Myomesin is a structural component of the M-band that is expressed in all types of striated muscle. Its primary function may be the maintenance of the thick filament lattice and its anchoring to the elastic filament system composed of titin. Different myomesin isoforms have been described in chicken and mice, but no particular function has been assigned to them. Here we investigate the spatio-temporal expression pattern of myomesin isoforms by means of reverse transcriptase-polymerase chain reaction and isoform-specific antibodies. We find that two alternative splicing events give rise to four myomesin isoforms in chicken contrary to only one splicing event with two possible isoforms in mice. A splicing event at the C terminus results in two splice variants termed H-myomesin and S-myomesin, which represent the major myomesin species in heart and skeletal muscle of avian species, respectively. In contrast, in mammalian heart and skeletal muscle only S-myomesin is expressed. In embryonic heart of birds and mammals, alternative splicing in the central part of the molecule gives rise to the isoform that we termed EH-myomesin. It represents the major myomesin isoform at early embryonic stages of heart but is rapidly down-regulated around birth. Thus, the strict developmental regulation of the EH-myomesin makes it an ideally suited marker for embryonic heart.

Striated muscles are characterized by a very precise organization of contractile proteins into repeating structural subunits, the sarcomeres. A sarcomere is defined as the region between two Z-discs that anchor the thin (actin) filaments, with the thick (myosin) filaments being anchored in the center via a structure called the M-band. Elastic filaments composed of the giant protein titin stretch from the Z-disc to the M-band and may serve as a template for sarcomeric assembly and to maintain resting tension during the contraction cycle (for a review see Ref. 1). Despite the striking similarity at the level of the electron microscope (2, 3), the different types of striated muscle are characterized by distinct contractile properties. These adaptations to specific physiological requirements are associated with an isoform diversity of sarcomeric proteins (4). Different isoforms either arise from several genes (5) or from different transcripts of the same gene that are generated either by alternative splicing of the primary transcript (6–9) or by alternative initiation of protein synthesis (10). Many isoforms show a tissue and developmental stage-specific expression pattern (11), but so far, only a few of the regulatory sequences involved have been identified.

It has recently been demonstrated that myomesin, an integral component of the M-band, is also expressed in several isoforms (8, 12). Myomesin is present in all kinds of vertebrate striated muscle and is thought to play an important role in the integration of thick filaments with titin, a hypothesis based on its ability to bind both proteins in vitro (13). Studies of myofibrillogenesis in developing chicken heart have shown that myomesin becomes localized in its characteristic pattern simultaneously with the appearance of the first sarcomeres (14).

The first indications that myomesin may be expressed in several isoforms resulted from the observation that immunoblots of chicken heart and skeletal muscle show bands of different molecular weight (15). Subsequently, two transcripts of different sizes were detected in chicken heart and skeletal muscle (8). The two isoforms differ only at their respective C termini, whereas the major part of the protein, which consists of a unique head domain followed by a conserved pattern of immunoglobulin-like and fibronectin type III domains, is identical. The smaller skeletal muscle isoform (with a calculated molecular mass of 174 kDa) is homologous to mammalian myomesin, whereas the bigger heart isoform (calculated molecular mass 182 kDa) includes an additional unique domain at the C terminus. These isoforms designated as S-1 and H-myomesin are schematically represented in Fig. 1.

Myomesin shares its modular structure with two other M-band associated proteins, M-protein and skelemin. All three proteins are closely related and have a common ancestor in evolution (16). The nearest relative of myomesin is skelemin, which was originally described as a protein localized at the periphery of the M-band in mouse skeletal muscle (17, 18). It has recently been shown that skelemin is yet another splice variant of myomesin that is characterized by the insertion of a serine/proline-rich domain between domains My6 and My7 (12). The position of this alternatively spliced domain marked as EH-segment is depicted in Fig. 1. However, the existence of an avian (chicken) counterpart to skelemin has not yet been confirmed, and the functional role of this myomesin isoform remains obscure.

In order to understand the significance of this myomesin isoform diversity in greater detail, we have investigated the expression pattern of different myomesin isoforms in vertebrates using a combination of RT-PCR analysis and isoform-specific antibodies. Our results indicate that in chicken two
alternative splicing events give rise to four myomesin isoforms, whereas in mammals, a single splicing event leads to only two isoforms. The expression of the different isoforms is strictly regulated in a tissue-specific and developmental stage-specific manner. In addition, we have identified a myomesin isoform that is specifically expressed during embryonic heart development in vertebrates, which we have termed EH (embryonic heart)-myomesin.

