Identification of Multiple Myotube and Myosin Heavy Chain Types During Embryonic Development of Chicken Skeletal Muscles

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Abstract

We used a panel of monoclonal antibodies to cardiac and skeletal muscle myosin heavy chains to study the composition of this protein and the embryonic development of chicken skeletal muscle myotubes. The presence of a distinct slow muscle-like embryonic isoform of myosin heavy chain (MHC) became apparent in some myotubes from their different reactivities with some of these antibodies. Four types of myotubes were identified in hindlimb muscles at 7 days *in ovo*. Muscle fibre type diversity was apparent as early as 5 days *in ovo* with SM1 and SM2 MHC being restricted to only some myotubes. The presence of ventricular MHC was observed in all skeletal muscles of the hindlimb although its distribution was restricted to only certain myotubes even during early stages of development. The slow embryonic MHC differed from the major fast muscle-like embryonic MHC in showing restricted distribution and not being recognised by antibodies to fast class of MHC by the immunoblotting procedure. The suppression of slow embryonic MHC was observed around day 15 *in ovo*.

Key Words: muscle, development, embryonic, myosin heavy chain.

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Most adult vertebrate skeletal muscles are composed of two major fibre types that express different isoforms of a number of contractile and regulatory muscle proteins and differ in their biochemical and physiologic properties [13]. The mechanisms by which these muscle fibre types emerge or evolve, however, are still not clear. Muscle fibre type specialisation may be triggered by intrinsic differences within myogenic cell lineages, or different fibre types may evolve from a single myotube type through positional information or through the influence of interacting tissues. Whereas the early *in vivo* or *in vitro* specification of myotubes indicates intrinsic differences within myogenic cells, the latter specification or the completion of the differentiation process during development may be influenced by changes in the cellular or hormonal environment. The initiation of differentiation has been studied extensively but differences in the timing of emergence of fibre types have been reported from different laboratories.

Myosin has been used extensively for assessment of the differentiation of muscle fibre types because this protein exists in cell and developmental stage-specific isoforms. In chicken, atrial and ventricular isoforms have been described in the cardiac muscle [15] and SM1 and SM2 isoforms in slow skeletal muscle [6, 11, 7]. Embryonic, neonatal and adult isoforms of MHC belonging to the fast class have also been described in both avian and mammalian skeletal muscles [20, 14, 2, 1, 10]. We undertook the present study to investigate the onset of diversity of myotube types in chicken thigh muscles and the possibility of existence of additional isoforms of MHC during early development. Four types of myotubes could be identified at 7 days *in ovo*, and the presence of a MHC with a distribution pattern different from that of all the known isoforms was apparent in some of the myotubes.

Materials and Methods

Muscle samples and immunochemical procedures.

White Leghorn chickens purchased from Wickham Laboratories (Winchester Road, Wickham, Hants. UK) and their eggs incubated at 37°C were used for all of the

