Lampeys Have a Single Gene Cluster for the Fast Skeletal Myosin Heavy Chain Gene Family

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Abstract

Muscle tissues contain the most classic sarcomeric myosin, called myosin II, which consists of 2 heavy chains (MYHs) and 4 light chains. In the case of humans (tetrapods), a total of 6 fast skeletal-type MYH genes (MYHs) are clustered on a single chromosome. In contrast, torafugu (teleost) contains at least 13 fast skeletal MYHs, which are distributed in 5 genomic regions; the MYHs are clustered in 3 of these regions. In the present study, the evolutionary relationship among fast skeletal MYHs is elucidated by comparing the MYHs of teleosts and tetrapods with those of cyclostome lampeys, one of two groups of extant jawless vertebrates (agnathans). We found that lampeys contain at least 3 fast skeletal MYHs, which are clustered in a head-to-tail manner in a single genomic region. Although there was apparent synteny in the corresponding MYH cluster regions between lampeys and tetrapods, phylogenetic analysis indicated that lamprey and tetrapod MYHs have independently duplicated and diversified. Subsequent transgenic approaches showed that the 5′-flanking sequences of Japanese lamprey fast skeletal MYHs function as a regulatory sequence to drive specific reporter gene expression in the fast skeletal muscle of zebrafish embryos. Although zebrafish MYH promoters showed apparent activity to direct reporter gene expression in myogenic cells derived from mice, promoters from Japanese lamprey MYHs had no activity. These results suggest that the muscle-specific regulatory mechanisms are partially conserved between teleosts and tetrapods but not between cyclostomes and tetrapods, despite the conserved synteny.

Citation: Ikeda D, Ono Y, Hirano S, Kan-no N, Watabe S (2013) Lampeys Have a Single Gene Cluster for the Fast Skeletal Myosin Heavy Chain Gene Family. PLoS ONE 8(12): e85500. doi:10.1371/journal.pone.0085500

Editor: Sylvie Rétaux, CNRS, France

Received October 3, 2013; Accepted December 5, 2013; Published December 20, 2013

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Funding: This work was supported by the Japan Society for the Promotion of Science KAKENHI Grant Number 23780214 (https://www.jsps.go.jp/ english/e-grants/). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing interests: The authors have declared that no competing interests exist.

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Introduction

Myosin is a ubiquitous actin-based motor protein that drives a wide range of motile processes in eukaryotic cells. Muscle tissues contain the most classic sarcomeric myosin, called myosin II, which consists of 2 heavy chains (MYHs) and 4 light chains (reviewed in 1). MYH contains 2 loop structures, loops 1 and 2, which are located in its N-terminal half, called the motor domain or subfragment-1 (S1), at the ATP- and actin-binding sites, respectively. MYH transduces chemical energy produced by splitting ATP to mechanical energy through the C-terminal half, called a rod, which has an α-helical coiled-coil structure.

Various types of sarcomeric MYH genes (MYHs) have been found in mammalian muscles. For example, 2 fast skeletal MYHs, called embryonic MYH3 and perinatal MYH8, are expressed during pre- and postnatal development, respectively [2]. Three MYHs, types IIa (MYH2), IIb (MYH4) and IId/x (MYH1), are expressed in adult fast muscle. Extraocular MYH (MYH13) is expressed in extrinsic eye muscle. These 6 fast skeletal MYHs are clustered on a single chromosome, and this chromosomal organization is highly conserved among diverse mammalian species [3,4], suggesting their functional importance for temporal and spatial expression during development. However, the regulatory mechanisms involved in the gene expression and functional differences of encoded myosin molecules remain mostly unknown.

More diverse sarcomeric MYHs have been found in the fast muscle of teleosts, such as the common carp Cyprinus carpio [5-8], zebrafish Danio rerio [9-12], medaka Oryzias latipes [13-15] and torafugu Takifugu rubripes [16-19], at the genomic and transcriptional levels. In addition, genomic structural and syntenic analyses have revealed that at least 13 torafugu fast skeletal MYHs are distributed in 5 genomic regions, 3 of which contain multiple MYHs forming clusters A, B and C [18] (Figure
S1). These clusters are also found in the green spotted pufferfish Tetraodon nigroviridis, zebrafish and medaka [12,15,18], implying that such genomic arrangement is conserved in teleosts. Interestingly, human fast skeletal MYHs form a single cluster, but their syntenic regions in torafugu, green spotted pufferfish and medaka are duplicated, although each locus contains a single MYH [18].

Based on phylogenetic, genomic structural and syntenic analyses, teleost MYHs in clusters A, B and C were defined as those of fast skeletal types A, B and C, respectively; non-clustered teleost MYHs and those in the human cluster were defined to be of the fast D type [18] (Figure S1). Moreover, mammals, including mouse [3], dog [20] and the amphibian Xenopus tropicalis [18] (tetrapod) have a single syntenic region similar to that of humans but not similar to that of teleosts. However, the evolutionary relationship of fast skeletal MYHs among vertebrates and the functional significance of their cluster formation have remained unknown.

Lampreys and hagfishes are two groups of extant jawless vertebrates (agnathans). These vertebrates belong to a monophyletic group of cyclostomes and are thought to have diversified from a common ancestor shared with gnathostomes (jawed vertebrates) 535-462 million years ago [21]. Due to their unique phylogenetic history among vertebrates, evolutionary and developmental studies have been carried out extensively on lampreys (reviewed in [21,22]). Two fast skeletal MYHs (LjMyHC1 and LjMyHC2) and 2 non-muscle MYHs (LjMyHC3 and LjMyHC4) have been identified from an embryonic cDNA library of Japanese lamprey Lethenteron camtschaticum (renamed from Lethenteron japonicum in the NCBI Taxonomy database) [23]. Although phylogenetic and gene expression analyses on Japanese lamprey MYHs were partially carried out [23], information about their gene structure and location in the genome that would be helpful in increasing our knowledge about the evolutionary relationship of vertebrate MYHs is not available.