MATERIALS AND METHODS

RT-PCR Analysis—Chicken embryos were staged according to Hamburger and Hamilton (19). Timed pregnant mice were obtained from the C57BL strain (Life Technologies, Inc.). The day of detection of the vaginal plug was considered as embryonic day 0.5. Total RNA was isolated from heart, leg, and brain of chicken and mouse embryos using the SV Total RNA isolation system (Promega, Wallisellen, Switzerland). RT-PCR was carried out on approximately 1 μg of total RNA with the Access RT-PCR system (Promega) using the standard RT-PCR protocol suggested by the manufacturer (45 min of reverse transcription followed by 40 amplification cycles). Primers specific for different chicken myomesin isoforms were derived from the chicken myomesin sequence (8). The approximate positions of all primer sets are shown in Fig. 1a. Primers sequences were as follows and are denoted 5′–3′. The forward primers used are as follows: P1, GGAAAGAACTGCAGCTTTACCA-CC; P3, TTGTGAGACTGTCGTTCCCGAGGTT; and P5, GGAAAGAC-TGTCACACAATC. The reverse primers used are as follows: P2, TTC-TCTGTTGTTGTTCT; P4, CCAAAATUTCCGCCACCTTGTT; and P6, TTCACCGACACCAACACATC. Primers used for the amplification of the central fragment of mouse myomesin were derived from the mouse skelelin sequence (18) and are as follows: forward primer, GCC-GAAATCCTGCCAATGAG; reverse primer, ATATAGGCTGTAACTCTGTC. The specificity of primers was confirmed by sequencing of PCR products. Primers specific for chicken and mouse α-tubulin were used to standardize the amount of RNA used in the RT-PCR.

Expression of Recombinant Myomesin Fragments—The H-segment of chicken myomesin was amplified from the original cDNA clone (8) and subcloned into the BamHI site of the bacterial expression plasmid pGEX-2T (Amersham Pharmacia Biotech). The EH-segment of chicken myomesin including parts of the surrounding fibronectin type III domains was amplified by RT-PCR from HH38 (Hamburger-Hamilton stage 38) embryonic chicken total RNA, subcloned into the EcoRI and XhoI sites of Bluescript II KS(+) (Stratagene, Amsterdam, Netherlands) and verified by sequencing. Consequently, the EH segment was amplified by PCR and subcloned into the BglII and EcoRI sites of pGEX-2T. The two recombinant fragments (H- and EH-segments) were expressed in the Escherichia coli strain BL-21, and soluble glutathione S-transferase fusion proteins were purified from crude bacterial lysates by affinity chromatography on glutathione-agarose (Sigma, Buchs, Switzerland). Expression stability and integrity of recombinant proteins were monitored by SDS-polyacrylamide gel electrophoresis.

Antibodies—Antibodies against recombinant myomesin fragments were generated by immunizing adult female rabbits either with the H-segment or the EH-segment fused to glutathione S-transferase. Antibody against the S-segment was generated by immunizing rabbits with a synthesized 20-mer peptide coupled to keyhole limpet hemocyanin antibody against the S-segment was generated by immunizing rabbits with the S-segment of chicken myomesin including parts of the surrounding fibronectin type III domains. The antibody specificity was further characterized using a mouse anti-sarcomeric antibody (clone 2B4) which is specific for the S-segment.

Sequence Analysis—GenBank™ release 112.0 was searched using the Blast program (26) at the National Center for Biotechnology Information. Sequence alignments were performed using the MegAlign software (DNASTAR Inc, Madison, WI). Protein motif predictions were done with the software Motif. The EST clone AA248352 was a kind gift of Dr. Mathias Gautel (Heidelberg, Germany). For immunoblotting, horseradish peroxidase-conjugated anti-rabbit IgG (Dako, Zug, Switzerland), anti-rat IgG (Dako), and anti-rabbit IgG (Calbiochem) were used as secondary antibodies. For immunofluorescence, secondary antibodies were fluorescein isothiocyanate-conjugated goat anti-rabbit IgG (Cappel, West Chester, PA) and Cy3-conjugated goat anti-mouse IgG (Jackson ImmunoResearch, West Grove, PA). Cy5-conjugated phallolidin was a generous gift of Prof. H. Faulstich (Heidelberg, Germany).

SDS-Polyacrylamide Gel Electrophoresis and Immunoblotting—Tissue samples (brain, gizzard, heart, and skeletal muscle) were carefully dissected from the animal, homogenized by freeze-slitting, resuspended in a modified version of SDS-sample buffer (3.7 μl urea, 134.6 mM Tris, pH 6.8, 5.4% SDS; 2.3% Nonidet P-40; 4.45% β-mercaptoethanol; 4% glycerol, and 6 mg/100 ml bromphenol blue), and boiled for 1 min. SDS samples were run on 6% polyacrylamide minigels (Bio-Rad) together with broad range molecular weight standards (Bio-Rad). Equal amounts of protein were loaded for the different tissue extracts as judged by Coomassie Blue staining of a twin gel. Blotting was carried out overnight onto nitrocellulose Hyb-Bond-C extra (Amersham Pharma

Cell Culture and Immunostaining—Hearts from 11-day-old chicken embryos were digested with collagenase (108 units/ml, Worthington) in ADS buffer (116 mM NaCl, 20 mM HEPES, 0.8 mM NaH₂PO₄, 1 g/liter glucose, 5.4 mM KCl, 0.8 mM MgSO₄, pH 7.35) and cultured as described (23). Cells were plated onto dishes coated with fibrinogen in plating medium (67% Dulbecco’s modified Eagle’s medium, 17% Medium M199 (Amied AG, Basel, Switzerland), 10% horse serum, 5% fetal calf serum, 1% penicillin/streptomycin (Life Technologies, Inc.)). After 1 day the medium was replaced with maintenance medium (78% Dulbecco’s modified Eagle’s medium, 2% Medium M199, 1% penicillin/streptomycin, 1% horse serum, and 10 ng/ml fibronectin) (Sigma). To reduce the number of contaminating fibroblasts, glutamine was left out, and cytosine arabinoside (10 μmol/liter, Sigma) was added to the culture media.