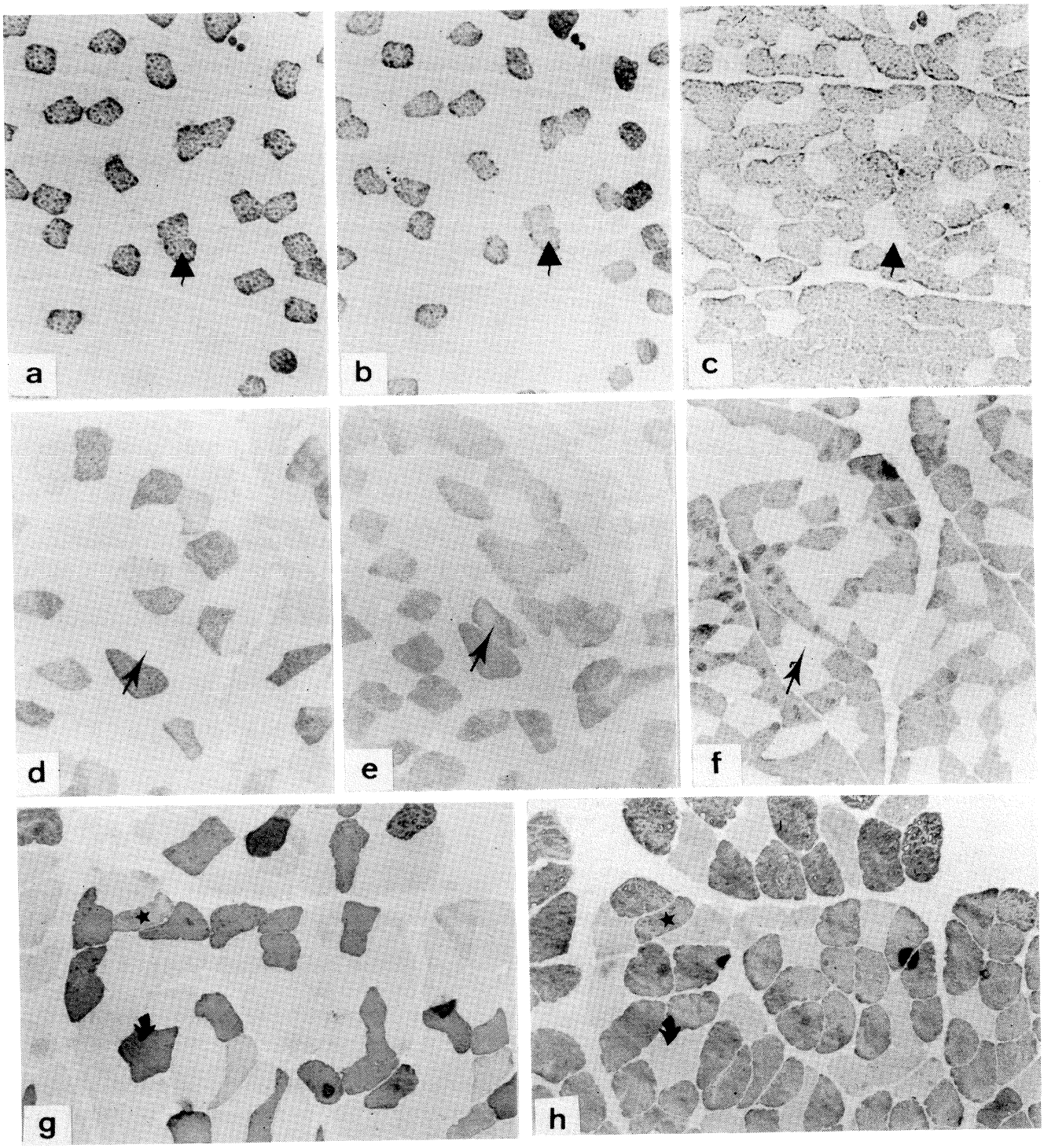


Figure 1. Specificities of antibodies for post-hatch or adult chicken skeletal muscles. Frozen sections of gastrocnemius (a-f) and ALD (g, h) muscles from 3 week (a-c) and adult (d-f, g, h) chickens stained by the immunoperoxidase procedure with antibodies 9812 (a, d, g), 83 (b, e, h) and LM5 (c & f). Identical cells in serial sections are labelled by similar arrows or symbols. a-c = x120, d-f = x48, g-h = x60.

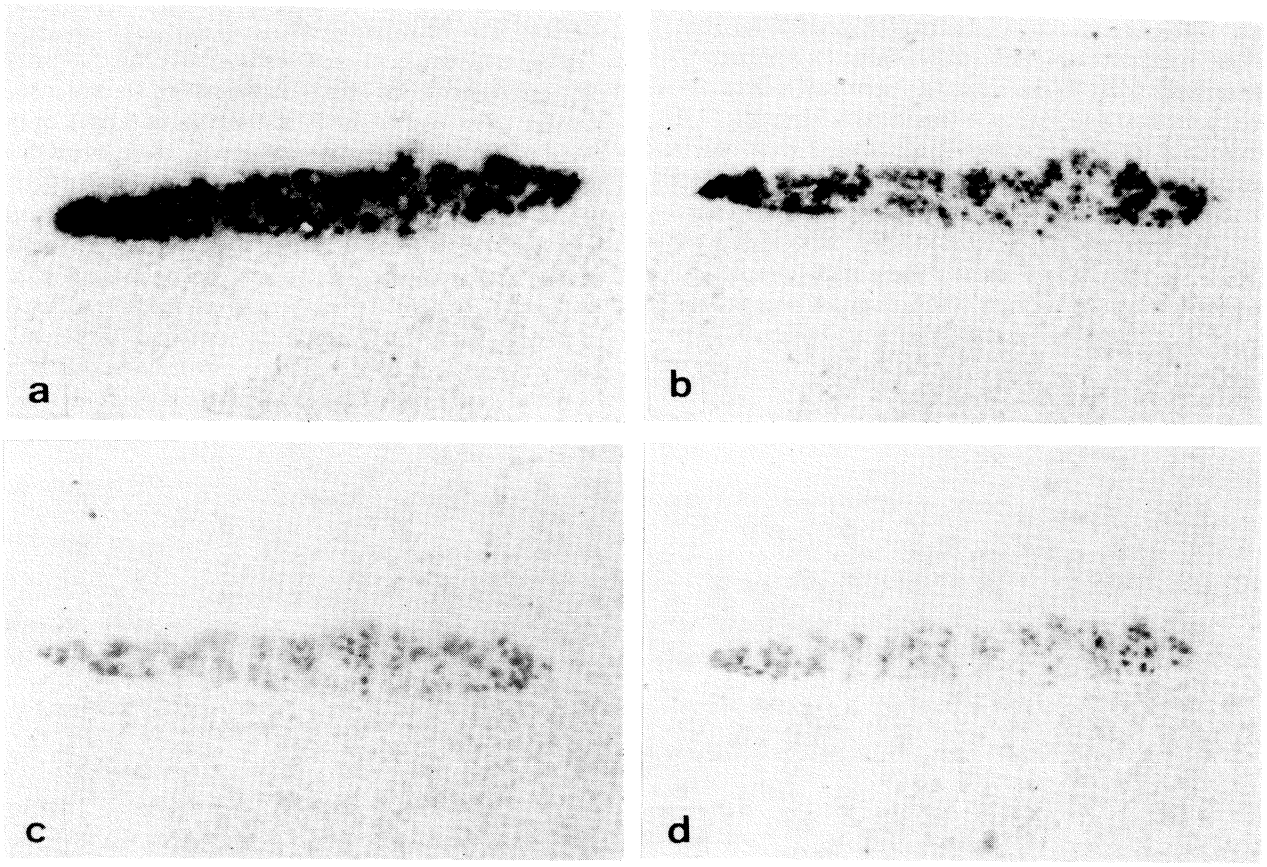


Figure 2. Frozen sections through the myotomal region of a 5 day chick embryo stained by the immunoperoxidase method with antibodies 96J (a), 18 (b), 9812 (c) and 83 (d). x110.

Table 1. Specificity of antibodies for chicken skeletal muscles by immunocytochemical and immunoblotting procedures.

