000b; Oana et al., 1995, 1998). To date, atrial (Oana et al., 1995, 1998; Yutzey et al., 1994) and ventricular (Bisaha and Bader, 1991; Machida et al., 2000b; Stewart et al., 1991) MHCs, have been characterized well. In this study, we determined the full cDNA sequence of chick slow skeletal MHC 2. Using an isolated cDNA fragment as a probe, we performed in situ hybridization analyses and found that the expression of slow skeletal MHC 2 mRNA was restricted to the subendocardial clusters of cells and around the blood vessels of the late embryonic and adult hearts. During development, at the protein level, slow skeletal MHC 2 was also localized in the same subendocardial structures but not in the myocardium. Based on various morphological criteria, the subendocardial cell clusters were determined to be Purkinje cells. Although the physiological significance of the slow skeletal MHC 2 expression in Purkinje cells remains unclear, this MHC isoform is an excellent molecular marker for terminally differentiated Purkinje cells.

2. Materials and methods

2.1. Animals

White Leghorn chicks and fertilized eggs were purchased from a commercial source (Saitama Experimental Animal Supply Co., Ltd, Saitama, Japan), and eggs were maintained under high humidity in a 38 °C incubator. Embryos were staged according to Hamburger and Hamilton (1951).

2.2. Isolation of the chick slow MHC 2 cDNA

The total RNA was extracted from ED 19 and adult chicken anterior latissimus dorsi (ALD) muscles as previously described (Chomczynski and Sacchi, 1987). The entire coding sequence of chick slow skeletal MHC 2 was determined from six overlapping cDNA clones, SSMHC2#1, SSMHC2#2, SSMHC2#3, SSMHC2#4, SSMHC2#5 and SSMHC2#6 (Fig. 1) that were isolated by the reverse transcription polymerase chain reaction (RT-PCR) method. To synthesize a first strand cDNA, 1 μg of the total RNA was reverse-transcribed with 200 U of Super Script II (GIBCO BRL, Rockville, MD, USA) using the random 9-mer primer (Takara, Kyoto, Japan). Since the sequence of the S-1 region of quail slow skeletal MHC 2 has been determined (DiMario and Stockdale, 1997), the primers for SSMHC2#1 and SSMHC2#2 were designed based on the quail sequence. The sequence of the rod portion of chick slow skeletal MHC 2 has been determined (Chen et al., 1997), and the primers for SSMHC2#4, SSMHC2#5 and SSMHC2#6 were designed on the basis of the chick sequence. The primers for SSMHC2#3 were designed based on SSMHC2#2 and SSMHC2#4 sequences. Shown below are the actual sequences of the primers used: SSMHC2#1 (forward primer: 5'-ATGTCTATGCTGGACATGAGCGAG-3', reverse primer: 5'-TGGTAGTAGATATGGTAGCTTCGC-3'), SSMHC2#2 (forward primer: 5'-TCAAGAGTGATTTTCCAGCA-ACCC-3', reverse primer: 5'-CGCTTCGACGGCCGGG-GTTTCTGG-3'), SSMHC2#3 (forward primer: 5'-GACAACCACCTTGGGAAGTCACCC-3', reverse primer: 5'-GTGGACTCGCGCTGCAGCGTCGCC-3'), SSMHC2#4 (forward primer: 5'-TGAAGCTGAGGAGAGACCTGG-AGG-3', reverse primer: 5'-TTGTTCTCCCTCTTGAGGGT-CTCC-3'), SSMHC2#5 (forward primer: 5'-AACGCCT-ATGAGGAGTCTCTGGAC-3', reverse primer: 5'-TCGTCCAGGCGCATCTGCAGGTCC-3'), and SSMHC2#6 (forward primer: 5'-GCGGATGAAGAAGAACATGG-AGCAGACC-3', reverse primer: 5'-GCACACCTCCATTT-ATTAACTCGGTGCG-3'). The PCR was carried out using PCR buffer (Perkin Elmer, Branchburg, NJ, USA), 3 U of Taq polymerase (Perkin Elmer), 200 ng of the primer and the first strand cDNA. The cycle times were 45 sec for denaturation (94 °C), 90 sec for annealing (60 °C) and 120 sec for synthesis (72 °C) for a total of 37 cycles, and 10 min for extension time (72 °C) at the end. Amplified products were analyzed in 1% agarose gels and purified for subcloning. cDNA fragments were subcloned into the pCR II vector using a TA cloning kit (Invitrogen, Carlsbad, CA, USA) and sequenced. The DNA sequence procedure was performed essentially as described (Machida et al., 2000a, 2000b). The nucleotide and deduced amino acid sequences were analyzed using a Genetyx-Mac 9.0 (Software Development, Tokyo, Japan).

