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The emergence of an adult muscle phenotype in urodelan amphibians: an immunohistochemical study

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ABSTRACT Electrophoretic techniques adapted for the analysis of muscles of lower invertebrates reveal four myosin heavy chain isoforms in the *dorsalis trunci* of *Pleurodeles waltlii*: two fast (MHC-IIA, MHC-IIB), and one slow (MHC-I) in the adult and one isoform (MHC-La) in the larvae. Polyclonal antibodies were prepared against the larval (anti-MHC-La) and one of the fast myosin (MHC-IIA) isoforms and their specificity was confirmed by western blot analysis. An immunohistochemical analysis was then carried out on frozen sections of the *dorsalis trunci* of *P. waltlii* at different stages of development. From stage 44 it was possible to demonstrate the presence of MHC-IIA in the small diameter fibers at the periphery of the muscle; the number and diameter of these fibers increased from stage 44 to stage 56 when anatomical metamorphosis had finished. By stage 56 these fibers could also be readily identified using standard histochemical techniques as type IIA fibers. We conclude that fast IIA myosin is expressed well before the final adult muscle phenotype has been established and its expression is therefore independent of thyroid hormone.

KEY WORDS: *urodelan amphibians, myosin, muscle, thyroid hormone, immunochemistry*

Introduction

In skeletal muscle a number of different myosin isoforms have been identified by their electrophoretic mobility in adult mammals (d'Albis *et al.*, 1979, 1987, 1989; Whalen *et al.*, 1981; Butler-Browne *et al.*, 1982, 1990; Butler-Browne and Whalen, 1984), birds, (Hoh *et al.*, 1976) amphibians, (Chanoine *et al.*, 1987, 1991) and fish, (Zawadowska and Karasinski, 1988; Martinez *et al.*, 1989) by their differing ATPase activities (Barany *et al.*, 1965; Barany, 1967; Brooke and Kaiser, 1970; Brooke *et al.*, 1971; Gauthier, 1986; Staron and Pette, 1986), and by their immunological properties (Masaki, 1974; Bruggmann and Jenny, 1975; Gauthier and Lowey, 1977; Schwartz *et al.*, 1977a,b, 1980; Butler-Browne *et al.*, 1982; Butler-Browne and Whalen, 1984; Thornell *et al.*, 1984). In adult mammals, four myosin heavy chains coded by distinct genes (Izumo *et al.*, 1986) have been identified by SDS-PAGE (Billeter *et al.*, 1981; Pierobon-Bormioli *et al.*, 1981; Fitzsimons and Hoh, 1983; Butler-Browne and Whalen, 1984; Betto *et al.*, 1986) and are expressed in the different fiber types: MHC-I is found in the slow oxidative type I fibers, MHC-IIA in the fast oxidative type IIA fiber and IIB in the fast glycolytic IIB fibers. Recently a third type of fast MHC has been described in mammals and has been called fast IIX (Schiaffino *et al.*, 1989) or IID (Bär and Pette, 1988).

By adapting the existing electrophoretic techniques for the analysis of muscles of lower vertebrates we have been able to demonstrate the existence of three adult myosin heavy chain isoforms, a fast (MHC-IIB), an intermediate (MHC-IIA), a slow (MHC-I) and one larval isoform. The ATPase activity of a muscle fiber is known to be correlated to the type of myosin heavy chain isoform expressed by that fiber (Staron and Pette, 1986).