In the present study, to elucidate the evolutionary relationship among vertebrate fast skeletal MYHs, we compared those from teleosts and tetrapods with their lamprey counterparts. We found that lampreys possess at least 3 fast skeletal MYHs, which are clustered on a single chromosome in a head-to-tail manner as in tetrapods. Subsequent transgenic and transfection studies on the function of the 5′-flanking regions of Japanese lamprey fast skeletal MYHs demonstrated that these genes have the same transcriptional regulation as those of teleost MYHs but different transcriptional regulation from those of tetrapod fast skeletal MYHs.

**Materials and Methods**

**Ethics Statement**

Zebrafish specimens were maintained under standard husbandry conditions at the School of Marine Biosciences, Kitasato University, and animal experiments were approved by the Animal Experimentation Committee of the School of Marine Biosciences, Kitasato University. The permit number for this study is 2231.

**Experimental Fish**

Mature specimens of the Japanese lamprey, Lethenteron camtschaticum, which were collected in a tributary of the Miomote River, Niigata Prefecture, Japan, during the breeding season in early June, were purchased from local fishermen. Fertilized eggs prepared from Japanese lamprey were incubated in freshwater at 10°C for embryonic development. Embryonic staging was based on the report by Tahara [24]. Zebrafish specimens were used for an in vivo promoter assay in which their embryos were incubated at 26°C.

**Analysis of a Contiguous Genomic Region Containing Fast Skeletal MYHs from the Sea Lamprey Genome**

The sea lamprey Petromyzon marinus genome database (Petromyzon_marinus-3.0) was screened with the full-length cDNA sequence of a fast skeletal MYH from Japanese lamprey [23] (AB126173) using the BLASTn program. A BLAST search was carried out on the genomic database using the Ensembl Genome Browser (http://www.ensembl.org/index.html). From various positive hits, we chose 4 supercontigs, which were found to contain partial sequences of MYHs. The exon-intron structures of these MYHs were predicted by manual inspection. The recently released sea lamprey genome database (Petromyzon_marinus-7.0) was also used to verify the results obtained with the Petromyzon_marinus-3.0 database.

Coding exons of torafugu, Takifugu rubripes, were predicted by manual inspection from the genomic sequence of scaffold_139 (database version FUG4; http://www.ensembl.org/index.html). Moreover, to compare the genomic organization of the MYHs with that of other chordate lineages, we also analyzed the genome databases of the amphioxus Branchiostoma floridae (database version Brafl1; http://genome.jgi-psf.org/Brafl1/Brafl1.home.html) and the tunicate Ciona savignyi (database version CSAV 2.0; http://www.ensembl.org/Ciona_savignyi/Info/Index).

**Shotgun Sequencing of the MYH Cluster Region in Japanese Lamprey**

DNA was extracted from the muscle of an individual of Japanese lamprey using a conventional phenol/chloroform method. The genomic region containing an MYH cluster in Japanese lamprey was divided into 14 parts by using the long PCR method. Primers (Table S1) were designed according to the sequences obtained from the sea lamprey genome database and cDNA sequences of Japanese lamprey MYHs [23] (AB126173 and AB126174). PCR was carried out with PrimeSTAR GXL DNA Polymerase (Takara, Otsu, Japan) at a reaction volume of 50 μl according to the manufacturer's instructions. To construct a shotgun library, PCR products were cut into short fragments with Nebulizers (Life Technologies). Randomly selected shotgun clones in each library were sequenced using a BigDye Terminator v3.1 Cycle Sequencing Kit (Life Technologies) and ABI PRISM 3730 and 3130 xl (Life Technologies). Sequences were assembled by using the Phred/Phrap/Consed [25–27] sequence analysis software.
package. The determined sequences were processed using Phred, trimmed for quality, screened for vector sequences and assembled using Phrap. Quality scores were assigned automatically, whereas electropherograms and assembly were viewed and verified for accuracy using Consed.

During manuscript preparation, Mehta et al. [28] published and released the entire genome sequence of the Japanese lamprey. Therefore, to identify the MYH cluster and neighbor genes in the genomic sequence, we analyzed the genome database of the Japanese lamprey (http://jangreypegenome.imcb.a-star.edu.sg/).

Expression Analysis of Japanese Lamprey MYHs

Total RNAs were extracted from the muscle tissue of an adult Japanese lamprey and embryos at stages 21, 25 and 27 using ISOGEN (Nippon Gene, Tokyo, Japan). RT-PCR was performed using primers specific to Japanese lamprey MYHs 1, 2 and 5 (Table S2A) as described previously [14]. The amplification of Japanese lamprey cytoplasmic actin LgCA1 (AB060287) was carried out to confirm first-strand cDNA synthesis.