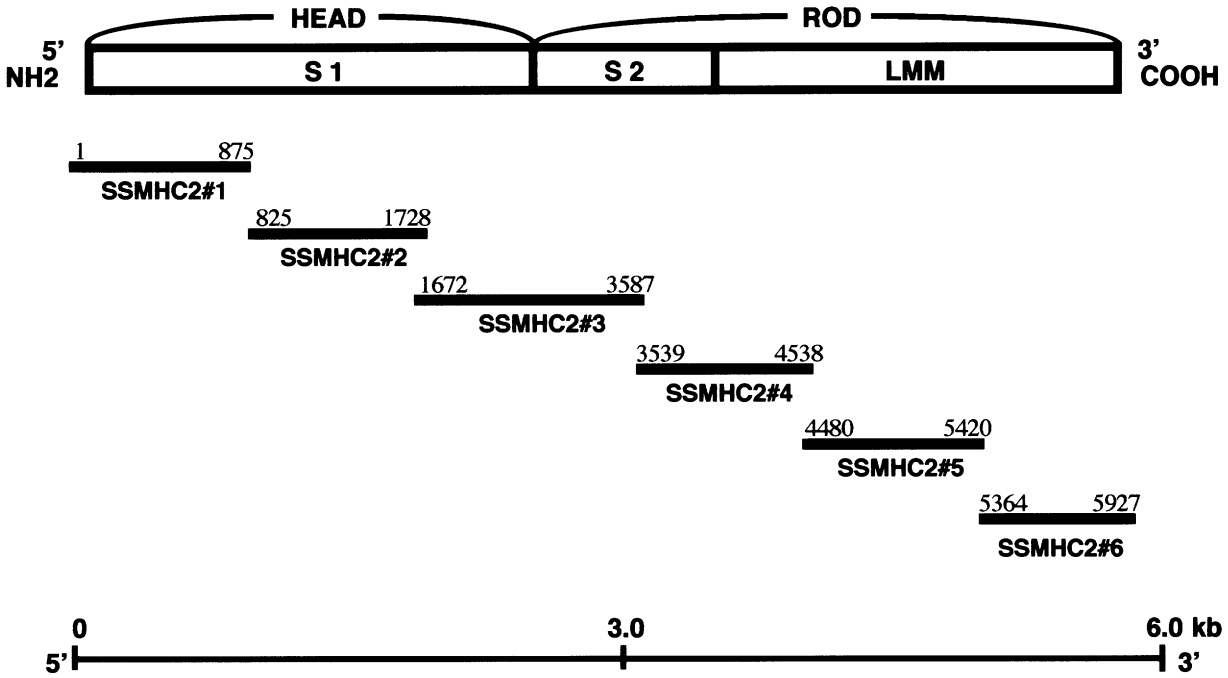

Fig. 1. Relationship of the six chick myosin heavy chain cDNA clones (SSMHC2#1, SSMHC2#2, SSMHC2#3, SSMHC2#4, SSMHC2#5, and SSMHC2#6) against the domain map of myosin heavy chain. The MHC domains shown are S1 head, S2 portion of the rod and the light meromyosin (LMM) portion of the rod. The relative lengths of S1, S2, LMM, and each clone are proportional to the nucleotide number. The numbers of each clone correspond to the nucleotide location within chick myosin heavy chain cDNA.

2.3. In situ hybridization

In situ hybridization was performed as described by Machida et al. (2000a). Digoxigenin-labeled RNA probes were synthesized from SSMHC2#6 in pCR II by cutting with Xba I and transcribing with Sp6 polymerase (anti-sense), or cutting with Hind III and transcribing with T7 polymerase (sense). SSMHC2#6 was used as slow skeletal MHC 2 specific probe because it contained the untranslated region that has little homology with other types of MHC mRNAs. The specificity of the probe was confirmed by northern blotting. To compare the spatial expression patterns of both the slow skeletal MHC 2 and the atrial (slow skeletal MHC 3) MHC mRNAs in chick embryos at the same stage, the atrial MHC digoxigenin RNA probe, which was previously described by Machida et al. (2000a) was also used. No signal was detected with the sense probe.

2.4. Immunohistochemistry

To determine the localization of slow MHC proteins in chick heart, four different mouse monoclonal antibodies against MHC (kindly provided by Dr. Bandman, University of California, Davis, CA, USA), which react specifically with different isoforms (Bourke et al., 1995; Lefeuvre et al., 1996) were used. These include a ventricular isoform and three isoforms found in the slow fibers of chick skeletal muscles, and these antibodies have been used to localize specific isoforms. The antibodies used in this paper and their reactivity with known chicken MHCs are summarized in Table 1(Bourke et al., 1995; Lefeuvre et al., 1996; Rosser et al., 1996). Immunohistochemical analysis was performed essentially as described previously (Machida et al., 2000a, 2000b).

For double-immunofluorescence labeling, both MHC monoclonal and desmin antibodies were used. A rabbit anti-

Table 1
Specificity of anti-MHC monoclonal antibodies against chick MHCs

Monoclonal antibody	Cardiac		Slow skeletal		Apparent specificity
	Atrial	Ventricular	1	2	
NA8	+	–	–	+	Atrial MHC and Slow skeletal MHC 2
NA3	–	–	–	+	Slow skeletal MHC 2
NA7	–	–	+	–	Slow skeletal MHC 1
HV11	–	+	–	–	Ventricular MHC

The specificity of these monoclonal antibodies against chick MHCs has been demonstrated previously (Bourke et al., 1995; Lefeuvre et al., 1996; Rosser et al., 1996).

chick desmin antibody (Chemicon International Inc, CA, USA) was used to identify myocytes and conduction tissue cells (Machida et al., 2000a; Thornell et al., 1985). The secondary antibodies used were fluorescein-linked anti-mouse Ig and Texas Red-labeled anti-rabbit Ig (Amersham, Bucks, UK).

3. Results

3.1. Determination of the full sequence of a chick slow MHC 2 cDNA

To study the molecular characteristics of different slow skeletal MHCs, we cloned chick MHC cDNA fragments. We previously determined the complete sequence of chick atrial MHC (slow skeletal MHC 3) (Oana et al., 1998). For chick slow skeletal MHC 2, only partial sequence data are available (Chen et al., 1997). The reported sequence has a 2.4 kb insert and encodes a small portion of subfragment 2 and the light meromyosin within the MHC rod domain as well as 105 bp of the 3'-untranslated portion of the mRNA. To obtain the complete sequence data on this isoform, we isolated six fragments of slow skeletal MHC 2 cDNA by the RT-PCR method. The relationship of these six overlapping cDNA clones is shown in Fig. 1. We found that the amino-acid sequences of SSMHC2#4, SSMHC2#5 and SSMHC2#6 were 100% identical to that published by Chen et al. (1997). A partial sequence of quail slow skeletal MHC 2 was reported by DiMario and Stockdale (1997). They isolated a clone that encoded a part of the S-1 region as well as the 5'-untranslated portion, and its transcripts were predominantly expressed in quail slow muscle during development (DiMario and Stockdale, 1997). The nucleotide sequences of our two chick cDNA clones (SSMHC2#1 and SSMHC2#2) and the reported quail slow skeletal MHC 2 (DiMario and Stockdale, 1997) showed high homology (95.8% identical), confirming that SSMHC2#1 and SSMHC2#2 indeed encode the chick slow skeletal MHC 2 gene. From the sequence data of these six clones, we obtained a 5927 base-long sequence representing the entire open reading frame ofthe chick slow skeletal MHC 2 gene. This open reading frame encodes a predicted protein consisting of 1941 amino acids. The nucleotide sequence data obtained in this study will appear in the DDBJ/EMBL/Gene Bank nucleotide sequence databases with the accession number AB057661.