For preparation of the skeletal muscle cell cultures, the breast muscles of 11-day-old chicken embryos were dissociated mechanically in the absence of Ca²⁺ by using a vortex. Cells were plated with a density of 10⁹/ml medium onto dishes coated with 0.1% gelatin in plating medium (M199, 10% horse serum, 2% chicken embryo extract, 1% glutamine, and 1% penicillin/streptomycin).

For immunofluorescence staining, 4-day-old cultures were fixed with 4% paraformaldehyde in PBS for 15 min at room temperature, blocked with 0.1% glycine in PBS for 5 min, and permeabilized in 0.2% Triton X-100/ PBS for 10 min. After blocking with 5% normal pre-immune goat serum and 1% bovine serum albumin in PBS for 20 min, primary antibodies were added and incubated for 1 h at room temperature. After washing with PBS, secondary antibodies were added for 45 min. The specimens were washed in PBS and mounted in 0.1 M Tris-HCl, pH 9.5, glycerol (3:7) containing 50 mg/ml x-propyl gallate as anti-fading reagent (24).

Frozen Sections of Chicken Embryos—Fertilized eggs from White Leghorn hens (Hungerbühler, Flawil, Switzerland) were incubated at 37 °C for about 7 days. Embryos were removed from the eggs, transferred to cold PBS, and staged according to Hamburger and Hamilton (19). After an overnight incubation in 30% sucrose in PBS, the embryos were frozen in liquid nitrogen and stored at −70 °C until sectioning. 10-μm thick sagittal sections through the whole embryos were cut on a cryostat (Reichert, Vienna, Austria) and collected on gelatin-coated glass slides. For immunofluorescence experiments, the sections were fixed, stained, and mounted as described above.

Microscopy—Images were recorded with a Leica inverted microscope DMR (Wetzlar, Germany), connected to a Leica true confocal scanner TCS NT. Leica PL APO 100×/1.4 oil or PL APO 63×/1.4 oil immersion objectives were used. The system was equipped with an argon/krypton mixed gas laser. Image processing was done on a Silicon Graphics workstation using the image processing software “Imaris” (Bitplane AG, Zurich, Switzerland) (25). The fluorescence images of the frozen sections were recorded on an inverted microscope (Zeiss, Oberkochen, Germany) with an attached CCD camera (Kappa, Videotec AG, Switzerland) using an Achrostop 5 x 0.5 objective.

Sequence Analysis—GenBank™ release 112.0 was searched using the Blast program (26) at the National Center for Biotechnology Information. Sequence alignments were performed using the MegAlign software (DNASTAR Inc, Madison, WI). Protein motif predictions were done with the software Motif. The EST clone AA248352 was a kind gift of Dr. C. C. Liew (Toronto, Ontario, Canada). The nucleotide sequences of chicken and human EH-segments have been deposited in the GenBank™ data base with accession numbers AF185572 and AF185573, respectively.
Myomesin Isoforms

Fig. 1. Schematic representation of myomesin isoforms. a, scheme of the myomesin cDNA. The position of the alternative EH exon, corresponding to the previously described Ser/Pro domain of mouse skelemin, is marked by a triangle. The alternatively spliced S- and H-exons giving rise to chicken skeletal- and heart-specific myomesin isoforms are shown at the 3' end. Positions of P1–P6 PCR primers that were used in this study are indicated by half-arrows. b, diagram of the myomesin protein. Myomesin is mainly composed of immunoglobulin-like (ellipses) and fibronectin type III domains (rectangles). The N-terminal domain has no homologies to other known proteins. Chicken S- and H-myomesin isoforms differ in their C terminus; the S-isoform has a short C-terminal sequence, which is very homologous to the mammalian variant of myomesin, whereas the H-isoform is characterized by a unique H-segment. The EH-isoform has an additional EH-segment inserted in the center of the molecule. Arrows indicate the positions of the epitopes of the antibodies, which were used in this study.

FIG. 2. Expression of myomesin isoform mRNAs in chicken is tissue- and developmental stage-specific. RT-PCR analysis of total RNA extracted from chicken tissues of different developmental stages (Fig. 2). By using the primers P1 and P2 located in the C-terminal H-segment of myomesin (Fig. 1), a 236-bp product was amplified from heart extracts of different embryonic stages, as well as from hatching stage and adult hearts (Fig. 2a, lanes 1–5). By contrast, no product was observed in RNA samples prepared from identical stages of skeletal muscle or adult brain (Fig. 2a, lanes 6–10). These results are consistent with earlier findings indicating that the expression of this isoform mRNA in chicken is restricted to the heart (8). Therefore, we will refer to this isoform as H-(heart)-myomesin, carrying the H-segment at the C terminus. By using a primer pair specific to the C-terminal sequence found in the skeletal isoform of myomesin (primers P3 and P4 in Fig. 1), a product of 434 bp was amplified from both heart and skeletal muscle samples (Fig. 2b, lanes 1–5 and 6–9, respectively); however, the intensity of the bands obtained from heart samples was found to be somewhat weaker than those from the skeletal muscle samples (Fig. 2b, lanes 1–5). Therefore, the isoform mRNA transcript previously termed “skeletal muscle-specific” (8) is expressed in both types of striated muscle in chicken, but its expression level is lower in the heart. This isoform is referred to as S-(skeletal)-myomesin because it is mainly found in chicken skeletal muscle, and the short sequence added to the C terminus by alternative splicing is referred to as S-segment (see Fig. 1).