In lower vertebrates (Chanoine *et al.*, 1987, 1989, 1992, 1994), several populations of muscle fibers have been characterized enzymehistochemically by their sensitivity following preincubation at acid or alkaline pH both in the adult (I, IIA and IIB) and in the animal undergoing metamorphosis (IIC). The *dorsalis trunci* of *P. waltlii* is a mixed muscle containing a predominance of fast muscle fibers. In the 8 month old adult there are 67% type IIA fibers, 20% type IIB fibers, 13% type I fibers and no type IIC fibers. In contrast in *P. waltlii* of the same age which are deprived of pituitary TSH, the type IIA fibers are absent and 70% of the fibers are type IIC transition fibers as in these metamorphosing animals at stage 55C (Chanoine *et al.*, 1987). The correlation between the percentage of type IIC fibers in the metamorphosing individual and in the hypophysectomized individuals confirms an absence or delay in the maturation of the type IIA fibers in the absence of thyroid hormone (Salles-Mourlan *et al.*, 1994). This would suggest that the maturation of the type IIA fibers is dependant on thyroid hormone whereas type I and type IIB muscle fibers seem to mature normally in the hypothyroid animal and were not therefore influenced by the thyroid hormone status.

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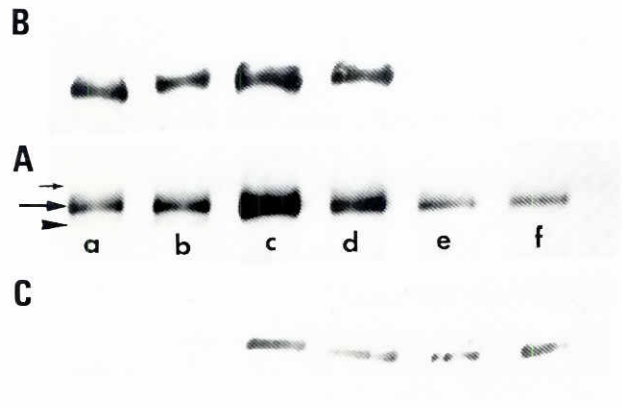


Fig. 1. Demonstration of antibody specificity by western blot analysis. **(A)** *SDS-PAGE of the myosin heavy chains present in the dorsalis trunci muscles of P. waltlii. a and b, adult muscle. c and d, a mixture of adult and larval myosin extracts. e and f, larval myosin extracts. Fast myosin heavy chain (MHC-IIB) (arrowhead). Intermediate heavy chain (MHC-IIA) (medium arrow). Slow myosin heavy chain (MHC-I) (small arrow).* **(B)** *Immunoblotting analysis of adult myosin with anti-MHC-IIA.* **(C)** *Immunoblotting analysis of larval myosin with anti-MHC-Larval.*

In *P. waltlii*, thyroxine (T4) is not detected in the serum until stage 54. A regular increase in its level is then observed during metamorphosis until stage 55 (Chanoine *et al.*, 1987) when the adult muscle phenotype is finally established. In view of these findings it was decided to study the early stages of development in order to define the exact chronology of the appearance of the IIA muscle fibers and to determine whether or not thyroid hormone is required for the expression of the MHC-IIA.

During development, standard histochemical techniques cannot be used to follow the appearance of the different fiber types since more than one type of myosin isoform is often present in the same muscle fiber. In these conditions it is always the major isoform which will predominate in the ATPase profile and the minor isoforms will remain undetected or give an ATPase reactivity which is difficult to interpret. In this study therefore polyclonal antibodies were made by immunizing rabbits with either adult fast or larval myosin. Two antibodies were obtained; one was shown to be specific for MHC-IIA, which is the predominant isoform present in the *dorsalis trunci*, and the other was specific for the larval isoform. Using an immunocytochemical approach, it was possible to follow the appearance of fast IIA fibers in the muscle during development and the gradual replacement of the larval by fast IIA myosin.

Results

The *dorsalis trunci* of *P.waltlii* is a predominantly fast muscle containing 87% fast fibers and 13% slow (Chanoine *et al.*, 1987). The slow myosin isoform is only seen as a faint band at the dilutions required to obtain the best separation in the gel system used in this study. In the metamorphosing animal this muscle contains up to 70% IIC transition fibers (Brooke *et al.*, 1971).