Phylogenetic Tree Construction

The deduced amino acid sequences of Japanese lamprey MYHs 1, 2 and 5 were aligned using the multiple sequence alignment program CLUSTAL W [29]. The neighbor-joining (NJ) tree was constructed using the Poisson correction model on Mega 5 software [30]. Bootstrap resampling analysis from 1000 replicates was used to evaluate internal branches. The maximum-likelihood (ML) tree was constructed by the quartet puzzling algorithm using TREE-PUZZLE software [31] with the following parameter settings and estimation methods: the JTT substitution model, the uniform rate heterogeneity model and exact and slow parameter estimation. Reliability values for respective internal branches were expressed as a percentage of how often the corresponding cluster was found among the 1000 intermediate trees. Lc MYHs from the Japanese lamprey L. camtschaticum were compared with Hs MYH1 (adult fast IId/x, AB720829), Hs MYH3 (embryonic fast, NP_002461), Hs MYH3 (embryonic fast, NP_002462), Hs MYH7 (beta cardiac, NP_000248) and Hs MYH14/7B (slow A, NP_065935) from human, Homo sapiens, and Ai MYH (striated muscle, CAA9247) from the bay scallop, Argopecten irradians. MYH sequences of the torafugu, Takifugu rubripes (Tr), were cited from our previous data [16-19].

Preparation of GFP and DsRed Constructs and Microinjection

Sequences in the 5′-flanking region of Japanese lamprey zeafish and mouse MYHs were amplified from genomic DNA by PCR and were cloned into the promoter-less pGL3-Basic plasmid vector (Promega, Madison, WI) with the In-Fusion HD Cloning Kit (Clontech) (primer sequences are listed in Table S2D). Cell cultures and transfections were performed as described by Meissner et al. [32] with some modifications. Mouse C2C12 myoblasts were provided by the RIKEN BRC through the National Bio-Resource Project of the Ministry of Education, Culture, Sports, Science and Technology, Japan. The cells were cultured on gelatinized culture plates in a growth medium consisting of complete Dulbecco's modified Eagle's medium (DMEM) with high glucose (Nacalai Tesque, Kyoto, Japan) supplemented with 10% fetal bovine serum (Biowest, Nuaille, France), 100 U/ml penicillin, 100 μg/ml streptomycin and 2.5 μg/ml amphotericin B. C2C12 cells were
transfected at 70-90% confluency with 0.5 μg of promoter DNA, 0.05 μg of pRL-TK (Promega) and 1.5 μl/μg DNA of X-tremeGENE 9 transfection reagent (Roche, Basel, Switzerland). The transfection medium was replaced after 24 h with a differentiation medium consisting of DMEM supplemented with 2% horse serum (Biowest). Myotubes were harvested after 3 or 5 days. Undifferentiated myoblasts were harvested 24 h after transfection.

Luciferase Assays

Cells were lysed in 100 μl of Passive Lysis Buffer (Promega). Twenty microliters of lysate was subjected to luciferase assay using the Dual-Luciferase Reporter Assay System (Promega) and a Lumat LB 9507 luminometer (Berthold Technologies, Bad Wildbad, Germany) according to the manufacturer’s instructions. All experiments were performed in triplicate, and the data are presented as the mean ± SD. Student’s t-test was used to determine statistical significance.

Multiple Alignment of the 5’ Flanking Sequences of MYHs

The 5’ flanking sequences of Japanese lamprey, zebrafish and mouse MYHs were aligned with the Shuffle-LAGAN program [33]. The alignments were compared with VISTA [34] using the Japanese lamprey MYH1 and mouse MYH1 (Ild/x) as references with Calc Window and Min Cons Width of 20 bp, respectively. To identify putative transcription factor binding sites (TFBSs) in the identified conserved non-coding elements (CNEs), we used the MATCH program to search the TRANSFAC database (http://www.gene-regulation.com/cgi-bin/pub/programs/match/bin/match.cgi) [35], where the parameters were set to the vertebrates matrix (high quality) and the cut-off was set to minimize false negative matches (minFN).

Results

Analysis of a Contiguous Genomic Region Containing Fast Skeletal MYHs from the Sea Lamprey Genome

We searched the sea lamprey genome database (Petromyzon_marinus-3.0) using the cDNA sequence of Japanese lamprey fast skeletal MYH as a probe and found 6 regions encoding the partial sequences of MYHs in 5 contigs (Figure S2). Although vertebrate sarcomeric MYHs have two 5’-untranslated exons [36], it was difficult to use these exons without sequencing their transcripts. Therefore, these exons were excluded from analysis. Moreover, contigs 6974 and 38608 were analyzed as one contig because these two partially overlap.

At the 3’-site of contig 2034 and the 5’-site of contig 15297, we found partial sequences that could be encoded by a single MYH referring to Japanese lamprey LjMyHC2 (accession #AB126174 in the DDBJ/EMBL/GenBank databases). We also found that the 3’-site of contigs 6974/38608 and the 5’-site of contig 39193 contained partial sequences that could be encoded by another MYH homologous to Japanese lamprey LjMyHC1 (AB126173). At the 3’-site of contigs 15297 and the 5’-site of contig 6974/38608, we found partial MYH sequences that had not been reported before. For convenience in the present study, we renamed LjMyHC1 and LjMyHC2 as Japanese lamprey MYH1 and MYH2, respectively. Because Kusakabe et al. [23] reported 2 non-skeletal MYHs (LjMyHC3 and LjMyHC4) in the Japanese lamprey, we named this newly identified MYH as MYH5. As summarized in Figure S2, sea lamprey fast skeletal MYHs are strongly suggested to be clustered on a single chromosome in a head-to-tail manner as in their mammalian counterparts.

In addition, we searched the recently released sea lamprey genome database (Petromyzon_marinus-7.0) to verify our results described above. Although we found no fast skeletal MYH other than those obtained with the Petromyzon_marinus-3.0 database, scaffold GL477325 contained not only 2 MYHs (MYH2 and MYH5) but also a growth arrest-specific protein 7 gene (GAS7) connected to the 3’-site of the MYH cluster (Figure S2). Scaffold GL477325 contains a region with a long sequence gap, and contigs 6974/38608 and 39193 are expected to be located in this gap region (Figure S2). Although the length of the intergenic region between MYH1 and GAS7 is estimated to be 20 kb, the presence of other genes between MYH1 and GAS7 remains a possibility. GAS7 is also located next to the 3’-site of the fast skeletal MYH cluster of tetrapods such as humans and mice and the 3’-site of non-clustered MYHs of torafugu, green spotted pufferfish and medaka [18] (Figure S1).