The analysis of cDNA and genomic sequences of mouse myomesin has shown that alternative splicing of an exon located between fibronectin type III domains My6 and My7 gives rise to another isoform that has been termed skelemin (12, 18). However, no such isoform had been previously reported in chicken (8) or human myomesin (27). To check this, we designed the primers P5 and P6, located in domains My6 and My7 of myomesin, which flank the alternatively spliced segment (Fig. 1a). Surprisingly, we found that the isoform mRNA containing the alternatively spliced segment is not only present in chicken but that its expression is developmentally regulated. At early embryonic stages a product of 636 bp, corresponding to the transcript that includes the alternatively spliced exon, was observed in heart, and a product of 327 bp was found at later stages (Fig. 2c). The size of the latter product corresponds to a myomesin isoform mRNA lacking the alternatively spliced segment between domains My6 and My7 as described previously (8). In the hearts of hatching stage embryos and in adult chicken, the 636-bp product was completely replaced by the 327-bp product (Fig. 2c). In order to confirm the presence of the alternatively spliced segment, the 636-bp product was cloned and sequenced, revealing a sequence stretch of 103 amino acids...
that was inserted between domains My6 and My7 of chicken myomesin. Sequence comparison with the alternatively spliced exon 17a of mouse skeleton (12) showed a high homology (see below and Fig. 10), thus confirming the presence of this alternative splice variant in chicken. Interestingly, RT-PCR analysis of chicken skeletal muscle samples of different stages using the primer pair P5 and P6 gave rise to the 327-bp product only (Fig. 2c). Neither product could be amplified from adult brain (Fig. 2c). The molecular weights of the myomesin isoforms are indeed accumulated in chicken heart and did not react with skeletal muscle, gizzard, or brain extracts. The anti-EH antibody recognizes a band of about 200 kDa in the embryonic heart extract only; it does not react with embryonic skeletal muscle, gizzard, brain, or any adult tissue extracts.

**Generation of Isoform-specific Antibodies**—In order to investigate the expression of different myomesin isoforms in chicken at the protein level, we generated isoform-specific polyclonal antibodies in rabbits. The following parts of myomesin were used as immunogens: the S- and H-segments at the C terminus of myomesin and the EH-segment. Accordingly, the antibodies were named anti-S, anti-H, and anti-EH (see Fig. 1). The specificity of the antibodies was checked by immunoblot analysis of chicken tissue extracts (Fig. 3). The anti-H antibody recognized a protein with a molecular mass of about 190 kDa in extracts of adult chicken heart and did not react with extracts of skeletal muscle (Fig. 3a). The anti-S antibody recognized a protein of about 180 kDa in skeletal muscle extracts but did not react with heart extracts (Fig. 3b). Although the RT-PCR analysis indicated that some amounts of the S-myomesin isoform mRNA are indeed accumulated in chicken heart, no protein product was detected suggesting that expression of S-myomesin protein in the heart is too low to be detected by immunoblotting if normal exposure times are used. The anti-EH antibody recognized a protein of almost 200 kDa in extracts of embryonic chicken heart and did not react with extracts of skeletal muscle (Fig. 3c). The molecular weights of the myomesin isoforms determined by immunoblotting are in good agreement with the calculated sizes for S- (174 kDa), H- (182 kDa) and EH-myomesin (192 kDa) (8). Note that the expression of all three myomesin isoforms is restricted to striated muscle and that none of the antibodies reacted with smooth muscle (gizzard extract) or non-muscle tissue (brain extract).

To confirm further their specificities, the antibodies were tested by immunofluorescence staining of cultured chicken embryonic cardiomyocytes (Fig. 4, a–f) and skeletal muscle cells (Fig. 5, a–l). All three isoform-specific antibodies label the M-band of the sarcomeres as demonstrated by the antiperiodic staining obtained with a monoclonal antibody against sarcomeric α-actinin (Fig. 4g, i). Arrowheads indicate the position of the heart; arrows indicate the position of the skeletal muscle anlage in the vicinity of the heart. The anti-H antibody specifically stains the heart and does not react with skeletal muscle (Fig. 4d). The anti-EH antibody strongly stains the first skeletal muscle anlage and to a smaller extent the heart (Fig. 4e). The anti-EH antibody specifically stains the heart and does not react with skeletal muscle or other tissues (Fig. 4g). Bar, 10 μm.
heart-specific antibody with S-myomesin which is too weak to be observed in immunoblots (see Fig. 3). The possibility that the faint signal in skeletal muscle cells is due to weak cross-reactivity is also supported by RT-PCR results where no H-isoform mRNA could be detected in skeletal muscle. Sequence comparisons between the H-segment and the S-segment identified several short elements consisting of three or four amino acids that occur in both sequences and that may lead to cross-reacting epitopes (8).

