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Distinct vascular and intestinal smooth muscle myosin heavy chain mRNAs are encoded by a single-copy gene in the chicken

Yoshio Hamada[1], Masashi Yanagisawa[2], Yoshinari Katsuragawa[2], John R. Coleman[1]*, Shuichi Nagata[3], Genji Matsuda[3] and Tomoh Masaki[2]#

[1]National Institute for Basic Biology, Okazaki, Aichi 444, Japan

[2]Institute of Basic Medical Sciences, University of Tsukuba, Tsukuba, Ibaraki 305, Japan

[3]Department of Biochemistry, Nagasaki University School of Medicine, Nagasaki, Nagasaki 852, Japan

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We examined whether the gizzard MHC gene is expressed in other smooth muscle tissues and, if so, whether there exist any smooth muscle MHC isoforms at the mRNA level. Northern blot analysis showed that the gizzard MHC gene was also expressed in the aorta and jejunum, but not in the pectoralis muscle or in fibroblasts. This indicates that striated muscle and non-muscle MHC isoforms are encoded in genes distinct from the smooth muscle MHC gene. Further, nuclease S1 mapping showed that the aortic smooth muscle MHC mRNA was distinct from the gizzard mRNA in the 5'-terminal coding region. Both of these mRNA species are expressed in the jejunum. These observations suggest that there exist at least two chicken smooth muscle MHC isoforms, vascular-type and intestinal-type, and that these isoforms are generated from a single-copy gene, probably by an alternative mRNA processing mechanism.

Myosin is an essential component of the contractile cytofilaments on striated muscle, smooth muscle and non-muscle tissues, and consists of two heavy chains of approximately 200 kDa and two pairs of light chains (1). The heavy chains possess the characteristic filamentous structure in the carboxyl-terminal halves of the polypeptide (the rod) and actin-activated ATPase activity in the amino terminal halves (the head). The existence and expression of structurally and functionally distinct isoforms of myosin heavy chain (MHC) have been extensively investigated in striated (skeletal and cardiac) muscle myosins (2,3). The sarcomeric MHC isoforms are individually encoded by the members of a highly conserved MHC multigene family in higher vertebrates; the expression of each isoform gene is regulated in a tissue specific manner according to the developmental and hormonal state(4). In smooth muscle, we and others also detected immunologically distinct MHCs in embryonic and adult chicken gizzard (5,6).

We have recently cloned and sequenced a full-length cDNA for chicken gizzard smooth muscle MHC (7). We have shown that the smooth muscle MHC is evolutionarily distinct from

* Present address: Division of Biology and Medicine, Brown University, Providence, RI 02912.

To whom correspondence should be addressed.

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

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sarcomeric MHCs and that it is encoded by a single-copy gene, i.e., there exists no multigene family comparable to that for the sarcomeric MHCs. In this study, we characterized the MHC mRNA from various chicken smooth muscle tissues by using gizzard cDNA fragments as probes. The analysis showed that the aortic MHC mRNA differs from the gizzard counterpart in its 5'-terminal coding sequence, thereby suggesting the existence of at least two smooth muscle MHC isoforms.

MATERIALS AND METHODS

Isolation of DNA and RNA

High molecular weight DNA was extracted from 10-day old chicken whole embryos by the proteinase K / phenol method (8). Smooth muscle tissues of gizzard, jejunum and aorta, as well as pectoralis skeletal muscle, were dissected from an adult chicken immediately after death and separated from connective tissues. Skin fibroblasts were obtained from 10-day old chicken embryos and cultured in monolayers in Eagle's minimum essential medium supplemented with 5% fetal calf serum at 37°C under 5% CO_2 / 95% air. Tissue and cellular RNA was extracted by the guanidinium / hot phenol method (9) and poly $(A)^+$ RNA was isolated by oligo (dT) cellulose column chromatography (8).