	Antibody				
	96J	18	83	9812	LM5
Myosin heavy chain					
Fast embryonic	-	-	-	-	+
Fast neonatal	-	-	-	-	+
Fast adult	-	-	-	-	+
Slow embryonic	+	-	-	-	-
Ventricular	+	+	-	-	-
Atrial	+	-	+	-	+
SM1	+	-	+	+	-
SM2	-	-	+	-	-

immunoblotting and immunohistochemical studies of embryonic and post-hatch muscles. For immunohistochemical investigation, 5µ thick sections of muscles frozen in liquid nitrogen were stained with antibodies by the immunoperoxidase procedure as described previously [8].

Whole muscle extracts for electrophoresis were prepared by homogenising the tissue samples in 10 vol. of extraction buffer containing 2% SDS, 0.62 M Tris pH 6.8 and 0.75 M mercaptoethanol. After boiling for 5 minutes, 10 µl of embryonic muscle extracts were separated in SDS polyacrylamide (5%) gels according to the procedure described by Laemmli (1970). The proteins from the gels were transferred to nitrocellulose sheets by the method of Towbin *et al* (1979). The Western blots were stained with antibodies diluted 1/1000-1/2500 in phosphate buffered saline containing 0.1% Tween 20. We used peroxidase-linked rabbit antibodies to mouse or rat immunoglobulins (Dakopatts) to localise the antibody staining [8].

Results

Specificity of the antibodies

Antibody LM5 in the chicken skeletal muscles recognises all the fast forms of MHC including embryonic, neonatal and adult isoforms [4]. Antibody 9812 is specific

for SM1 [8], 18 for ventricular MHC [18] and 96J for SM1, cardiac and slow embryonic MHC (Table 1). Antibody 83 was raised to MHC isolated from chicken atrium but it also recognises SM1 and SM2 isoforms in skeletal muscle [17]. Whereas antibodies 96J and 9812 reacted with only a proportion of the type I or slow cells in most adult chicken hindlimb muscles, antibody 83 as expected, stained all of the type I cells recognised by acid stable myosin ATPase (Fig. 1). In the chicken anterior latissimus dorsi (ALD) muscle, however, antibody 83 did not stain a large proportion of the cells stained for SM1 with antibodies 9812 or 96J (Fig. 1g, h). This indicates possible heterogeneity in SM1 composition of ALD muscle.

Study of developing muscles

In immunocytochemical investigations of hindlimb muscles, antibodies LM5 (not shown) and 96J stained a large number of myotubes in chick embryos at 5 days *in ovo* (Fig. 2). The staining with antibody 18 to ventricular myosin heavy chain (VMHC) at this stage seemed generally similar and only slightly lighter than that observed with LM5 or 96J (Fig. 2a and b). The number of myotubes stained with antibodies 9812 and 83, recognising SM1 and SM2 MHC, in contrast was very much lower at this stage of development (Fig. 2c and d). SM1 and SM2 myosin heavy chains thus appeared to be restricted to only some of the myotubes at 5 day and to only presumptive slow muscle cell types during subsequent embryonic develop-