To better understand the properties of the chick slow skeletal MHC 2, we compared its sequence with other chick sarcomeric MHCs. Five chick sarcomeric MHC isoforms have been fully sequenced (Chao and Bandman, 1997; Machida et al., 2000a, 2000b; Molina et al., 1987; Oana et al., 1998). The amino acid sequence of slow skeletal MHC 2 is 81.8% identical to that of chick ventricular MHC (Machida et al., 2000b) and is 75.6% identical to that of chick atrial MHC (Oana et al., 1998). Furthermore, the chick slow skeletal MHC 2 amino acid sequence shows 75.3%, 76.9%, and 75.0% identity with chick embryonic fast skeletal MHC (Molina et al., 1987), chick neonatal fast skeletal MHC (Machida et al., 2000a), and chick adult fast skeletal MHC (Chao and Bandman, 1997), respectively.

3.2. Expression of slow MHC mRNAs in the developing conduction system

To study the expression of the slow skeletal MHC 2 gene, we employed in situ hybridization analyses. Slow skeletal MHC 2 mRNA was expressed strongly in the adult ALD (Figs. 2A, 2B). The sense probe did not hybridize to serial sections of the same muscle sample (Fig. 2C), demonstrating the specificity of the anti-sense probe binding. We then investigated the expression of slow skeletal MHC 2 mRNA in the chick heart. Although its expression was detected in neither atrial nor ventricular myocardium (Fig. 3A), hybridization signals were present around the blood vessels (Figs. 3B, 3C) and in the subendocardial structure (Figs. 3D, 3E) of the ventricle. This expression was observed from a late embryonic stage to adult (Figs. 3F, 3G, 3H).

Using S1-nuclease mapping analyses, we have earlier shown that atrial MHC (slow skeletal MHC 3) is expressed in the developing but not in adult ventricle (Oana et al., 1998). Immunohistochemical studies have suggested that atrial MHC is present in the adult conduction system of the ventricle (de Groot et al, 1987; Sanders et al, 1986). We reinvestigated using in situ hybridization if the atrial MHC gene was indeed expressed in the adult conduction system of the ventricle. Our results showed that slow skeletal MHC 3 mRNA was expressed strongly in the atrium (Fig. 3I) and that in the ventricle, its expression was in the subendocardium structures (Figs. 3J, 3K) from the late embryonic to adult stages. Based on their histological appearances, we suggest that these structures are the conduction system of the ventricle. Thus, we tentatively conclude that two of the slow skeletal MHC genes, slow skeletal MHC 2 and slow skeletal MHC 3 (atrial MHC), are expressed in the cells of the ventricular conduction system.

3.3. Expression of slow MHC proteins in the developing conduction system

To demonstrate specific expression of various MHC isoforms including the two slow skeletal MHCs in the conduction cells, we performed immunohistochemical staining using several monoclonal antibodies (Table 1). At the early embryonic stages, little immunoreactivity was detected in the myocardium with NA3 (data not shown), which reacts specifically with the chick slow skeletal MHC 2 isoform. However, we found some NA3-positive cells in the subendocardial structures and around blood vessels (Fig. 4A), and by ED 18, many NA3-positive cells were clearly found in these areas (Fig. 4B). In the adult ventricle, the same NA3-positive cells were present in the subendocardial conduction fiber-like structures (Fig. 4C) as well as around blood vessels