Preparation of cDNA Probes

Chicken gizzard smooth muscle MHC cDNA clone GMH-5 / GMH-6 (7) was digested with appropriate restriction endonucleases (see text) and the restriction fragments were separated by agarose gel electrophoresis. Desired fragments were purified from the gels with GeneClean DNA purification kits (Bio-101, Inc.). For Southern and Northern analysis, the cDNA fragments were labeled to a specific activity of 5 x 10^8 cpm/mg with [α-^{32}P]dCTP (3,000 Ci/mmol; Amersham) by the random-primed labeling method (10). For S1 nuclease mapping, 5'-end labeling of the cDNA fragments was carried out with the T4 polynucleotide kinase and [γ-^{32}P] ATP (3,000 Ci/mmol; Amersham) to a specific activity of 5x10^6 cpm/μg. 3'-end labeling of the 5'-terminal *Bam*HI fragment was carried out by T4 DNA polymerase with [α-^{32}P] dCTP to a specific activity of 4x10^6 cpm/μg.

Southern and Northern Blot Analysis

Genomic DNA (10 μg) was digested to completion with restriction endonucleases, electrophoresed through 0.8 % agarose gels, and transferred to GeneScreen Plus membranes (New England Nuclear) in 0.4 M NaOH / 0.6 M NaCl (11). Poly$(A)^+$ RNA (1 μg) was electrophoresed through formaldehyde / 1 % agarose gels (8) and transferred to GeneScreen Plus membranes as recommended by the manufacturer. The membranes were hybridized to the labeled cDNA probes (40 ng/ml) at 42°C for 16 hr in a hybridization solution containing 50 % formamide / 1 M NaCl / 1 % $NaDodSO_4$ / 250 μg/ml sonicated salmon sperm DNA. The membranes were washed in 0.3 M NaCl / 30 mM Na citrate (pH 7.5) / 1 % NaDodSo4 at 65°C and autoradiographed with intensifying screens for 12-72 hr at -70°C.

S1 Nuclease Mapping

Poly $(A)^+$ RNA (5-20 mg) was combined with 10^5 cpm of 5'-labeled cDNA probe, coprecipitated with ethanol, and dissolved in 20 μl of hybridization solution containing 80 % formamide / 0.4 M NaCl / 50 mM Pipes (pH 6.4) / 1 mM EDTA. The samples were heated to 85°C for 10 min and subsequently incubated at 48°C for 8 hr. They were then adjusted to 0.2 M NaCl / 2 mM $ZnSO_4$ / 30 mM NaOAc (pH 4.6) / 10,000 U/ml S1 nuclease (Bœhringer) in a reaction volume of 200 μl, and further incubated at 37°C for 30 min. Yeast tRNA (10 μg) was added as carrier and nucleic acids were precipitated with ethanol, dissolved in 90 % formamide, heated at 90°C for 3 min, and electrophoresed through 7.5 M Urea / 6 % polyacrylamide gels. The gels were placed on Whatman 3MM papers, dried under vacuum and autoradiographed for 12-24 hr at room temperature.

RESULTS AND DISCUSSION

To reconfirm that chicken gizzard smooth muscle MHC is encoded by a single-copy gene, genomic Southern blots were probed with a short restriction fragment of chicken gizzard MHC

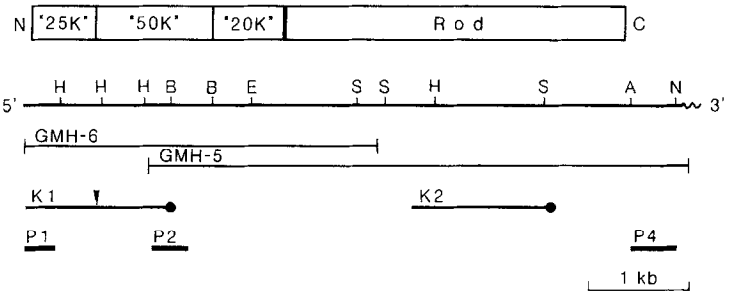

Figure 1. Chicken gizzard smooth muscle MHC cDNA. The extent of the cDNA clones (GMH-5 and GMH-6) and of the probe fragments used in this study (K1 and K2 for S1 nuclease mapping; P1, P2 and P4 for DNA/RNA blot analysis) is shown under the restriction map of the cDNA (7). Closed circles represent the labeled 5'-ends of the anti-sense strands of S1-mapping probes; the cleavage site of probe K1 (see text and Fig. 4b) is marked by an arrowhead. H, *Hind*III; B, *Bam*HI; E, *Eco*RI; S, *Sst*I; A, *Acc*I; N, *Nco*I. The MHC polypeptide domains are shown above the map; 25K, 50K and 20K represent proteolytic subfragments of the MHC head.