Analysis of a Contiguous Genomic Region Containing Sarcomeric MYHs from the Amphioxus and Tunicate Genomes

We searched the Branchiostoma floridae and Ciona savignyi genome databases using the cDNA sequence of Japanese lamprey fast skeletal MYH as a probe and found that both B. floridae and C. savignyi possessed MYH clusters (Figure S1). As shown in Figure S1, at least 12 MYHs are clustered in scaffold_192 of the B. floridae genome database. Furthermore, C. savignyi MYHs are distributed in 2 genomic regions, both of which contain at least 2 MYHs (Figure S1). However, there is no synteny among vertebrate, amphioxus and tunicate MYH cluster regions (Figure S1).

Exon-Intron Organization of Japanese Lamprey MYHs

As shown in Figure S2, the sequences obtained from the sea lamprey genome database only fragmentarily covered the MYH cluster region. To analyze the full length of this region, we sequenced a 106-kb region containing all 3 fast skeletal MYHs of Japanese lamprey. Fourteen fragments in the MYH cluster region were amplified by the long PCR method (PCR primer sequences, their target regions and product sizes are listed in Table S1), and DNA sequences of respective fragments totally covering the full length of the MYH cluster region were determined by shotgun sequencing (AB720829). The resulting physical map and gene organization of MYH2-MYH5-MYH1 are shown in Figure 1.

Detailed analysis revealed that Japanese lamprey MYHs 1, 2 and 3, each consisting of 39 coding exons, were 20 kb, 27 kb and 41 kb in length (start to stop codon), respectively (Figure 1)
and Table S3). As shown in Table S3, the exon structures of fast skeletal MYHs of Japanese lamprey, humans and teleosts are highly conserved. The pattern of split codons (intron phase) is identical among all fast skeletal MYHs. Moreover, the respective exon sizes are identical except in 4 regions (exons 3, 7, 16-17 and 40-41) (Table S3). As reported previously, exons 3 and 40/41 encode the N- and C-termini, respectively [36]. The C-terminal exon 40 in human MYHs 1, 2, 4 and 8 corresponds to exons 40-41 in human MYHs 3 and 13 and teleost MYHs (Table S3) [36]. All Japanese lamprey MYHs possess exons 40-41, as is the case in human MYHs 3 and 13 and teleost MYHs (Table S3). The varied sizes of exons 7 and 16-17 encode variable lengths of loops 1 and 2, respectively [1,36].

Although Japanese lamprey and sea lamprey are closely related, their genomic sequences are not identical. Therefore, we compared coding sequences of each MYH from Japanese lamprey and sea lamprey. The alignment used to calculate the sequence identities is provided in FASTA format (Dataset S1). Due to the lack of sea lamprey genome sequences, exons 12-20, 23-26 and 35-37 of MYH1; exons 26 and 35 of MYH2; and exons 7-9, 17, 18, 20, 27 and 32 of MYH5 were excluded from analysis. The coding sequence identities of Japanese lamprey and sea lamprey MYHs 1, 2 and 5 are 97.1, 97.3 and 96.2%, respectively.

During manuscript preparation, Mehta et al. [28] published and released the whole genome sequence of the Japanese lamprey. We found that scaffold00065 contained the fast skeletal MYH cluster (Figure S1). However, due to sequence gap regions, exons 2-15 of MYH1 and exons 6, 17 and 21-23 of MYH5 were not found in scaffold00065. We also found that MAP2K4 and GAS7 are located next to the 5′-site and 3′-site of the fast skeletal MYH cluster, respectively (Figure S1). MAP2K4 is also located to the 5′-site of the fast skeletal MYH cluster of tetrapods and the 5′-site of non-clustered MYHs of teleosts [18] (Figure S1).

**Expression Analysis of Japanese Lamprey MYHs**

Although RT-PCR was carried out to detect the expression of Japanese lamprey MYH5, no transcript was observed in adult muscle or whole embryos at developmental stages 21, 25 and 27 (Figure S3). In contrast, the primers used for MYH1, MYH2 and cytoplasmic actin LjCA1 could detect the transcripts in all samples tested (Figure S3).

**Phylogenetic Analysis**

The full-length amino acid sequences deduced from Japanese lamprey and human MYHs were subjected to phylogenetic analysis. We used amino acid sequences of Japanese lamprey MYHs deduced from the full length of the MYH cluster region (AB720829). Because the full sequences of fast A (Tr MYH_M86)-, B (Tr MYH_M2528 and M1034)-, C (Tr MYH_M743)- and D (Tr MYH_M454)-type MYHs were only available from torafugu [18], these were cited as references (Figure 2). In addition, the cardiac and slow skeletal MYHs of humans and torafugu and the sarcomeric MYH of bay scallop were used as outgroups.

The alignment used to construct the phylogenetic tree is provided in FASTA format (Dataset S2). The topology obtained...
from the neighbor-joining tree is identical to that obtained from the maximum-likelihood tree, implying that the trees obtained are highly reliable (Figure 2). Phylogenetic analysis indicated that the 3 Japanese lamprey MYHs, including the newly identified Lc MYH5, are classified as a fast skeletal type but not as cardiac or slow skeletal types (Figure 2). Fast skeletal MYHs of humans, torafugu and Japanese lamprey formed 2 major clades, gnathostomes and cyclostomes, in the phylogenetic tree obtained in the present study (Figure 2). Therefore, there is no strict orthology between any Japanese lamprey MYH and any gnathostome MYH.