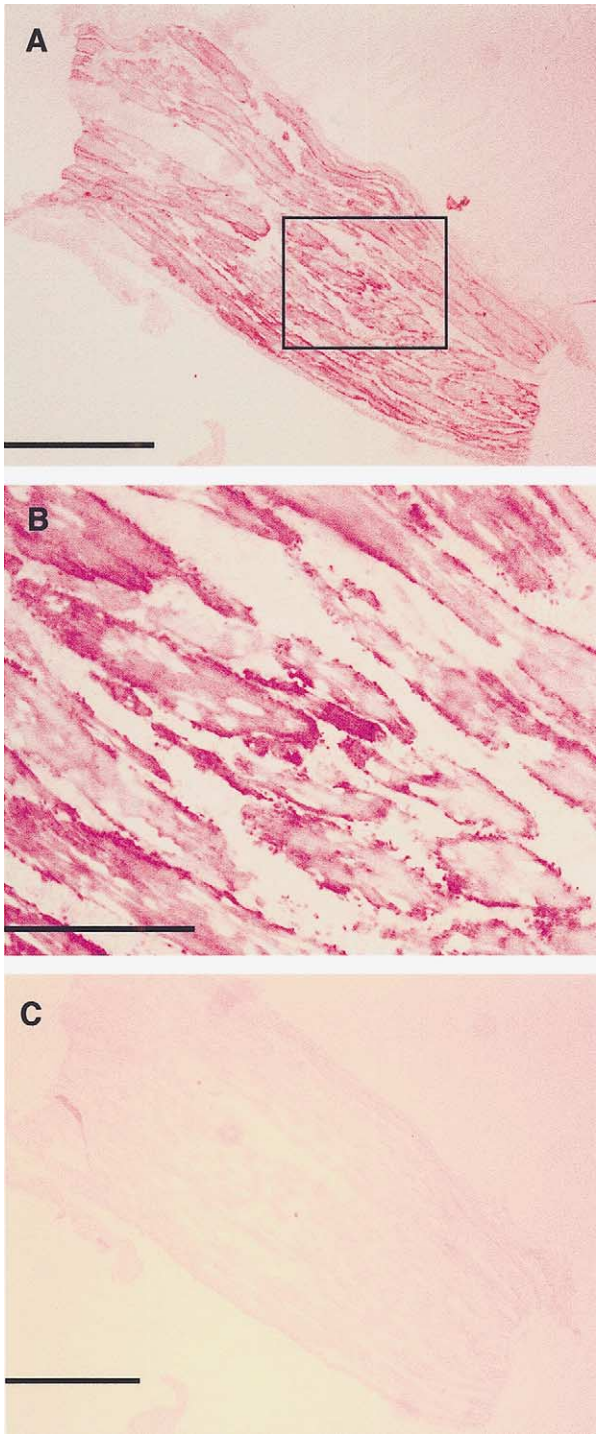

Fig. 2. In situ hybridization analyses for slow skeletal MHC 2 mRNA on frozen sections of adult anterior latissimus dorsi (ALD). A), A section incubated with the digoxigenin-labeled slow skeletal MHC 2 probe (antisense). B), A higher magnification of the boxed area in A. C), A section incubated with the sense probe. Slow skeletal MHC 2 mRNA is expressed strongly in adult ALD (A and B). The sense probe did not hybridize to a serial section (C). Bars: 1000 µm (A and C), 400 µm (B).

(Fig. 4D). In the atrium, at the late embryonic and adult stages, many of the NA3-positive cells were recognized and were located in the subendocardial conduction fiber-like structures (Fig. 4E) and around blood vessels (Fig. 4F). NA3-positive cells were not found in the proximal components of the conduction system (atrioventricular node and His bundle).

NA8 which reacts with both chick atrial MHC (slow skeletal MHC 3) and slow skeletal MHC 2 isoforms stained strongly the atrial myocardium of early embryos (Fig. 5A), whereas it stained the ventricular myocardium only very weakly (Figs. 5A, 5B). At ED 13, some NA8-positive cells were recognized in the subendocardial structures in the ventricle (Figs. 5C, 5D), and by ED 18, many strongly NA8-positive cells were present in the subendocardial conduction fiber-like structures (Fig. 5E) and around the blood vessels of the ventricular myocardium (Fig. 5F). The similar staining patterns were seen in the adult ventricle (Fig. 5G). HV11 which reacts specifically with the adult chicken ventricular MHC isoform stained ventricular myocytes uniformly, but the subendocardial conduction fiber-like structures and the cells around blood vessels strongly in the ventricular myocardium of adult chickens (Fig. 5H). No reactivity was found with NA7, suggesting that slow skeletal MHC 1 was not expressed in the developing and adult heart.

Previous immunohistochemical studies have suggested that the NA3- and NA8-positive cells are those of the conduction system (Gonzalez-Sanchez and Bader, 1985). This was based on the general morphology of the stained structure. To verify that the subendocardial cells that stained with NA3 and other anti-MHC reagents are indeed the conduction tissue cells, we performed immunofluorecent double-staining of heart sections using antibodies to NA3 (Fig. 6A) or NA8 (Fig. 6B) and desmin (Figs. 6C, 6D). Desmin is the intermediate filament type present in all types of muscle cells. It is particularly abundant in Purkinje cells, especially in birds and larger mammals (Filogamo et al., 1990; Machida et al., 2000a, Thornell et al., 1985) and is a good marker for these cells. Figs. 6A and 6B show the staining patterns of a day 7 neonate chick ventricle with the two MHC antibodies. Both NA3 (Fig. 6A) and NA8 (Fig. 6B) labeled only a population of cells located in the subendocardium. Anti-desmin stained the same ventricle sections diffusely, but more importantly it strongly labeled the structures present in the subendocardium (Figs. 6C, 6D). Both the appearance and anti-desmin expression of the subendocardial structures indicate that they are conduction fibers. When the staining patterns by anti-MHC (green) and anti-desmin (red) were superimposed, these subendocardial structures became yellow (Figs. 6E, 6F), indicating co-localization of slow skeletal MHC and desmin. These results show that the linear structures stained with the anti-MHC antibodies are conduction fibers.

4. Discussion

In this study, we determined the full nucleotide sequence of the chick slow skeletal MHC 2 mRNA. The primary sequence of this myosin type allowed us to determine the structural features of this isoform and its phylogenetic position within the sarcomeric MHC multigene family.