cDNA. The probe was the 0.36-kb *Bgl*II(1198) / *Pst*I(1561) fragment of GMH-6 (nucleotide residues are numbered according to (7)), which encodes a portion of the 50 K subfragment of the MHC head (P2 in Fig. 1). As expected, a single restriction fragment of genomic DNA was detected in each lane (Fig. 2a). The possible existence of tandemly repeated multiple loci (like those encoding rat alpha- and beta-cardiac MHC (12)) can be virtually excluded, since the detected genomic fragments were relatively short in comparison with the size of the mRNA. In contrast, 7-10 genomic fragments were detected when the same membrane was rehybridized with a corresponding short fragment of chicken skeletal MHC cDNA under the same stringency (not shown). These findings indicate that gizzard MHC is indeed encoded by a single-copy gene and that there exists no highly conserved multigene family for chicken smooth muscle MHC.

To examine whether this MHC gene (GMH locus) is expressed in tissues other than gizzard smooth muscle, Northern blots were probed with a fragment containing the 3' non-coding region of gizzard MHC cDNA (*Acc*I(5999) / *Nco*I(6442) fragment of GMH-5; P4 in Fig. 1). A hybridizing transcript of approximately 6.9 kb was detected in the gizzard, aorta and jejunum, but not in the pectoralis muscle or in fibroblasts (Fig. 3a). Taken in conjunction with the results shown in Fig. 2a, it is clear that the signal represents transcripts from the GMH locus. This point was further confirmed by the S1 nuclease analysis (see below). Similar amounts of the transcript were detected in the three smooth muscle tissues (Fig. 3a), suggesting that this mRNA encodes the most abundant species of MHC not only in the gizzard (7) but also in the aorta and jejunum. These observations indicate that the GMH locus is distinct from the non-muscle MHC gene and probably is the only smooth muscle MHC gene in the chicken.

We performed nuclease S1 mapping to characterize further the GMH-transcript in these smooth muscle tissues. S1 analysis with the 5'- end labeled *Rsa*I(3793) / *Nar*I(5213) fragment of GMH-5, which encodes for a middle portion of the MHC rod (K2 in Fig. 1), demonstrated that the gizzard, aortic and intestinal mRNAs can fully protect the probe (Fig. 4a). This confirms our conclusion that the same (GMH) locus is expressed in these tissues. However, similar analyses with the 5'-terminal *Bam*HI(1385) fragment of GMH-6 (K1 in fig.1) demonstrated tissue specific