The anti-S antibody specifically stains chicken skeletal myotubes (Fig. 4i) and, although more weakly, also cardiomyocytes (Fig. 4c). We believe that this staining is specific because the RT-PCR results already pointed to an expression of S-myomesin in chicken heart (see Fig. 2a). The staining obtained with the anti-S antibody on cardiomyocytes was very weak, so that the signal had to be amplified by image processing resulting in some nonspecific background (e.g., weak nuclear staining in Fig. 4c).

The anti-EH antibody reacts exclusively with embryonic chicken cardiomyocytes (Fig. 4c) but not with embryonic chicken skeletal muscle cells (Fig. 4k).

These stainings confirm that all antibodies specifically recognize the respective myomesin isoforms in the M-band. In addition, it can be concluded that in cultured skeletal muscle cells only S-myomesin is present, whereas all three different isoforms are co-expressed in cultured embryonic cardiomyocytes. Since sarcomeric α-actinin is present in Z-discs throughout the sarcomere, it is also possible to investigate whether any of the myomesin isoforms is incorporated only in a subset of sarcomeres within a given cell by comparing the staining of sarcomeric α-actinin with that of the isoform-specific antibodies. Comparison of the two stainings in Fig. 4 shows that regardless of the expression level, all three isoform-specific antibodies uniformly stain the M-bands of all myofibrils in cultured cardiomyocytes. Thus, as judged by light microscopy, all myomesin isoforms appear to be incorporated equally well into the M-bands of myofibrils.

The Isoform Expression Pattern in the Chicken Embryo Is Tissue-specific—By having established the specificity of the generated antibodies and the localization of their corresponding antigens, we analyzed the distribution of myomesin isoforms in situ during embryonic development. Cryosections of stage 29 chicken embryos were stained with isoform-specific antibodies in combination with antibodies against sarcomeric α-actinin and phalloidin (Fig. 5). The distribution and relative amounts of different myomesin isoforms can be estimated here directly by comparing the staining intensities of heart (arrowheads) and skeletal muscle anlagen (arrows). The anti-H and anti-EH antibodies exclusively stain embryonic heart (Fig. 5, a and g), respectively, whereas the anti-S antibody strongly stains skeletal muscle tissue and, to a weaker extent, the heart (Fig. 5d). No difference in the intensity in which the different types of striated muscles were stained is observed with the antibody against sarcomeric α-actinin (Fig. 5, b, e, and h) or with F-actin staining (Fig. 5, c, f, and i). These findings confirm the conclusions drawn from the RT-PCR analysis and the stainings of isolated myocytes, namely that in embryonic skeletal muscle cells only one myomesin isoform is expressed, whereas all splice variants of myomesin can be detected in the embryonic chicken heart.

The Expression of H-myomesin Is a Characteristic Feature of Avian Cardiac Tissue—H-myomesin is expressed in the heart of chicken throughout all stages of development. To investigate whether the presence of this distinct isoform in heart is a common feature of avian species, we performed immunoblotting on heart and skeletal muscle extracts of goose, pigeon, and ostrich with antibodies against chicken myomesin isoforms (Fig. 6). Of these three species the goose is the closest, the pigeon is more distant, and the ostrich is the most distant species (among currently living birds) from the chicken in the evolutionary tree (28). The anti-H and anti-S antibodies were used together with the antibody B4 that recognizes an epitope in the domain My12 (see Fig. 1) and therefore cross-reacts with all myomesin isoforms. Surprisingly, the anti-H antibody does not react with the heart extracts of the majority of the birds that were tested, with the notable exception of a weak reaction visible in the extract from goose, which is closely related to chicken (Fig. 6). In contrast, the anti-S antibody reacts with the skeletal muscle extracts of all birds investigated, indicating that among avian species, the S-segment is much more conserved than the H-segment. However, the fact that the anti-S antibody does not react with any of the heart extracts clearly shows that the C terminus of avian H-myomesin differs from that of S-myomesin. This observation is confirmed by immunoblots with the B4 antibody showing that the mobilities between heart and skeletal muscle myomesin differ in all avian species investigated (Fig. 6). These results indicate that all birds seem to express an isoform of higher molecular weight in the heart and that the higher molecular weight is probably due to the presence of an alternatively spliced domain at the C terminus. Nevertheless, the sequence of this H-segment is not conserved between distantly related avian species as shown by the lack of cross-reactivity with the anti-H antibodies.

Only One Myomesin Isoform Can Be Detected in Striated Muscle of Adult Mammals and Reptiles—The finding that a heart-specific isoform of myomesin is present in all avian species that we investigated raises the question whether such an isoform may also be present in the hearts of mammalian species. We consequently analyzed the reactivity of heart and skeletal muscle extracts of several vertebrates using all available antibodies against myomesin (Fig. 7). Of these extracts, only chicken heart showed a specific reaction with the anti-H antibody (data not shown), whereas no reaction with the antibodies against S-myomesin was observed. In contrast, the anti-S antibody reacted strongly with both heart and skeletal extracts of several mammals and a reptile species (lizard), yielding bands of the same apparent molecular weight (Fig. 7). From these results, we conclude that in these species, only one isoform appears to be expressed in both adult heart and skeletal muscle. We also tested two lower
The H-myomesin isoform is not present in all vertebrates. Striated muscle samples of different vertebrate species were probed by immunoblot with anti-H antibody (S) or an antibody My190-Nrt that recognizes all myomesin isoforms (My-N). H, proteins extracted from heart; Sk, proteins extracted from skeletal muscle. In contrast to birds, only one myomesin isoform can be found in mammalian and reptilian tissues because the anti-S antibody recognizes myomesin in both heart and skeletal extracts of mammals and reptiles (row S). The analysis of the heart and skeletal extracts of different animals shows that mammals and reptiles have only one myomesin isoform in the heart and skeletal muscle, whereas the heart myomesin in birds, amphibians, and fish have a much higher mobility than the skeletal myomesin from the same species. This suggests that some lower vertebrates may possess a heart-specific C-terminal domain.