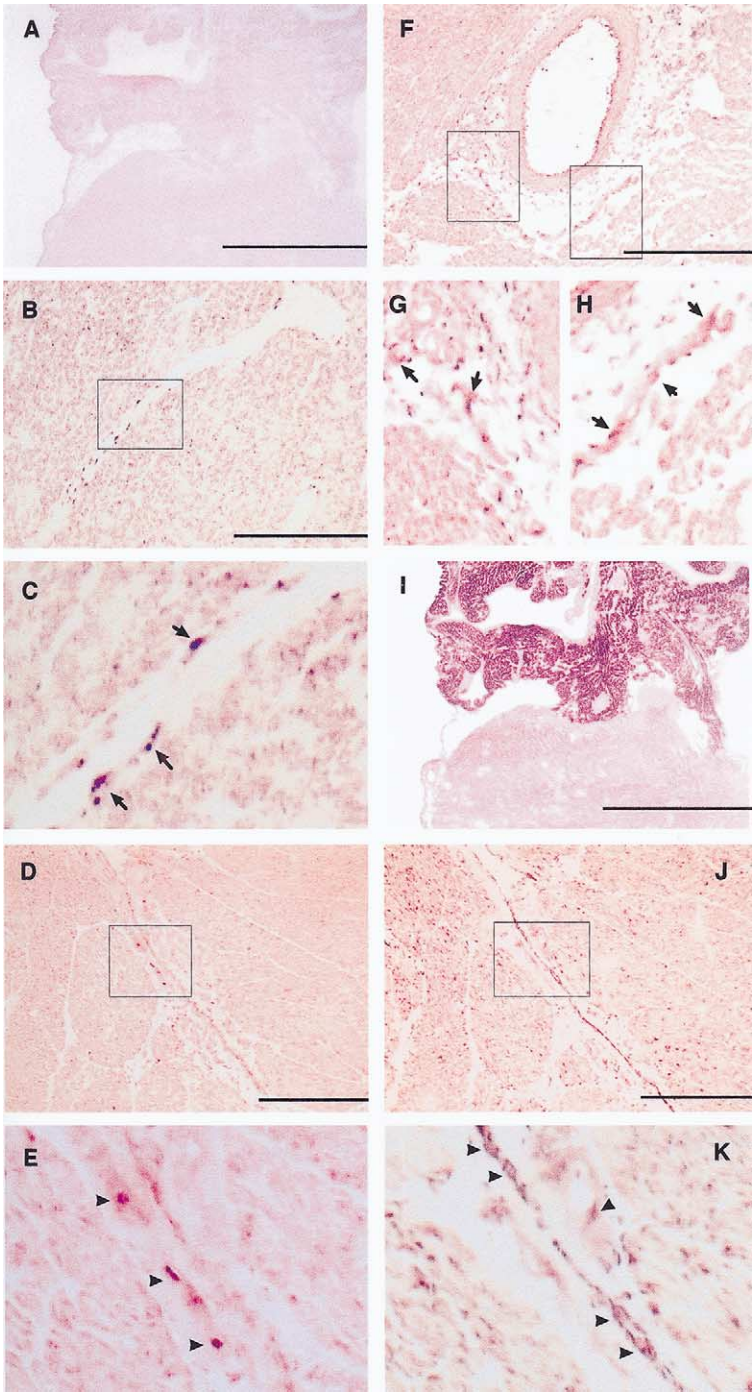

Fig. 3. In situ hybridization analysis for slow skeletal MHC mRNAs on frozen sections of late embryonic and adult chick heart. Sections are from ED 19.5 (A and I), neonatal day 0 (B and C), and adult (D, E, F, G, H, J and K) hearts. They were incubated with the digoxigenin-labeled slow skeletal MHC 2 (A through H) or atrial MHC mRNA (I through K) probe. The boxed areas in B, D, F, and J are shown in higher magnifications in C, E, G, H, and K, respectively. Specific hybridization signals were not detected in the myocardium (A). In the ventricle, signals were detected around blood vessels (C, G and H, arrows) and in subendocardial structures (E, arrowheads). Atrial MHC mRNA was expressed strongly in the atrium (I). It was also expressed in the subendocardial structures (K, arrowheads) of the ventricular myocardium. Bars: 1000 μm (A and I), 400 μm (B), 250 μm (D and J), 200 μm (F).

4.1. Determination of the full sequence of a chick slow skeletal MHC 2 cDNA

In mammals, the complexity and diversity of MHC have been extensively studied in multiple species, including rat, mouse, and human (Weiss et al., 1999). Comparative studies of the mammalian MHC gene family have provided unique insights into the evolutionary relationships among the divergent MHC genes. The vertebrate MHC multigene family has been subdivided into fast skeletal muscle and cardiac/slow skeletal muscle subfamilies (Stedman et al., 1990). At present, nine distinct chick sarcomeric MHC isoforms have