Expression of Transgenes Containing the 5′-Flanking Sequences of Japanese Lamprey MYHs in Zebrafish

The 5′-flanking sequences of the medaka muscle actin genes are known to drive the expression of the reporter gene encoding green fluorescent protein (GFP) in the striated muscles of Japanese lamprey embryos [37]. These results imply that the 5′-flanking sequences of Japanese lamprey muscle-specific MYHs also drive the reporter gene expression in teleosts. To examine this possibility, the 5′-flanking sequences of Japanese lamprey fast skeletal MYHs were fused to the gene encoding GFP or DsRed and introduced into fertilized zebrafish eggs by microinjection. We used the regions of approximately 5, 3 and 2 kb from the start codon for Japanese lamprey MYHs 1 and 2, all the sequences functioned as the promoter with comparable activity in zebrafish muscle fibers (Figures 3 and S4 and Table S4). Furthermore, no obvious ectopic expression was observed. Therefore, we selected the sequence containing 3 kb from the start codon for further analysis. In addition, the regions -5, -3 and -2 kb from MYH5 were unable to function as the promoter (Table S4).

The -3 kb 5′-flanking sequences of Japanese lamprey MYH1 (MYH1-3kb-hrGFP) and MYH2 (MYH2-3kb-DsRed) were able to drive the transient expression of GFP and DsRed, respectively, both in the epaxial and hypaxial trunk muscles at 1 and 3 days post-fertilization (dpf) (Figure 3A-C). Furthermore, the MYH1-3kb-hrGFP and MYH2-3kb-DsRed transgenes were co-expressed in at least 2 muscle fibers in the same cells.
The MYH1-3kb-hrGFP transgene was expressed in trunk muscles as well as in cranial muscles (Figure 3E-G). Immunohistological observations were then conducted to localize the muscle fiber types that express the reporter gene. An embryo expressing the MYH1-3kb-hrGFP transgene was stained with an antibody against GFP and F59 antibody raised against chicken slow skeletal MYH [38]. As shown in Figure 3K, superficial slow muscle fibers were labeled by F59, and the MYH1-3kb-hrGFP transgene was found to be expressed in fast muscle fibers.

Expression of Transgenes Containing the 5′-Flanking Sequences of Japanese Lamprey MYHs in Mouse Myoblasts

To examine the promoter activity of Japanese lamprey MYHs in tetrapod muscles, the -3 kb 5′-flanking sequences of
Japanese lamprey MYHs 1 and 2 were fused to the luciferase gene, introduced into mouse myogenic C2C12 cells and examined for luciferase activity. As previously reported [32], the activity of the mouse -2.8 kb MYH1 promoter connected to the luciferase gene was significantly higher (70-fold) in differentiated myotubes than in proliferating myoblasts (Figure 4A). However, the luciferase activity of the constructs containing Japanese lamprey MYH1 and 2 promoters were almost the same as or less than that of a promoter-less control pGL3 vector in myotubes (Figure 4A). In contrast, the zebrafish -3 kb myhz2 [10] and myhc4 [11] promoters had significant activity, approximately one-half that of the mouse -2.8 kb MYH1, in myotubes (Figure 4B).

Conserved Non-coding Elements (CNEs) in the 5′-Flanking Sequences of Vertebrate MYHs

The 5′-flanking sequences of cyclostome Japanese lamprey MYHs 1 and 2 fused to the reporter gene drove specific reporter gene expression in the fast skeletal muscle of zebrafish embryo (Figure 3), suggesting that the regulatory mechanisms involved in the transcriptional expression of fast skeletal MYHs are conserved between cyclostomes and teleosts. However, in contrast to the case of the proximal promoter of mouse MYH1 and zebrafish myhz2 and myhc4, the Japanese lamprey MYH promoters did not direct the reporter gene expression in differentiated mouse C2C12 myotubes (Figure 4). These results suggest that the muscle-specific regulatory mechanisms are partially conserved between teleosts and tetrapods but not between cyclostomes and tetrapods. To identify candidates for functionally conserved non-coding elements (CNEs) for muscle-specific expression between cyclostomes, teleosts and tetrapods, the 5′-flanking sequences of the MYHs of Japanese lamprey (the -3 kb MYHs 1 and 2 promoters used for the transgenic and the transfection assays), zebrafish (the -3 kb myhz2 and myhc4 promoters used for the transfection assay) and mouse (the -2.8 kb MYH1 promoter used for the transfection assay) were aligned with Shuffle-LAGAN [33] and visualized with mVISTA [34] (Dataset S3). Shuffle-LAGAN alignment did not identify even coding exons between Japanese lamprey and mouse MYH sequences (Figure S5). When the promoters of Japanese lamprey MYH2, zebrafish myhz2 and myhc4 were aligned with that of Japanese lamprey MYH1 as a baseline, we found 7, 11 and 11 CNEs, respectively (Figure S5A). Moreover, when the promoters of zebrafish myhz2 and myhc4 were aligned with