The Expression of EH-myomesin in Heart Is Developmentally Regulated—The results of our RT-PCR analysis indicate that there is a change in myomesin isoform expression during embryonic heart development. In order to confirm that this is also the case at the protein level, we performed immunobLOTS on chicken heart and skeletal muscle tissue of different developmental stages using our isoform-specific antibodies (Fig. 8).

With the anti-H antibody, we detected a shift of molecular weight for H-myomesin in heart extracts of different stages of development. In embryonic hearts a high molecular weight band was predominant, and at hatching stage, it becomes replaced by a lower molecular weight band (Fig. 8, row H, lanes 1–6). A similar shift in molecular weight of S-myomesin is detected by the anti-S antibody on heart extracts (Fig. 8, row S, lanes 1–6). Thus, at early stages, H-myomesin and S-myomesin are co-expressed in the developing heart, with the level of S-myomesin being significantly lower since the immunoblot had to be overexposed in order to reveal the signal for S-myomesin. A rough comparison of the band intensities obtained from heart and skeletal extracts indicates that the expression level of S-myomesin in heart is at least 1 order of magnitude lower than in skeletal muscle (data not shown). In skeletal muscle S-myomesin is the only isoform expressed at every developmental stage. Also, there is no transition in molecular weight (Fig. 8, row S, lanes 8–11).

Immunoblots with the anti-EH antibody reveal that, at the early developmental stages, the upper band of the doublet is due to an inclusion of the EH-segment. Because of the low abundance of the S-myomesin only the H-myomesin containing the EH-segment is visible on the blot (Fig. 8, row EH, lanes 1–6). At the time of birth the reactivity with the anti-EH antibody decreases, and only a very weak signal can be detected in the adult heart (Fig. 8, row EH, lane 7). Comparison of rows H, S, and EH suggests that the co-expressed isoforms, the high abundant H-myomesin as well as low abundant S-myomesin, include the EH-segment during early embryonic heart development but that this segment is spliced out at the time of hatching. No signal can be detected with the anti-EH antibody in skeletal muscle at any stage (Fig. 8, row EH, lanes 8–11). Immunoblots with the general My190-Nrt antibody confirm these results, since a similar transition to lower molecular weight was detected in the same heart extracts (Fig. 8, row My-N, lanes 1–7) but not in skeletal muscle (lanes 8–11). Note that this antibody only reveals the H-myomesin but not S-myomesin in the heart, since its expression is too low to be detected at this exposure time. Therefore, the inclusion of the EH-segment into the different myomesin isoforms, referred to as EH-myomesin, seems to be characteristic for embryonic chicken heart.

To determine whether the EH-myomesin isoform is also expressed in embryonic hearts of mammalian species, we analyzed mouse hearts of different developmental stages by RT-PCR. For the study of evolutionary distantly related species, the antibody My190-Nrt, which was generated against the head domain of human myomesin (21), proved to be more effective. Despite the fact that the head domain sequence is rather heterogeneous between vertebrates (8), the antibody My190-Nrt recognizes myomesin in all vertebrate muscle extracts tested, although very weakly in fish (Fig. 7). In heart and skeletal muscle extracts of mammals and reptiles, the My190-Nrt antibody recognized the same bands of identical molecular weight as the anti-S antibody, which suggests that these species express only the S-myomesin isoform in their heart and skeletal muscles. However, in skeletal muscle extracts of chicken, frog, and trout, the myomesin isoform detected was apparently smaller than in the corresponding heart extracts. Since none of the antibodies generated against the alternatively spliced segments of myomesin reacts with extracts of chicken, frog, and trout, the myomesin isoform detected was apparently smaller than in the corresponding heart extracts. Therefore, the inclusion of the EH-segment into the different myomesin isoforms, referred to as EH-myomesin, seems to be characteristic for embryonic chicken heart.

To determine whether the EH-myomesin isoform is also expressed in embryonic hearts of mammalian species, we analyzed mouse hearts of different developmental stages by RT-PCR.
PCR (Fig. 9a) and immunoblotting (Fig. 9b). In embryonic mouse heart two amplification products were observed with primers located in domains My6 and My7 of myomesin (Fig. 9a, lanes 1 and 2). The upper band completely disappears at the time of birth and at adult stages (Fig. 9a, lanes 3 and 4, respectively) and also cannot be detected in skeletal muscles of any stage. Immunoblot analysis confirms that the upper band is due to the inclusion of the EH-segment. In embryonic mouse heart a high molecular weight band is recognized both by the antibody My190-Nrt and the anti-EH antibody (Fig. 9b, lanes 1 and 2). Around birth, a second band of lower molecular weight appears (Fig. 9b, lane 3). In adult heart, as well as in skeletal muscle, this is the only detectable isoform (Fig. 9b, lanes 4 and 5–7). This isoform does not contain the EH-segment since it is not recognized by the anti-EH antibody. Thus, we conclude that the presence of an embryonic isoform of myomesin in heart, termed EH-myomesin, is characteristic for avian as well as for mammalian species and is therefore a universal marker for embryonic heart.