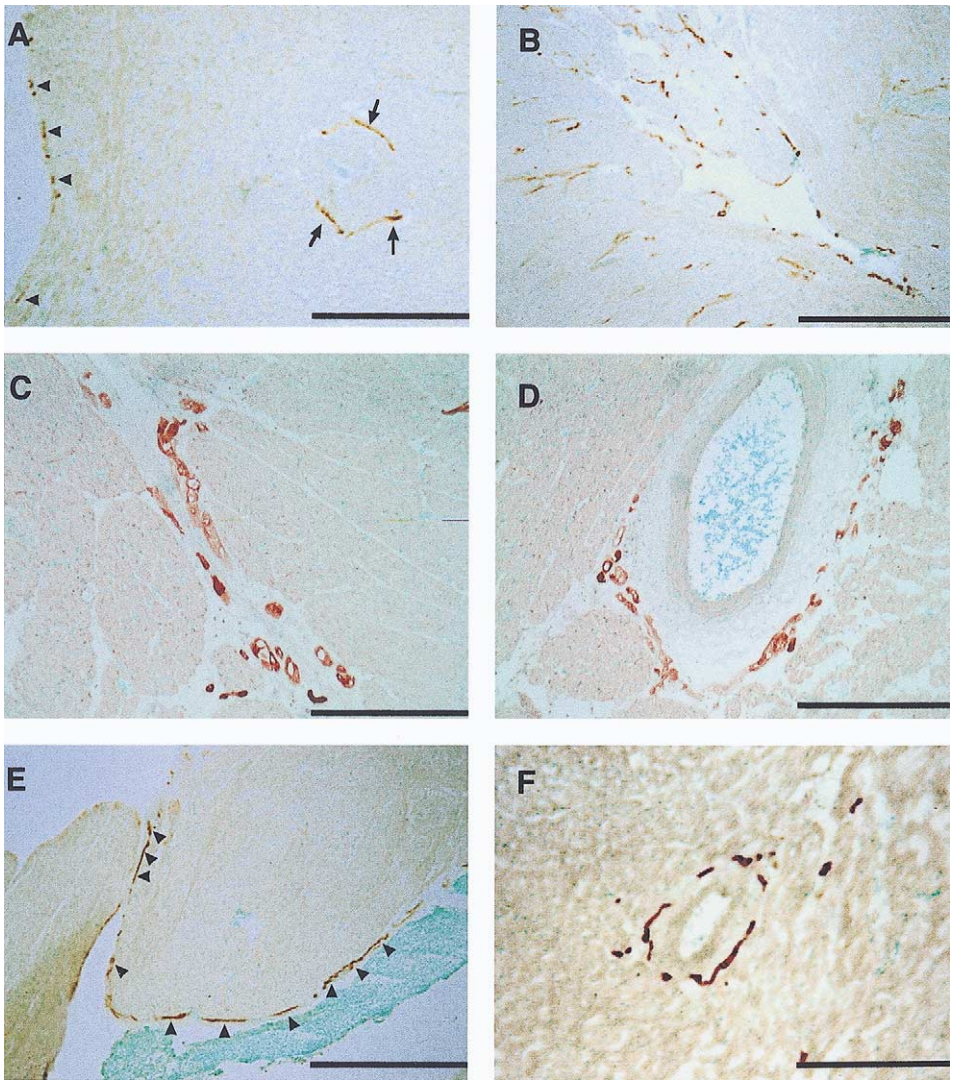

Fig. 4. Immunohistochemical staining of frozen heart sections with anti-slow skeletal MHC 2. Sections are from ED 15 (A), ED 18 (B and E), and adult (C, D and F) hearts. They were incubated with the NA3 antibody followed by a horseradish peroxidase-linked secondary antibody. Although the myocardium was not stained with NA3 at early embryonic stages, some intensely NA3-positive cells began to appear as subendocardial clusters (A, arrowheads) and around the blood vessels in the ventricular myocardium (A, arrows) in later developmental stages. At ED 18, many NA3-positive cells appeared in the same locations (B). In the adult, intensely NA3-positive cells were detectable within the subendocardial structures (C) and around the blood vessels of the ventricular myocardium (D). In atria, in late embryos (E) and adults (F), many of the NA3-positive cells were found in the subendocardial layer (E, arrowheads) and around the blood vessels of the atrial myocardium (F). Bars: 400 μm (A, B and E), 200 μm (C, D and F).

been described (Bandman and Rosser, 2000). The full coding regions of six, including slow skeletal MHC 2 in this study, of the nine chick isoforms have been sequenced (Chao and Bandman, 1997; Machida et al., 2000a, 2000b; Molina et al., 1987; Oana et al., 1998). To investigate the relationships between slow skeletal MHC 2 and other MHC isoforms, we have compared the amino acid sequence data of various MHC types. Slow skeletal MHC 2 was 81.8% identical to chick ventricular MHC and 75.6% identical to chick atrial MHC. The mammalian heart expresses α and β/slow MHCs (Mahdavi et al., 1982). The α type is predominantly expressed in the adult atria and the β/slow isoform is found mainly in the adult ventricle (Lompre et al., 1984). The β/slow type of MHC is the isoform expressed in adult slow skeletal muscle (Lompre et al., 1984). The amino acid sequences of the α and β/slow MHCs in mammals show a high homology (99%) and are conserved well (Matsuoka et al., 1991). In contrast, homology among chick cardiac MHCs is considerably less.

4.2. Expression of slow skeletal MHC 2 in the developing conduction system

It is well known that Purkinje fibers are distinguished from heart muscle cells by a distinct localization pattern of myofibrillar proteins (Mikawa and Fischman, 1996; Moorman et al., 1998; Schiaffino, 1997). Gonzalez-Sanchez and Bader (1985) reported that the differentiated conduction cells of chick heart expressed a slow tonic type of skeletal MHC, which was not present in the myocardium. Immunohis-