By denaturing gel electrophoresis three myosin heavy chains: fast IIA, fast IIB and slow I could be detected in the adult back muscle. In the larval muscles a single band corresponding to the larval myosin heavy chain was detected (Fig. 1A).

Characterization of the antibodies

Both the anti-MHC-IIA and anti-MHC-La were characterized by immunohistochemistry and immunoblotting. On transverse frozen sections the adult MHC antibody stained the type IIA muscle fibers exclusively and immunoblotting confirmed that this antibody also reacted specifically with the fast IIA myosin heavy chain and showed no cross reactivity with the other types of MHC (Fig. 1B). This was also true for the larval MHC antibody which recognized specifically the larval myosin heavy chain and showed no cross reactivity with the adult isoforms (Fig. 1C).

Expression of the larval myosin heavy chain

At hatching (stage 34) the anti-MHC-La reacted in a homogeneous manner with all of the myotubes. This homogeneous pattern of staining was maintained in the muscle fibers from stage 34 up until stage 55a, the stage which marks the beginning of pleurodelan metamorphosis. During metamorphosis the IIA fibers were the first to eliminate the MHC-La isoform.

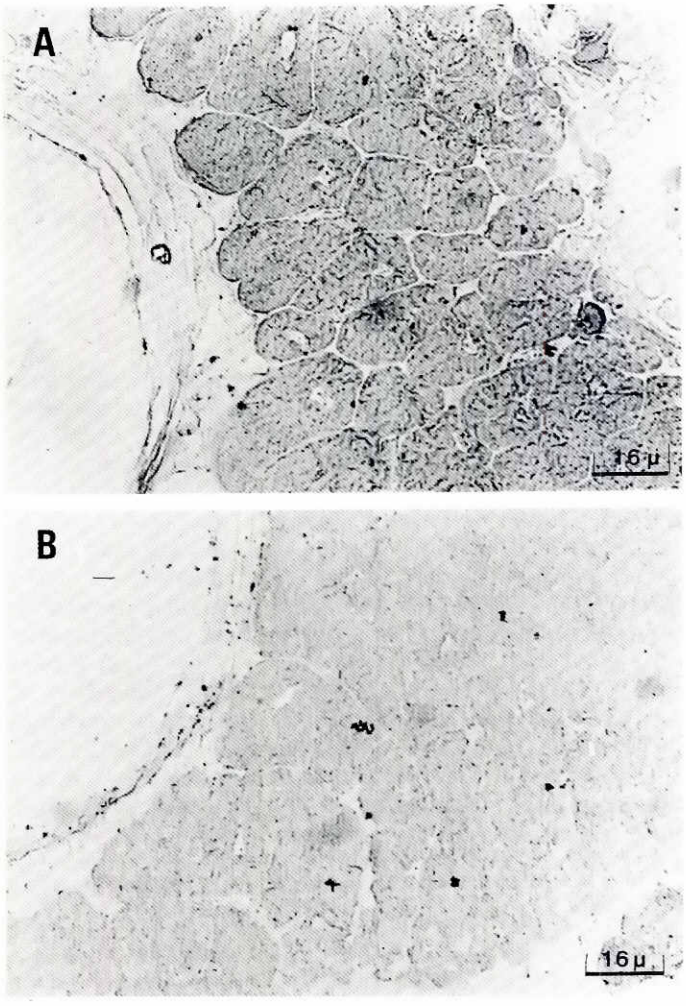


Fig. 2. Immunohistochemistry on transverse sections of the *dorsalis trunci* at stage 39 with antibodies directed against the larval MHC and the adult fast MHC-IIA. **(A)** *Positive reaction with the anti-MHC-Larval;* **(B)** *negative reaction with the anti-MHC-IIA.*