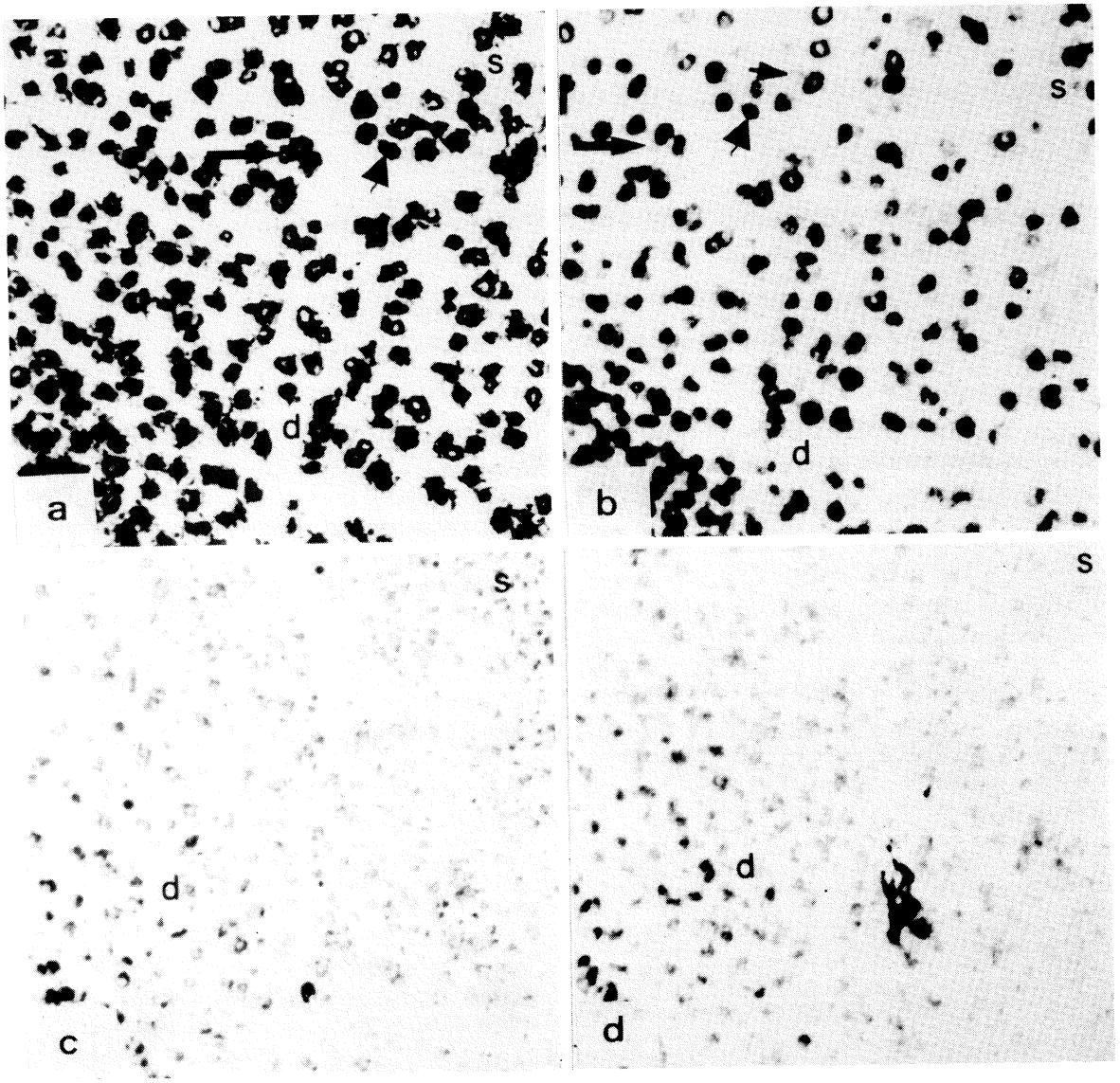


Figure 3. Frozen sections of femorotibialis medius muscle from a 7 day chick embryo stained by the immunoperoxidase procedure with antibodies 96J (a), 18 (b), 9812 (c) and 83 (d). Identical myotubes in sections a and b are labelled with similar arrows; d = deeper, s = superficial. x240.

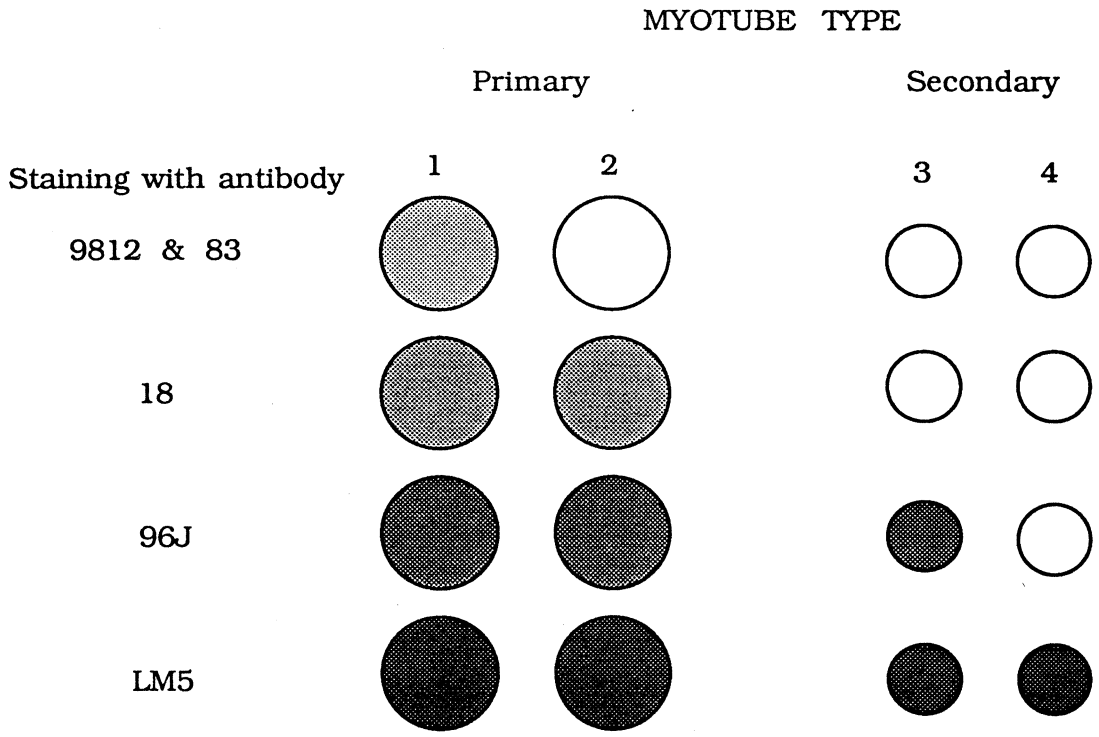


Figure 4. Schematic representation of staining of 4 myotube types with different antibodies in a 7 day chick embryo.