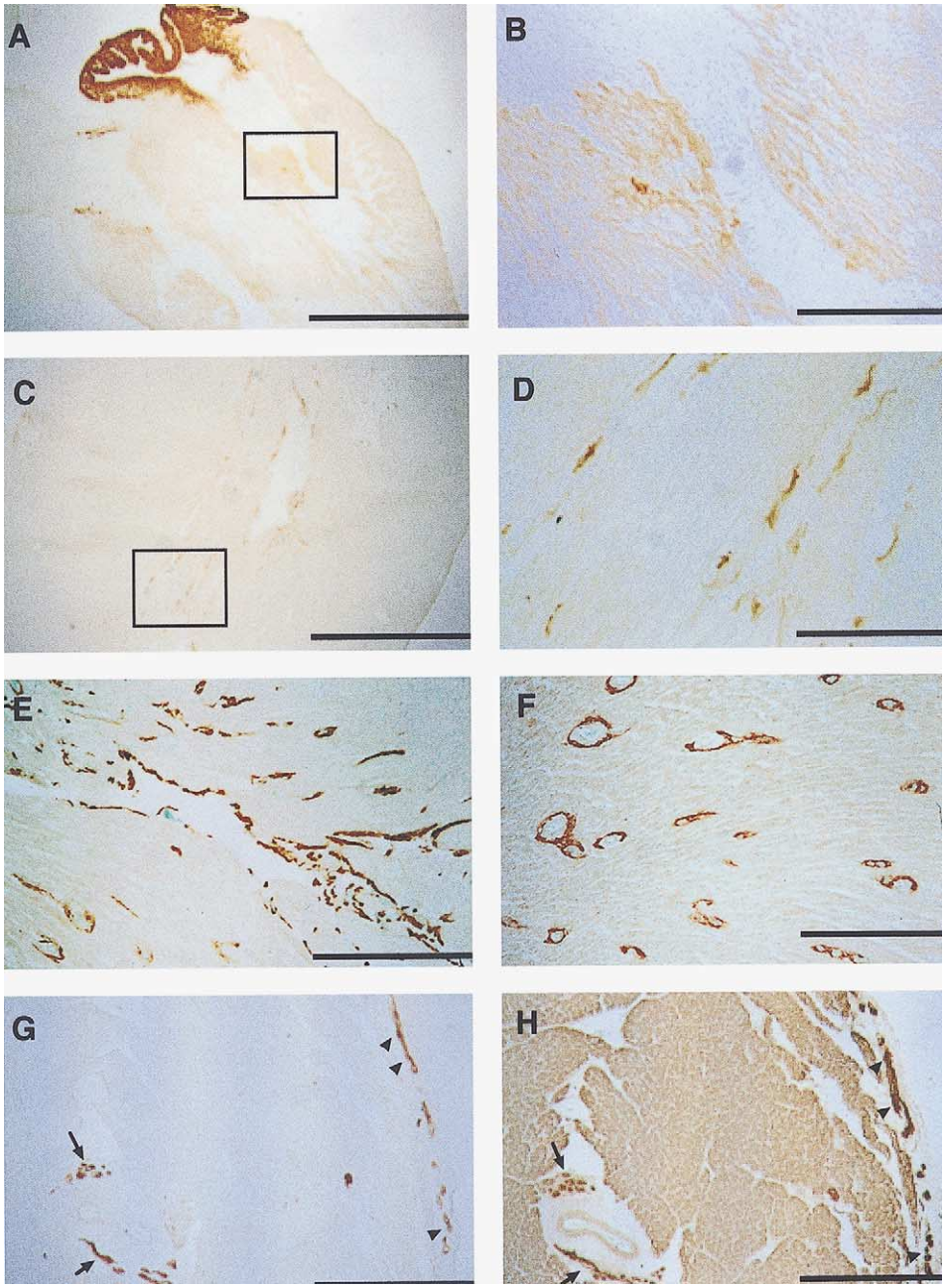

Fig. 5. Immunohistochemical staining of frozen sections of ventricles at various developmental stages with the NA8 antibody. Sections are from ED 8 (A and B), ED 13 (C and D), ED 18 (E and F), and adult (G) hearts. They were incubated with NA8 antibody and then with a horseradish peroxidase-linked secondary antibody. A section of an adult ventricle (H) was stained with the HV11 antibody. The boxed areas in A and C are shown in higher magnification in B and D. At ED 8, the atrial myocardium stained strongly with NA8 (A). In contrast, the ventricle showed weak reactivity with NA8 (A and B). At ED 13, some intensely NA8-positive cells were detectable in the ventricle (C and D). At a later embryonic stage (ED 18), many of the NA8-positive cells were seen in the ventricle (E and F). These intensely NA8-positive cells were located in the subendocardial layer (E) and around the blood vessels in the ventricular myocardium (F). At the adult stage, NA8-positive cells were in the subendocardial layer (G, arrowheads) and around the blood vessels of the ventricular myocardium (G, arrows). The HV11 antibody appeared to react uniformly with ventricular myocytes and the subendocardium layer (H, arrowheads) and around blood vessels of the ventricular myocardium (H, arrows). Bars: 1000 μm (A and C), 400 μm (E, F, G and H), 200 μm (B and D).

tochemical analyses have provided evidence that atrial MHC is present in the adult conduction system of the ventricle (de Groot et al., 1987; Sanders et al., 1986). In the avian slow skeletal muscle, three different slow MHC isoforms have been identified, which, according to their appearance during development, have been called slow skeletal MHC 3 (atrial MHC), slow skeletal MHC 1, and slow skeletal MHC 2 isoforms (Page et al., 1992). However, the type of slow MHCs expressed in the chick conduction system has not been fully characterized. We demonstrated here that slow skeletal MHC 2 and atrial MHC (slow skeletal MHC 3) are expressed in the Purkinje fiber of the chick heart. The expres-