![Figure 4. Activity of Japanese lamprey, zebrafish and mouse myosin heavy chain gene (MYH) promoter in C2C12 cells. A: C2C12 myoblasts were transfected with a promoter-less control pGL3 vector (control), -3 kb Japanese lamprey MYH1 (MYH1) or MYH2 (MYH2) and -2.8 kb mouse MYH1 (Ild/x) (mouse MYH1) promoter luciferase constructs along with pRL-TK as a transfection control. B: C2C12 myoblasts were transfected with a promoter-less control pGL3 vector (control), -3 kb zebrafish myhz2 (myhz2) or myhc4 (myhc4) and -2.8 kb mouse MYH1 (Ild/x) (mouse MYH1) promoter luciferase constructs along with pRL-TK as a transfection control. Cells were grown for 24 h in a growth medium, and myoblasts were harvested for luciferase assays. Other cells were cultured for an additional 4 days in differentiation medium, and myotubes were harvested for luciferase assays. The results are expressed as firefly luciferase activity per Renilla luciferase activity from each sample. The data represent the average of triplicate data, and all bars are the mean ± SD. Data were analyzed using Student's t test (*, P < 0.05; **, P < 0.01). doi: 10.1371/journal.pone.0085500.g004]
that of mouse MYH1 as a baseline, we found 4 and 7CNEs, respectively (Figure S5B). The identified CNEs were analyzed for conserved putative transcription factor binding sites (TFBSs) using the MATCH program based on the TRANSFAC database [35]. A cut-off to minimize false negative matches (minFN) was applied for searching putative TFBSs, as the minFN cut-off is useful for the detailed analysis of relatively short DNA fragments (see the on-line help information for the MATCH program) [35]. We found several conserved TFBSs in the CNEs, whereas the typical muscle-specific TFBSs, such as MyoD and myogenin, were not identified (Table S5). In contrast, conserved TFBSs for FOXD3 and Oct-1/POU2F1 were clearly identified (Table S5). We identified 10 FOXD3 and 5 Oct-1/POU2F1 binding sites in the Japanese lamprey/zebrafish CNEs (Table S5B, C). However, only 2 out of 10 FOXD3 and 2 out of 5 Oct-1/POU2F1 binding sites were identified with relatively high probability scores (core similarity >0.9, matrix similarity >0.85) (Tables S5B and C). By contrast, the FOXD3 binding site was not identified in the mouse/zebrafish CNEs (Table S5D, E). Although the probability score was not high, we identified 7 Oct-1/POU2F1 binding sites in the mouse/zebrafish CNEs (Table S5D, E).

**Discussion**

Like teleost and tetrapod MYHs, lamprey fast skeletal MYHs are tandemly arrayed (Figures 1, S1 and S2). Moreover, taking into consideration the synteny of lamprey MYHs with tetrapod and teleost counterparts, it was predicted that vertebrate MYHs evolved from a common ancestor of cyclostomes and gnathostomes. This hypothesis is strongly supported by the fact that the exon structure of fast skeletal MYHs is highly conserved between cyclostomes and gnathostomes (Table S3). Furthermore, all fast skeletal MYHs of Japanese lamprey and teleosts, as well as human MYHs 3 and 13, are encoded by 41 exons, implying that the common ancestor of vertebrate fast skeletal MYH was encoded by 41 exons. Human MYHs 1, 2, 4 and 8, which are encoded by 40 exons, are likely to have lost intron 40 after divergence from the common ancestor of human MYH3 and MYHs 1, 2, 4 and 8 (Figure 2).

The newly identified Japanese lamprey MYH5 is not expressed either in whole embryo or adult muscle tissue (Figure S3). However, a phylogenetic analysis indicated that the 3 Japanese lamprey MYHs, including the newly identified MYH5, were classified as a fast skeletal type but not as cardiac or slow skeletal types (Figure 2). Moreover, in the present study, strict orthology was not found in MYHs between cyclostomes and gnathostomes (Figure 2). It is possible that the tandem duplication of fast skeletal MYH occurred in the lamprey lineage after splitting from the gnathostome lineage and that fast skeletal MYH clustering was not an early event in vertebrate evolution. Indeed, the phylogenetic tree indicates that there was a single fast skeletal MYH in the common ancestor of lampreys, teleost fish and mammals and that the MYHs underwent independent duplications in the different lineages (Figure 2). Although other chordate lineages, such as the amphioxus *B. floridæ* and the tunicate *C. savignyi*, do possess at least 1 MYH cluster (Figure S1), no conserved synteny was found between vertebrate and other chordate MYH clusters (Figure S1). In addition, McGuigan et al. have shown that tunicate MYHs are phylogenetically distinct from diverse MYHs in vertebrates [12]. However, MYH clusters obviously exist in amphioxus and tunicates, implying that the formation of MYH clusters was a common event and is not a novel feature established in the vertebrate lineage but rather is shared by the broader taxa of animals. The functional significance in the formation of MYH clusters for chordate evolution remains an open question.