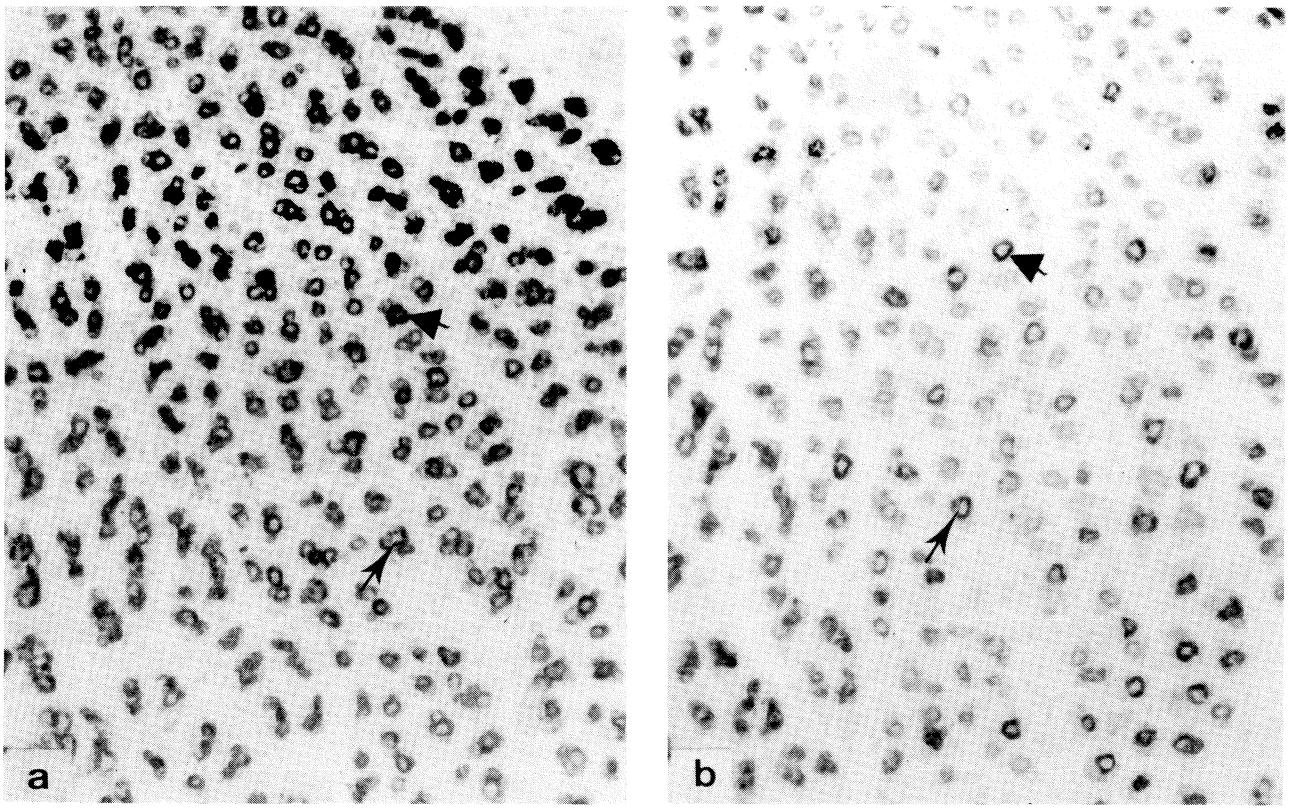


Figure 5. Serial sections of femorotibialis medius muscle from a 9 day chick embryo stained by the immunoperoxidase procedure with antibodies 96J (a) and 18 (b). Identical cells are labelled with similar arrows. x130.

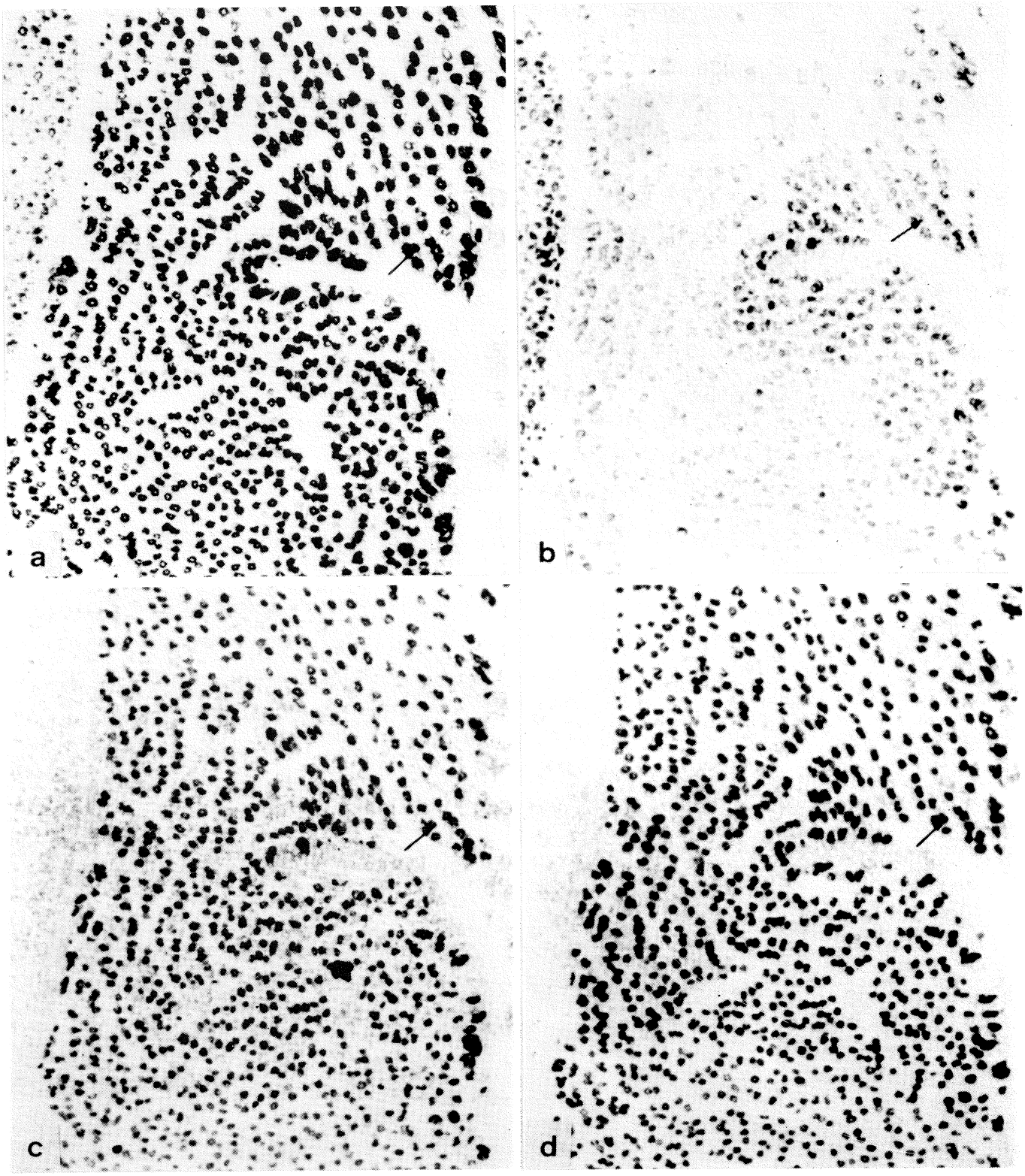


Figure 6. Serial sections of femorotibialis internus muscle from 12 day chick embryo stained by the immunoperoxidase procedure with antibodies 96J (a), 18 (b), 9812 (c) and 83 (d). An identical cell in serial sections is labelled with an arrow. x95.