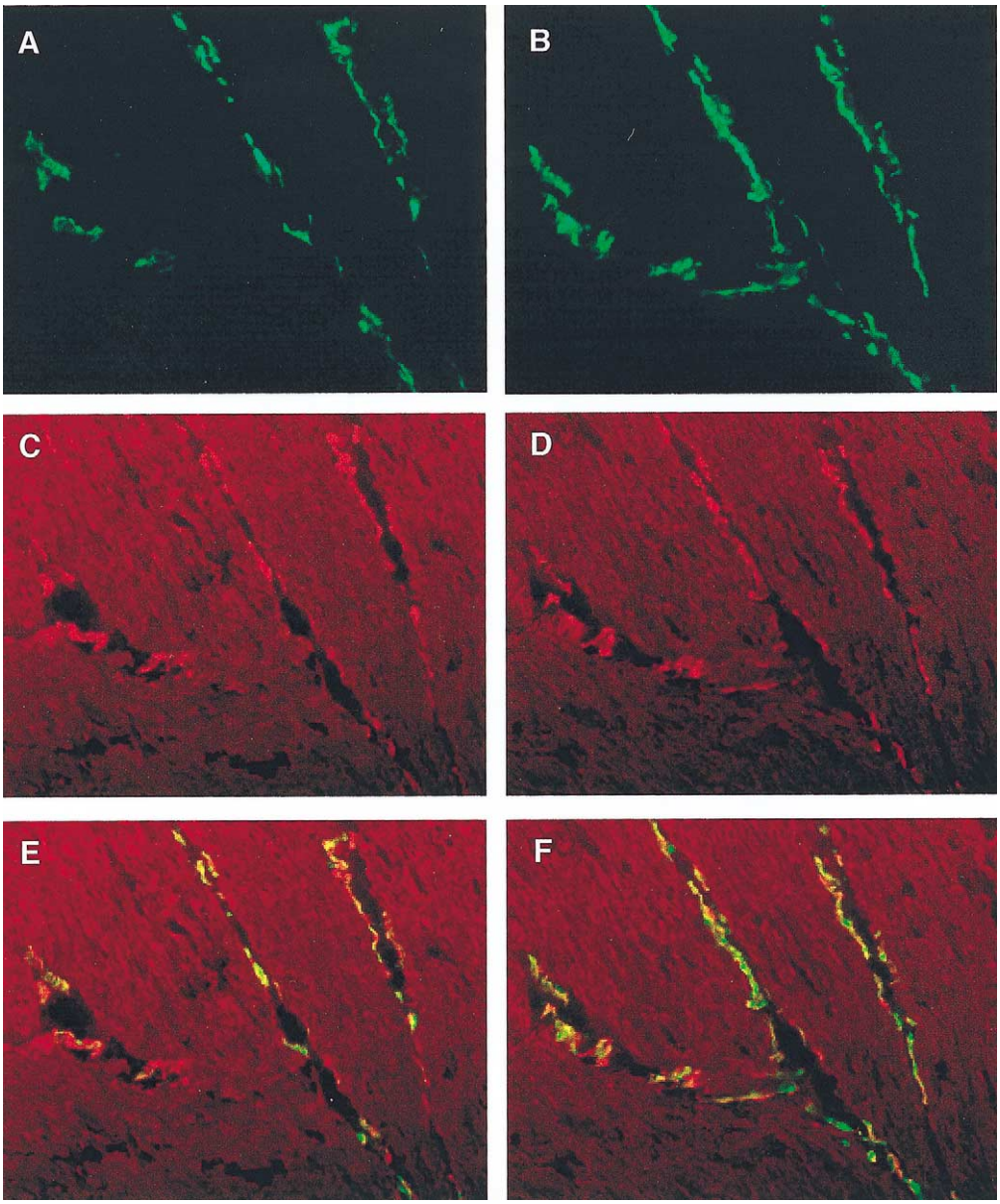

Fig. 6. Double-immunofluorescence staining of frozen chick ventricle sections at neonatal day 7 with antibodies against slow skeletal MHCs and desmin. Frozen sections were incubated with NA3 (A) or NA8 (B) and anti-desmin (C and D), followed by an FITC-conjugated (A and B) or a Texas Red-conjugated (C and D) secondary antibody. The NA3- or NA8-positive cells were located in the subendocardium of the ventricle (A and B). Anti-desmin stained uniformly the ventricular myocytes but also the same subendocardial structures (C and D). The FITC and Texas Red images were superimposed (E and F), showing that the NA3- and NA8-positive cells contained a high level of desmin.

sion of slow skeletal MHC 2 was detected beginning from the late embryonic stage to adult. This MHC isoform appeared to be expressed exclusively in Purkinje cells as its presence was not detected in the myocardium. Based on our in situ hybridization and immunohistochemistry data, we suggest that the previously uncharacterized slow tonic MHC localized in the conduction system (Gonzalez-Sanchez and Bader, 1985) is slow skeletal MHC 2.

It is difficult to define the functional significance of the expression of the slow skeletal MHC 2 in Purkinje cells. This MHC isoform is abundantly expressed in adult slow skeletal muscles (Page et al., 1992). DiMario and Stockdale (1997) have shown that when muscle fibers derived from myoblasts of the slow muscle are cocultured with the neural tube, they express slow skeletal MHC 2, while muscle fibers formed from myoblasts of the fast muscle origin continue to express only fast MHC. They demonstrated that slow skeletal MHC 2 gene expression in vitro was regulated by a combination of cell lineage (intrinsic mechanisms) and innervation (extrinsic mechanisms). Interestingly, slow skeletal MHC 2 expression was delayed or absent in denervated avian slow muscle in vivo (Lefeuvre et al., 1996). Kirby et al. (1980) have reported that during sympathetic innervation to the chick heart, nerves are first seen (in the heart) at ED 10. Autonomic innervation

of the conduction system in the developing chick heart may trigger the expression of slow skeletal MHC proteins in this system.

4.3. Expression of atrial MHC (slow skeletal MHC 3) in the developing conduction system

Our study showed that atrial (slow skeletal MHC 3) MHC was present in the early embryonic myocardium and then in ventricular Purkinje cells. Gourdie et al. (1995), using retroviral cell tagging procedure, have demonstrated that peripheral elements of the chick cardiac conduction tissue are derived directly from the embryonic myocardium. Based on the expression pattern of atrial MHC, we suggest a possibility that the cells expressing this MHC in the primary ventricular myocardium differentiate into conduction cells. Indeed, it is interesting to note that sympathetic innervation in developing chick heart correlates with or slightly precedes the appearance of the NA8- or NA3-positive cells (Figs. 4A, 5D).

4.4. Expression of multi-MHCs in the developing conduction system

Purkinje cells of the adult heart express not only two of the slow skeletal MHCs (atrial MHC and slow skeletal MHC 2) but also ventricular MHC. In addition, chick embryonic skeletal MHC was localized in adult Purkinje cells (unpublished data). Our previous study demonstrated that chick neonatal fast skeletal MHC appeared during early cardiogenesis and then was localized in the Purkinje fibers at the late embryonic and newly hatched stages, but not in the adult stage (Machida et al., 2000a). Thus these data indicate that the chick ventricle initially co-expresses ventricular MHC, atrial MHC and developmental types (embryonic and neonatal type) of skeletal MHCs but that later, the atrial and developmental types of skeletal MHCs are completely down-regulated. However, this regulation does not seem to occur in the ventricular conduction tissue (Purkinje myocytes). At the present time, the mechanisms involved in this down-regulation in the ventricular myocytes and in its absence in the Purkinje cells during development are unknown. Activities of various transcription factors in differentiating Purkinje cells have not been carefully analysed.

In summary, we have demonstrated a unique expression pattern of the slow skeletal MHC 2, distinct from atrial, ventricular and developmental types of skeletal MHCs in the conduction systems. The slow skeletal MHC 2 was exclusively expressed in differentiated conduction cells during development. It is essential to define the molecular mechanisms for the regulation of chick slow skeletal MHC 2 gene expression in chick conduction systems. Identification of the transcription factors of this gene is one of the urgent tasks.

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