Kusakabe et al. [37] showed that the GFP reporter gene was expressed predominantly in the striated muscles of Japanese lamprey embryos when driven by the 5′-flanking sequence of the medaka muscle actin gene. Accordingly, the authors hypothesized that the regulatory mechanisms involved in the muscle-specific gene expression had evolved before the divergence between cyclostomes and gnathostomes [37]. Our finding that the 5′-flanking sequence of cyclostome Japanese lamprey MYHs 1 and 2 fused to the reporter gene drove the expression of the reporter gene in the fast skeletal muscle of gnathostome zebrafish (see Figure 3) clearly supports their hypothesis. The transcripts of Japanese lamprey MYHs 1 and 2 showed an almost identical spatiotemporal expression pattern in whole-mount in situ hybridization [23]. Similarly, we found no apparent differences in the expression patterns of the reporter genes driven by MYHs 1 and 2 (see Figure 3A-C). In fact, the MYH1-3kb-hrGFP and MYH2-3kb-DsRed transgenes were co-expressed in at least 2 muscle fibers in the same cells (see Figure 3D). These results suggest that the regulatory mechanisms involved in the transcriptional expression of fast skeletal MYHs are conserved between cyclostomes and teleosts. However, it should also be noted that the expression patterns of Japanese lamprey fast skeletal MYH1 do not precisely correspond to the spatial location of muscle fiber types found in gnathostomes such as zebrafish. At embryonic stage 25, MYH1 (LjMyHC1) is expressed in the myotomal cells except the medial part of the myotome adjacent to the notochord, which might represent the Japanese lamprey adaxial cells [39]. Later, at stage 28, MYH1 is expressed in the adaxial cells but not in the deeper region of the somite [39]. It is well known that the adaxial cells are fated to give rise to slow skeletal muscle fiber in zebrafish [39]. Therefore, according to the endogenous expression pattern, Japanese lamprey fast skeletal MYH1 has the potential to be expressed both in slow and fast skeletal muscle fibers. To address whether the regulatory mechanisms involved in the transcriptional expression of fast skeletal MYHs are exactly conserved between cyclostomes and teleosts, the establishment of stable transgenic lines is needed. It was also noted that Japanese lamprey MYH1 promoter drove GFP expression in zebrafish cranial muscle derived from the head mesoderm, such as the intermandibularis anterior, intermandibularis posterior and interhyoideus (see Figure 3E-D) [7,40,41]. This pattern is in marked contrast to the fact that the expression of MYHs 1 and 2 is absent in head mesoderm derivatives [23,39,42]. It is possible that the -3 kb 5′-flanking sequence of Japanese lamprey MYH1 does not carry regulatory elements sufficient to
recapitulate the endogenous MYH1 expression. GFP reporter analyses on lamprey embryos will provide conclusive results.

The present study reveals that there is obvious synteny between lamprey and tetrapod MYH cluster regions (see Figures S1 and S2). Thus, the 5′-flanking sequences of lamprey MYHs 1 and 2 were expected to drive the reporter gene expression in tetrapod myogenic cells. However, in contrast to the proximal promoter of mouse MYH1, the Japanese lamprey MYH promoters did not direct the reporter gene expression in differentiated mouse C2C12 myotubes (see Figure 4A). These results suggest that although the common ancestor of teleosts and tetrapods shared the same muscle-specific regulatory mechanisms, tetrapods have not retained these regulatory mechanisms.

It was evident that the proximal promoters of Japanese lamprey fast skeletal MYHs drove the reporter gene expression in fast skeletal fibers of zebrafish embryos where endogenous MYHs were expressed. In zebrafish, at least 3 fast skeletal MYHs, myh1, myh2 and myh4, are dominantly expressed in fast fibers of embryos [9-11]; orthologs of torafugu [18], medaka [14] and common carp [7,8] are also expressed in embryos from the respective fish species. In addition, the proximal promoters of the above-listed MYHs from torafugu [43,44] and medaka [15] drive the reporter gene expression in the fast fibers of zebrafish embryos. These teleost MYHs are all classified as fast C type [18], suggesting that the proximal promoters of fast skeletal MYHs of lampreys and fast C-type MYHs of teleosts have a common ancestor and share common regulatory mechanisms, even though no synteny (see Figures S1 and S2) [18] or strict orthology (see Figure 2) was found between lamprey and teleost MYHs. Indeed, we found CNEs between promoters of Japanese lamprey MYHs 1 and 2 as well as between Japanese lamprey MYH1 and zebrafish myhz2 and myhc4 (Figure S5A). As the zebrafish myhz2 and myhc4 are among the dominantly expressed MYHs in fast fibers of embryos [9-11], these CNEs have the potential to direct gene expression in zebrafish embryos. We identified 10 FOXD3 and 5 Oct-1/POU2F1 binding sites in the Japanese lamprey/zebrafish CNEs (Table S5B, C).

In zebrafish, FOXD3 mediates the expression of myf5, one of the muscle regulatory factors, and affects myogenesis [45]. Flounder FOXD3 is also involved in myogenesis by regulating the expression of myf5 and MyoD [46]. Conserved TFBSs for FOXD3 are remarkably identified in the Japanese lamprey/zebrafish CNEs (Table S5B, C), implying that fast skeletal MYHs of lamprey and zebrafish are also regulated by FOXD3. Moreover, the muscle-specific expression of fast skeletal MYHs in mice is at least partially controlled by the transcription factors MEF2 and Oct-1/POU2F1 [47,48]. However, only 2 out of 10 FOXD3 and 2 out of 5 Oct-1/POU2F1 binding sites in the Japanese lamprey/zebrafish CNEs were identified with high probability scores (core similarity >0.9, matrix similarity >0.85) (Tables S5B and C). Therefore, transgenic analysis using the deletion constructs of these CNEs is needed to evaluate the significance of these CNEs for gene expression in zebrafish.

To determine whether the regulatory mechanisms involved in the transcriptional expression of fast skeletal MYHs are conserved between teleosts and tetrapods, the promoter activities of zebrafish myhz2 and myhc4 were examined in C2C12 myogenic cells (Figure 4B). In contrast to Japanese lamprey promoters, the zebrafish promoters exhibited significant activity in differentiated mouse C2C12 myotubes (Figure 4B). In fact, Shuffle-LAGAN alignment did not identify even coding exons between Japanese lamprey and mouse MYH sequences (Figure S5). These results suggest that the muscle-specific regulatory mechanisms are partially conserved between teleosts and tetrapods but not between lampreys and tetrapods.