By searching the EST data base (29) using the sequence of the mouse EH-segment, we were able to identify one human EST clone (GenBank™ accession number AA248352) which was originally isolated from a cDNA library of human fetal heart. Sequencing indicates that this clone represents the complete sequence of the human EH-segment. The deduced amino acid sequence is aligned with the mouse and chicken EH-segment (Fig. 10). The highest homology occurs between the human and mouse EH sequences (65% identity and 76% similarity), whereas the chicken sequence is more divergent (36% identity and 48% similarity compared with mouse). The presence of the EH-segment in a human EST clone originating from a fetal heart library clearly confirms our RT-PCR and immunoblot data for chicken and mouse and suggests that the expression of EH-myomesin can indeed serve as a marker for embryonic heart.

**DISCUSSION**

The spatio-temporal expression pattern of different myomesin isoforms in birds as well as in mammals was established using RT-PCR, immunofluorescence, and immunoblot analysis. Our results indicate that the smallest myomesin isoform, namely S-myomesin seems to be able to fulfill the basic function of the myomesin molecule, since it is the only myomesin isoform expressed in striated muscle of adult mammals, reptiles, and also in avian skeletal muscle. The alternatively spliced H-segment at the C terminus gives rise to H-myomesin which represents the major myomesin species in avian cardiac muscle. Inclusion of the EH-segment in the central part of the myomesin molecule leads to the EH-myomesin isoform, which is characteristic for the embryonic heart of mammals and birds.

We find that the C-terminal splice variant H-myomesin is expressed in hearts of all tested bird species. This isoform has a higher molecular weight and does not react with the anti-S antibody that could detect S-myomesin in skeletal muscle extracts from the same species. Quite surprisingly, the anti-H antibody did not react with other H-myomesins except with the closely related goose protein. Thus the primary sequence of the H-segment is not conserved between different avian species, but the heart specificity of the splice machinery must remain intact and thus allow the successful splicing. We speculate that the presence of the H-segment determines mechanical or biochemical properties of myomesin in avian cardiac muscle that are preserved in the diverging primary sequences. A similar observation was made in the case of the myomesin head domain. According to biochemical data the head domain contains a myosin-binding site (13, 31), but at the same time its sequence differs significantly from one species to another (8).

At present it is still not clear whether H-myomesin is accumulated in other vertebrate species. In the closest relatives of birds, reptiles, and mammals, only S-myomesin is expressed in both heart and skeletal muscles. Thus, the appearance of H-myomesin may be part of adaptive mechanisms that arose after
birds evolved from reptiles. However, going further down the evolutionary tree we find that myomesin isoforms of heart and skeletal muscle in amphibian and fish differ in their apparent molecular masses. In this case we could not establish whether this difference is due to an additional C-terminal segment because neither the anti-S antibody nor the anti-H antibody reacted with extracts of these species. Different mobility might also be due to other splicing events or to posttranslational modifications such as phosphorylation. To clarify whether the difference in molecular weight is indeed due to an additional C-terminal segment, the sequences of the myomesins of reptiles, amphibian, or fish species have to be compared. In addition to H-myomesin, minor amounts of S-myomesin can be detected in chicken heart; however, we believe that this is due to the leakage of the splicing mechanism and that S-myomesin does not play a major functional role in cardiac muscle of the chicken.

During embryonic development a specific myomesin isoform is expressed in heart that is characterized by the inclusion of the EH-segment. Sequence comparison showed that EH-myomesin corresponds exactly to a mouse splice variant of myomesin, previously called “skelemin” (12, 17), including a serine/proline-rich sequence between domains My6 and My7, which we have termed EH-segment. We could now show that this splice variant exists also in chicken and man. However, we were unable to detect myomesin isoforms containing the EH-segment either by RT-PCR or by immunoblotting in skeletal muscle at any developmental stage, which is in contradiction with the fact that the mouse skelemin cDNA was cloned from adult skeletal muscle (18). A possible explanation might be the insufficient sensitivity of our RT-PCR analysis to detect minor amounts of EH-segment containing transcripts present in skeletal muscle or that there is some expression of EH-myomesin in regenerating muscles.