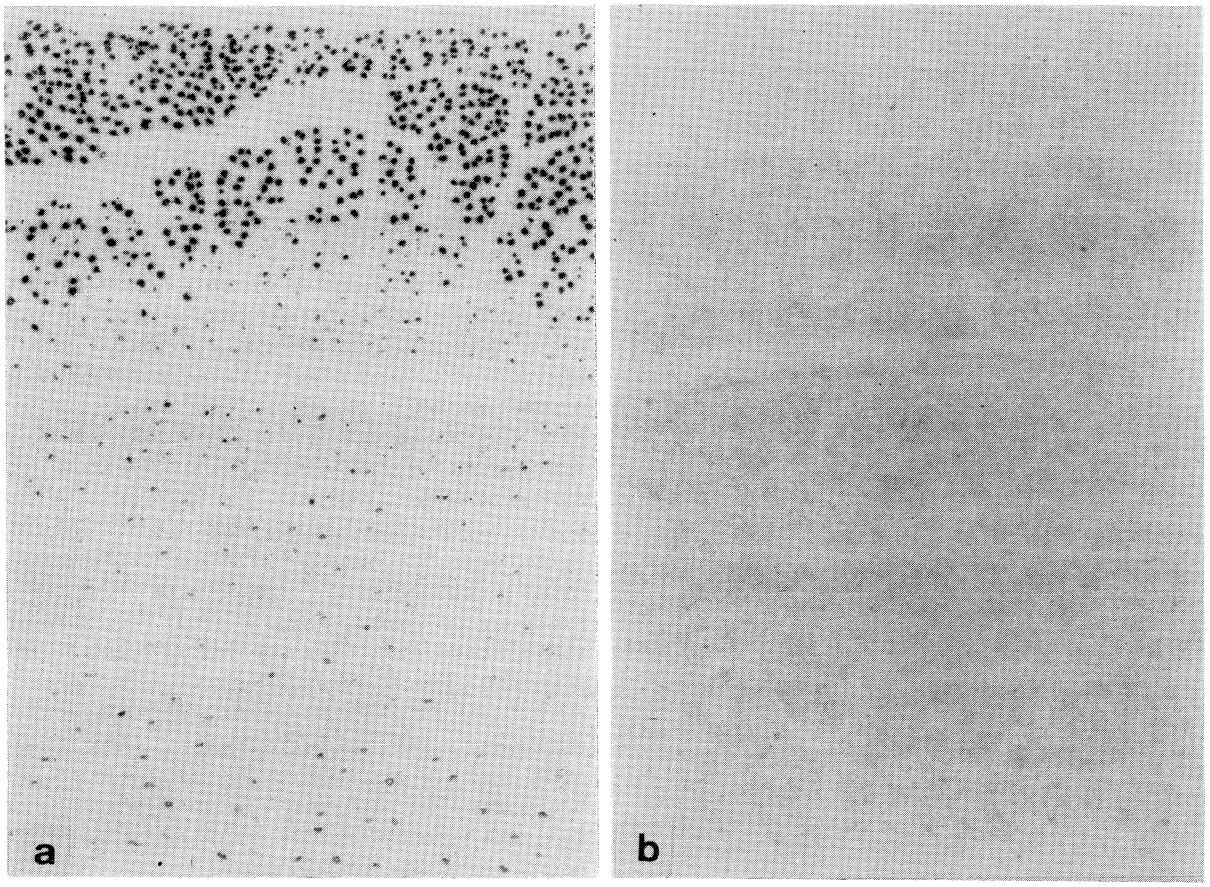
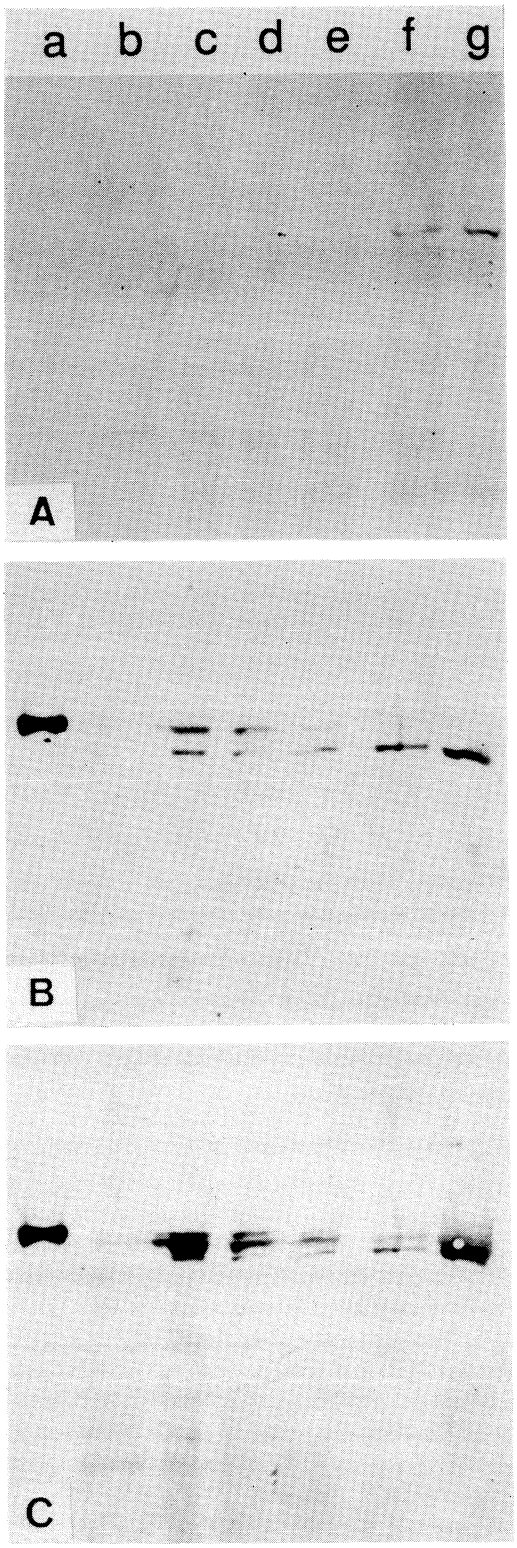