We found 11 CNEs conserved between promoters of mouse and zebrafish MYHs (Figure S5B). As the zebrafish promoters exhibited significant activity in differentiated mouse C2C12 myotubes (Figure 4B), these CNEs have the potential to direct the reporter gene expression in tetrapods. In contrast to the mouse/zebrafish CNEs, the FOXD3 binding site was not identified, but 7 Oct-1/POU2F1 binding sites were identified (Table S5D, E). This finding implies that Oct-1/POU2F1 binding sites, rather than FOXD3 binding sites, are important for the transcriptional expression of fast skeletal MYHs in tetrapods. However, the probability scores of Oct-1/POU2F1 binding sites in the mouse/zebrafish CNEs were not significant (Table S5D, E). Again, transfection analysis using the deletion constructs of these CNEs is needed to evaluate the significance of these CNEs for the gene expression in mouse C2C12 cells. However, our conclusion that tetrapods do not retain the same muscle-specific regulatory mechanisms as in teleosts is based on the finding that Japanese lamprey MYH promoters were not activated in C2C12 myogenic cells. Naturally, gene sets transcribed in the myogenic cell lines are different from those transcribed in myotomes of developing mammalian embryos. LacZ reporter analyses on mouse embryos will provide conclusive results.

Supporting Information

Table S1. PCR primer sequences, their target regions and product sizes.

Table S2. Nucleotide sequence of primers used in the present study.

Table S3. Intron phases and the coding exon sizes of vertebrate myosin heavy chain genes (MYHs).

Table S4. Quantification of GFP or DsRed expressing zebrafish embryos.

Table S5. Conserved non-coding elements (CNEs) identified by Shuffle-LAGAN.
Figure S1. Physical maps of Japanese and sea lampreys, human and torafugu syntenic regions containing fast skeletal myosin heavy chain genes (MYHs). Branchiostoma floridae and Ciona savignyi genomic regions containing sarcomeric MYHs are also presented. Gray and open rectangles indicate MYHs and non-syntenic genes, respectively. Syntenic genes MAP2K4, TMEM220, ADPRM, SCO1 and GAS7 are color-coded. Arrows indicate the directions of gene transcription. Five non-syntenic genes between human MAP2K4 and TMEM220 are not shown. Note that as the presence of other genes between MYH1 and GAS7 remains a possibility in the sea lamprey genome, the intergenic region is shown by a dashed line. Asterisks (*) indicate that there is no orthologous relationship between lamprey and human MYH1/MYH2.

(EPS)

Figure S2. Schematic representation of the location of the sea lamprey myosin heavy chain genes (MYHs) and growth arrest-specific protein 7 gene (GAS7) on the genome. The relative positions of contigs and a scaffold are shown. Contigs 2034, 15297, 6974, 38608 and 39193 and scaffold GL477325 were extracted from the sea lamprey genome databases Petromyzon_marinus-3.0 and -7.0, respectively. Arrows indicate the directions of transcription of the genes. A region with a long sequence gap in scaffold GL477325 is shown by a dashed line, and contigs 6974/38608 and 39193 are expected to be located on this gap region. At present (November 2013), the Petromyzon_marinus-3.0 database is obtainable from the Genome Institute at Washington University (http://genome.wustl.edu/pub/organism/ Other_Vertebrates/Petromyzon_marinus/).

(EPS)

Figure S3. Expression analysis of Japanese lamprey myosin heavy chain genes (MYHs). The target regions for amplification of each MYH are shown in brackets. The gene encoding cytoplasmic actin LjCA1 was used as the positive control.

(TIF)

Figure S4. Transient expression of the Japanese lamprey MYH1-2kb-hrGFP, MYH1-5kb-hrGFP, MYH2-2kb-DsRed and MYH2-5kb-DsRed transgenes in zebrafish embryos. Lateral view showing the expression of the MYH1-2kb-hrGFP (A), MYH2-2kb-DsRed (B), MYH1-5kb-hrGFP (C) and MYH2-5kb-DsRed (D) in the trunk myotome of zebrafish embryos at 3 days post-fertilization. Arrowheads indicate autofluorescence due to pigment cells on the embryo surface.

(TIF)

Figure S5. Shuffle-LAGAN alignment of the 5' flanking sequences of myosin heavy chain genes (MYHs). The 5' flanking sequences of Japanese lamprey MYH1 and MYH2, zebrafish myhz2 and myhc4 and mouse MYH1 are aligned with Shuffle-LAGAN and visualized with mVISTA. The 5' flanking sequences of Japanese lamprey MYH1 (A) and mouse MYH1 (B) are used as baselines. Coding (blue) and non-coding (light blue) exons are annotated. Pink peaks represent non-coding conservation above 70% over at least 20 bp.

(TIF)

Dataset S1. Coding sequences of Japanese lamprey and sea lamprey myosin heavy chain genes (MYHs) 1, 2 and 5.

(TXT)

Dataset S2. Amino acid sequence alignment of myosin heavy chains (MYHs) from Japanese lamprey, torafugu and humans.

(TXT)

Dataset S3. 5'-flanking sequences of the myosin heavy chain genes (MYHs) of Japanese lamprey, zebrafish and mouse.

(TXT)

Acknowledgements

We thank Dr. Yuko Mimori-Kiyosue, the RIKEN Center for Developmental Biology and the Olympus Corporation for their kind help in the use of microscopy equipment. The F59 antibody developed by F.E. Stockdale was obtained from the Developmental Studies Hybridoma Bank maintained by the Department of Biological Science, University of Iowa (Iowa City, IA).

Author Contributions

Conceived and designed the experiments: DI SW. Performed the experiments: DI YO SH. Analyzed the data: DI SW. Contributed reagents/materials/analysis tools: SH NK. Wrote the manuscript: DI SW.

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