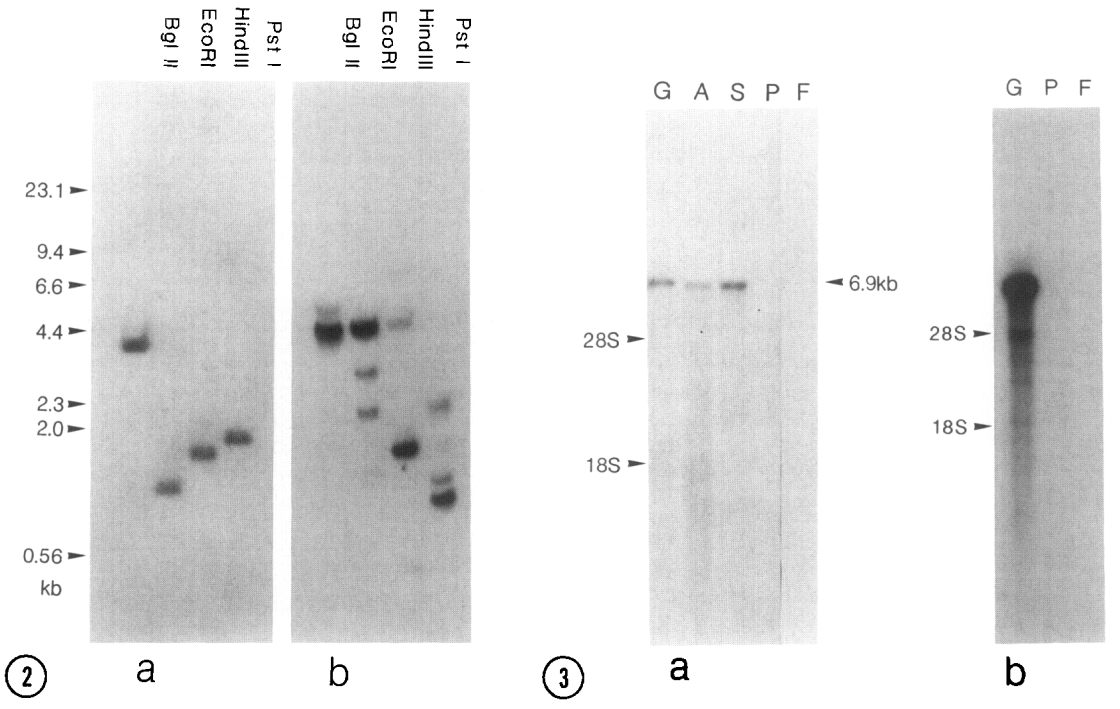

Figure 2. Southern blot analysis of chicken genomic DNA probed with chicken gizzard MHC cDNA P2 (a) and P1 (b). See Fig. 1 and text for description of probes. Lambda DNA *Hind*III digest was used as molecular weight markers.

Figure 3. Northern blot analysis of RNA from various chicken tissues. Poly (A)+RNA (1 mg) from adult chicken gizzard (G), aorta (A), jejunum (S for Small intestine), pectoralis muscle (P), and cultured skin fibroblasts (F) were elctrophoresed and hybridized with chicken gizzard MHC probe P4 (a) and P1 (b, overexposed) described in Fig. 1.

transcription of the MHC gene. As expected, the fully protected probe was detected in the gizzard and jejunal samples with the 5'-end labeled probe. However, the aortic mRNA protected only an approximately 750-base portion of the probe. This 750-base signal was also detected in the jejunal and weakly in the gizzard samples (Fig. 4b). In order to examine whether the MHC mRNA found in the aorta is generated from the single GMH gene locus by alternative splicing, we performed S1 mapping with the same BamHI fragment (K1) where the 3'-end instead of the 5'-end was labeled (Fig. 4c). Aortic mRNA protected a 700-base portion of the probe. This result indicates that the aortic MHC mRNA has identical 700 base long sequences with the gizzard MHC mRNA in the 5'-terminal region. The fully protected probe was detected also in this case. We speculate that it was caused by DNA-DNA duplex formation because the probe labeled by T4 DNA polymerase is more resistant to S1-nuclease than a kinase-treated probe at the protruding end. We conclude from these observations that two distinct types of smooth muscle MHC mRNA, intestinal (Gizzard) and vascular (aortic), were generated tissue-specifically from the single GMH locus.

It is most probable from the findings above that there exist alternative exon(s) and/or alternative transcription initiation sites in the 5'-terminal region of the GMH locus. To characterize further this region of the GMH locus, we probed a genomic Southern blot with a fragment