Several functions have been proposed for skelemin as follows: first it was suggested that it might act as a linker between intermediate filaments and the M-band (18); second an interaction of skelemin and β-integrin was proposed, based on an interaction between these proteins in a yeast two-hybrid assay (32). Thus, it was suggested that the function of skelemin in the M-band might be different from that of myomesin. Here, we provide strong evidence that skelemin, or EH-myomesin, is the only isoform of myomesin that is present in the M-band of early embryonic heart. Therefore, the principal role of EH-myomesin must be the same as of conventional myomesin, e.g. the maintenance of an ordered thick filament lattice in the M-band. Confusingly, the anti-skelemin antibody used by Reddy and co-workers (32) recognizes a protein not only in muscle tissue but also in Chinese hamster ovary cells, platelets, and even endothelial cells. A possible explanation for these conflicting results may be the binding of the antibody to a cross-reacting antigen. Indeed, their antibody recognizes a protein with a molecular mass of 205–210 kDa (32), which is in contradiction to the calculated molecular mass of 174 kDa and the mobility of myomesin as observed in previous studies (8, 15, 27, 33, 34). A cross-reacting antibody would also explain the detection of skelemin in smooth muscle (17). Previous investigations on myomesin expression by several laboratories have characterized myomesin as a sarcomeric protein and also reported its expression exclusively in striated muscle (8, 12, 15, 27, 31, 34, 35). In the present study we confirm by the means of RT-PCR and immunodetection that the expression of all myomesin isoforms is restricted to heart and skeletal muscle.

In contrast to H-myomesin, which was found only in avian cardiac muscle, the EH-isofrom of myomesin is present in the developing heart of both mammals and birds. Therefore, EH-myomesin could serve as an invaluable marker for the embryonic vertebrate heart, because no other myofibrillar protein expression is restricted to embryonic heart in such a tightly controlled manner (4). Sequence analysis showed that the EH-segments of mouse and human are rather similar, whereas the chicken EH-segment amino acid sequence is more divergent with only 36% identity and 48% similarity between the chicken and mouse EH-segments. This is a relatively small value as opposed to the 75% identity of the rod portion of myomesin, consisting of immunoglobulin and fibronectin domains (8).

Both mouse and human EH-segments are rich in serine/proline residues, hence the original name serine/proline rich domain, but this feature does not seem to be essential since the chicken counterpart contains fewer serine and proline residues, making it resemble an immunoglobulin-like domain much more closely. Despite the rather low sequence homology between mammals and birds, analysis of the secondary structure of all three sequences (Karplus-Schulz algorithm) predicts an increased flexibility of the EH-segment with regard to the immunoglobulin domains that make up the larger part of the molecule. In agreement with this prediction, we found that the circular dichroism spectrum of recombinant chicken EH-segment shows the characteristics of a largely unfolded protein with residual secondary structure (data not shown). Therefore, this segment could function as an additional flexible elastic stretch in the middle part of the myomesin molecule. The structure of EH-myomesin resembles in this aspect the structure of titin, which is also made up of two principally distinct regions, stretches of Ig modules separated by a unique segment of elusive secondary structure, the PEVK domain (36). The elasticity of this PEVK-segment allows titin to extend fully reversibly at physiological forces, without the need to unfold the Ig domains, which would be catastrophic in beating cardiomyocytes (37, 38).

What can be the physiological need for additional elasticity of the M-bands in embryonic heart? Embryonic cardiomyocytes differ in principle from skeletal muscle cells and adult cardiomyocytes by their ability to divide although already possessing all the contractile machinery. Presently, it is not clear what happens exactly to the myofibrillar apparatus during cell division, although there are some indications of partial disassembly of myofibrils, particularly the Z-disks (39). However, it was unequivocally demonstrated that some sarcomeres within isolated cardiomyocytes persist throughout mitosis (40, 41). Moreover, a recent study indicates that most myosin filaments remain bundled with myomesin in mitotic myocytes (42). This suggests that the M-bands in sarcomeres may be exposed to rather strong mechanical stress during the formation of the cleavage furrow and separation of the dividing cells. The additional elastic element in the middle of the myomesin molecule would therefore serve as a safety device, analogous to the PEVK domain of titin, thus preventing the irreversible unfolding of Ig domains.

Although our understanding of the functional significance of EH-myomesin is still incomplete, electron microscopic studies of the developing heart provide interesting insights. They reveal that heart sarcomeres acquire their characteristic morphology including an electron-dense M-band and stringently aligned thick filaments only around birth (43). The electron-dense M-band has been ascribed to the presence of muscle creatine kinase (44). Indeed, the appearance of muscle creatine kinase is correlated with the appearance of an electron-dense M-line in electron microscopic preparations, but muscle creatine kinase does not seem to play an essential structural role in the M-band since the muscle creatine kinase-deficient mouse exhibits no obvious abnormalities in sarcomeric structure (45). The increasing order leading to a perfect register of the thick
filaments may, however, be explained by the replacement of the more flexible EH-myomesin isoform by the adult myomesin, reducing the imprecision of the thick filament alignment in the M-band. This hypothesis is corroborated by studies of developing chicken skeletal muscle where no EH-myomesin is expressed, and perfectly aligned thick filaments in sarcomeres were observed in muscle of day 12 embryos (46).

The re-expression of embryonic or fetal genes was used as a molecular marker for hypertrophy (47). Cardiomyocytes from hypertrophic hearts are often characterized by myofibrillar disarray, and the sarcomeres are not as strictly registered as they are in the healthy heart, possibly indicating the reversion to more embryonic sarcomeric structures. Preliminary experiments have shown re-expression of EH-myomesin in cardiomyocytes from muscle LIM protein knock-out mice and from tropomodulin-overexpressing mice.2 Interestingly, both mouse strains show a phenotype of dilated cardiomyopathy (48, 49). However, future investigations will have to show to what extent the re-expression of EH-myomesin can be associated with myofibrillar disarray during other types of pathological hypertrophy.

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