Figure 7. Serial sections through parts of iliofibularis (top) and iliotibialis posterior (bottom) muscles from 14 day chick embryo stained by the immunoperoxidase procedure with antibodies 96J (a) and 18 (b). x48.

ment. Fast MHC recognised by antibody LM5, on the other hand, was detected in all myotubes until its suppression which led to non detection in presumptive slow muscle cells around day 15 *in ovo* (not shown). It is assumed that antibody LM5 during early development recognises only fast embryonic MHC although it also reacts with neonatal and adult isoforms of this protein.

At 7 days *in ovo* (Fig. 3 and 4), only a small proportion of the myotubes stained with antibodies 9812 and 83 and all these myotubes were present in presumptive slow muscle cell regions (Fig. 3c and d). At this stage, antibody 18 to VMHC still stained a large number of myotubes, although this number was lower than that with antibody 96J (Fig. 3a and b). There was also marked variation in the staining intensity of different myotubes with antibody 18. With the possible exception of a few myotubes, a large majority of the myotubes of small diameter either did not stain or stained only weakly with antibody 18. Antibody 96J stained a large number of myotubes, although a small proportion of myotubes stained with antibody LM5 for fast MHC exclusively (not shown) and were thus unstained with antibodies 9812, 83, 18 and 96J. The proportion of myotubes stained with antibody 18 decreased further during subsequent embryonic development. The staining of 9 day chick embryonic leg muscles (Fig. 5) was generally similar to that observed for day 7, except that the proportion of cells stained with antibody 18 at this stage was further lowered compared with that of antibody 96J. This was particularly apparent in muscles such as the femorotibialis medius (FTM), femorotibialis internus (FTI) and iliofibularis. The number of myotubes stained with antibody 18 decreased rapidly and only a small proportion of the myotubes stained in most muscles by 12 days *in ovo* (Fig. 6). In some muscles like the FTM, FTI, femorotibialis externus (FTE) and adductor exterior, fibres close to connective tissue sheaths or septa stained darker than in central regions (see Fig. 6b). Most of the myotubes stained with antibody 18 were usually large and therefore appeared to be primary generation fibres, with the exception of a few small diameter fibres in the peripheral regions which may be either newly formed fibres or growing ends of muscle fibres where the new sarcomeres are added during growth. Antibody 96J, in addition to recognising SM1 MHC in presumptive slow muscle cells in deeper regions, still stained fibres in superficial regions of many muscles and its overall staining pattern at this stage differed markedly not only from that with 9812 and 83 but also from that observed with antibody 18.



By 14 days *in ovo*, usually no staining was observed with antibody 18 (Fig. 7) in most hindlimb muscles although a weak staining in some samples could be observed at the edges of some muscles. By 15-16 days *in ovo*, VMHC was undetectable in all muscles. The staining with antibody 96J by this stage seemed generally similar to that with 9812 and 83. The fast class of MHC recognised by antibody LM5 was also undetectable in presumptive type I fibres (not shown).

The distribution pattern of myosin heavy chains detected with antibody 96J in the immunocytochemical studies above suggests that this antibody recognises at least three myosin heavy chains in early chicken embryonic skeletal muscles i.e. SM1, ventricular MHC and an embryonic MHC described here as slow embryonic. Muscle samples were therefore analysed further by immunoblotting procedure using SDS polyacrylamide (5%) gel electrophoresis. With this procedure, antibody 9812 detected SM1 MHC in embryonic leg muscles during late development with only a trace amount being apparent at day 10 *in ovo* (Fig. 8A.e). In the same samples, antibody 96J, in contrast, stained a band in the position of SM1 not only in 10-16 day (Fig. 8B.e-g) but also in 7 and 8 day (Fig. 8B.c, d) chicken embryonic leg muscle samples. As expected, antibody 96J during early embryonic development stained another MHC band with electrophoretic mobility similar to that of ventricular MHC. Only two MHC bands were thus identified with antibody 96J when we used the present electrophoretic procedure. One of the myosin heavy chains (slow embryonic) may have coelectrophoresed with either SM1 or the ventricular isoform. Ventricular myosin heavy chain is believed to have the same electrophoretic mobility as SM2 [11]. Antibodies to the fast class of MHC stained a single band of MHC in these embryonic samples. When Western blots stained with antibody 96J were treated with antibodies to fast MHC, this MHC (fast embryonic) showed electrophoretic mobility intermediate to that of SM1 and ventricular myosin heavy chain (Fig. 8C).