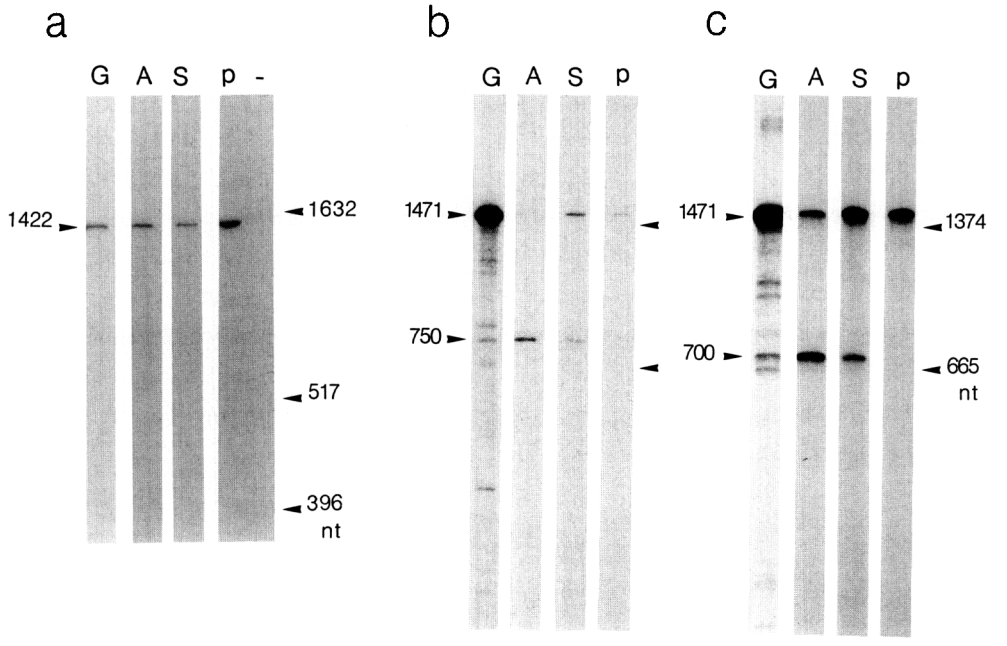

Figure 4. S1 nuclease mapping of RNA from various smooth muscle tissues. Poly(A)+RNA from adult chicken gizzard(G), aorta(A) and jejunum(S) were subjected to S1 mapping as described in Materials and Methods with gizzard MHC cDNA probe K2(a), K1(5'-end labeled)(b) or K1(3'-end labeled)(c). See Fig. 1 and text for description of probe. p, no S1 nuclease added (full length probe); -, yeast tRNA added instead of poly(A)+RNA (negative control). Kinase-labeled pBR322 *Hind*I(a) and *Sau*3AI (b and c) digests were used as molecular weight markers.

restricted to the proposed alternative region of the MHC cDNA (Fig. 2b). the probe used here was the 0.29-kb 5'-terminal *Eco*RV(220) fragment of GMH-6, which includes the 5' non-coding region and the coding sequence for the amino-terminal 74 amino acid residues of the MHC (P1 in Fig. 1). In contrast to the results shown in Fig. 2a, in addition to one strongly hybridizing fragment, this probe detected one or more segments that hybridized more weakly. The amino-terminal polypeptide encoded in this probe is the least conserved peptide region within the gizzard MHC head, when aligned with other known MHC sequences (7,13-15). Therefore, considered together with the findings shown in Fig. 2a, the more weakly hybridizing signals in Fig. 2b are unlikely to represent other MHC loci unrelated to the GMH locus (e.g., a non-muscle MHC gene). In fact, no transcripts capable of hybridizing to probe P1 were detected by Northern analysis of pectoralis or firoblast RNA (Fig.3b). Although further characterization of the GMH locus is necessary for definitive conclusions, it is most likely that these signals represent tandemly located alternative exon(s) in the 5'-region of the smooth muscle MHC gene.

Recently two types of smooth muscle MHC cDNA were detected in rabbit uterus which correspond to the two isoforms SM_1 and SM_2 at the protein level (16). These two types have different carboxyl termini. The two types are expressed also in adult rabbit aorta (17). However, the two types of chicken smooth muscle MHC cDNA described in the present article have different sequences in head region of the MHC.

In the context of an alternative mRNA processing hypothesis, it is worth noting that the boundary of the alternative and fixed regions of the smooth muscle MHC mRNA coincides (at the protein level) with the junction of the 25K and 50K domains of the MHC head (i.e., the K1-cleavage site, Fig. 1). Exon/intron junctions are known to be located often at the boundaries of protein domains (18), and an exon/intron junction is actually located at the 25K / 50K domain boundary in several vertebrate MHC genes of which the sequences are available (14,15). Since the proposed alternative regions of the smooth muscle MHC mRNA include the coding sequence for approximately 220 residues of the amino-terminus of the MHC, it is likely that not only the nucleotide sequences but also the encoded amino acid sequences are actually different between the vascular- and intestinal-type mRNAs. As the 25K domain constitutes a major part of the ATPase site of MHC (7), variations in the primary structure of this region would contribute to differences in the physiological properties of vascular and intestinal smooth muscles.

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