Discussion

Based on the immunocytochemical observations, four types of myotubes could be identified in the chicken embryonic skeletal muscles by 7 days *in ovo*. Myotube type 1 stained for fast embryonic (LM5), ventricular [18], SM1 and SM2 myosin heavy chains (9812 and 83), and myotube type 2 for fast embryonic (LM5) and ventricular MHC [18]. Myotube type 3 stained for both fast and slow embryonic MHC (LM5 and 96J), and myotube type 4 for fast

Figure 8. Reaction of some monoclonal antibodies with adult cardiac and developing chicken skeletal muscles. Western blots of extracts of identical muscle samples separated in SDS polyacrylamide (5%) gels stained by the immunoperoxidase procedure with antibodies 9812 (A) and 96J (B). After photographing, immunoblot B was restained with a monoclonal antibody to fast class of myosin heavy chain (C). Muscle samples: a = chicken ventricle; b-g = leg muscles from chick embryos at 5, 7, 8, 10, 13 and 16 day in ovo. The protein band with the fastest electrophoretic mobility is SM1 (A-C) and that with the slowest mobility is ventricular MHC (B, C). The fast embryonic MHC shows mobility intermediate to that of SM1 and VMHC (C).

embryonic MHC (LM5) only. SM1 and SM2 myosin heavy chains appeared to be expressed in only one of the four myotube types i.e. the presumptive slow (myotube type 1). All myotubes expressed fast embryonic MHC until 15-16 days *in ovo*, although only one myotube type of small diameter (secondary) expressed it exclusively. At 7 day *in ovo*, only two of the four myotube types expressed ventricular MHC and both these appeared to be primary generation myotubes. At this stage, slow embryonic MHC could be detected definitively in only one of the four myotube types, i.e. myotube type 3, but may also have been present in myotube types 1 and 2 in which SM1, SM2 and VMHC is also present. A myotube type containing only the slow isoforms of MHC [16] was not observed with these antibodies in the present study.

Muscle fibre type diversity in chicken embryos was apparent as early as 5 day *in ovo* with SM1 and SM2 myosin heavy chains being restricted to only some myotubes at this stage. This early fibre type differentiation is compatible with studies reported by Stockdale and colleagues [16]. In the present study we did not investigate stages earlier than 5 day *in ovo* when all myotubes are reported to express a common MHC phenotype in both fast and slow wing muscles [18]. With antibody 83, which recognises both SM1 and SM2 myosin heavy chains in most leg muscles, the distribution of these myosin heavy chains appeared to be restricted to the same myotubes that stained with SM1 MHC specific antibody 9812. Antibody specific for atrial MHC was not used in the present study although antibody 83 recognises this MHC in addition to SM1 and SM2 MHC [17]. If atrial MHC is present in embryonic skeletal muscles it must be present in the same myotubes that contained SM1 and SM2 myosin heavy chains (myotube type 1) as antibody 83 did not stain any additional myotubes.

Ventricular MHC during early embryonic development was observed in a large number of the myotubes (both presumptive fast and slow) in all thigh muscles. The timing of its suppression, however, varied not only in different muscles and myotubes, but also in different regions of the same muscle. Generally, its level of expression decreased first in central regions of most muscles. Nondetection of VMHC in some myotubes or regions of muscle was a gradual process. Unlike the fast class of MHC that was found to be present in all myotubes until 15-16 days *in ovo*, the relatively restricted distribution pattern of VMHC was already apparent by day 7 *in ovo*. In a small proportion of the myotubes which from their generally small size appeared to be secondary generation fibres, VMHC was either undetectable or was present in only small amounts, although the appearance of secondary generation fibres in the chick embryo has been reported not to occur until about 12 days *in ovo* [12]. In the present study, a few myotubes of small diameter around large myotubes presumed to be primary generation myotubes in some muscles could already be detected by day 7 *in ovo*. As has also been reported for posterior latissimus dorsi (PLD) and ALD [18], the synthesis of VMHC in hindlimb muscles thus appeared to be restricted to primarily large (primary generation) cells though not only presumptive slow but also presumptive fast muscle cells. Although VMHC was present even in those muscles that did not contain any slow fibres, its distribution during later stages e.g. at 12 days *in ovo* appeared to correlate with a subpopulation of slow myotubes in those muscles in which these cells were present. By this time some myotubes in the peripheral regions or near connective tissue sheaths or septa also stained moderately darkly in some muscles in the hindlimb. This variation in the staining of VMHC may be related either to areas of greater growth or to points of muscle insertion where the new sarcomeres are added at the ends of existing myotubes.

During embryonic development, a proportion of the myotubes clearly stained with antibody 96J, but not with antibodies to slow or cardiac myosin heavy chains. Although antibody 96J recognises both atrial and ventricular myosin heavy chains [5], the staining of these myotubes is probably not due to the presence of cardiac myosin heavy chains because these cells were not stained by either antibody 18 (ventricular) or 83 (atrial) unless these monoclonal antibodies recognised only a subset of the cardiac myosin heavy chains. This embryonic MHC (slow embryonic) therefore was recognised by antibody 96J in those cells that did not stain for SM1, SM2 and atrial or ventricular MHC although they stained for fast embryonic MHC with which antibody 96J does not react as is demonstrated by immunoblotting.

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References

[1] Bader D, Masaki T, Fishman DA: Immunochemical analysis of myosin heavy chain during embryonic myogenesis *in vivo* and *in vitro*. *J Cell Biol* 1982; 95: 763-770.

[2] Bandman E, Matsuda R, Strohman RC: Developmental appearance of myosin heavy and light chain isoforms *in vivo* and *in vitro* in chicken skeletal muscle. *Dev Biol* 1982; 93: 508-518.

[3] Crow MT, Stockdale FE: Myosin expression and specialisation among the earliest muscle fibers of the developing avian limb. *Dev Biol* 1986; 113: 238-254.

[4] Dhoot GK: Changes in the distribution of slow skeletal myosin heavy chain SM1 in developing