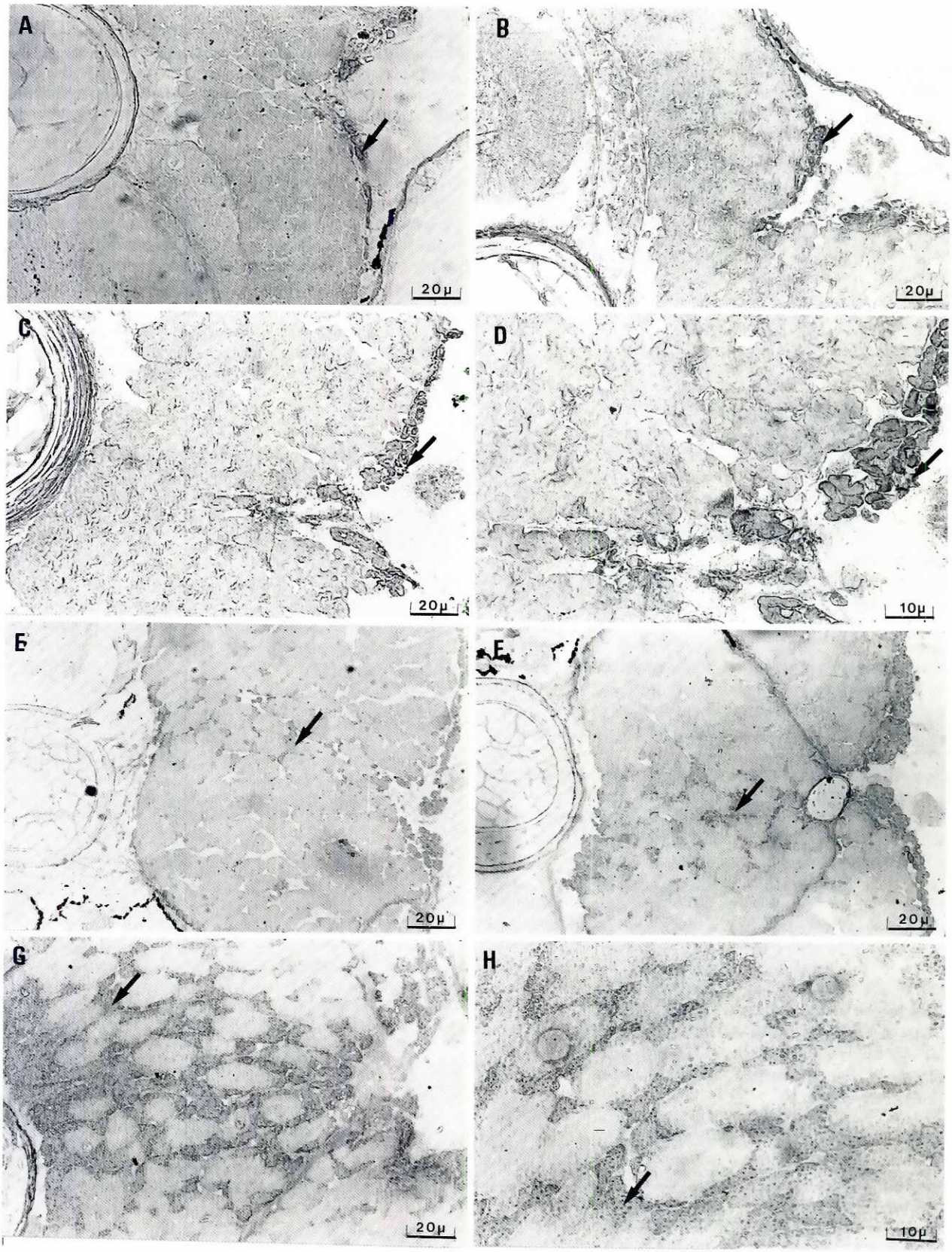


Fig. 3. Immunohistochemical analysis of transverse sections of the *dorsalis trunci* with antibodies directed against the MHC-IIA. *The gradual appearance of MHC-IIA in the potential type IIA muscle fibers is shown at different stages of development (***A***, stage 44;* **B***, stage 49;* **C** *and* **D***, stage 50;* **E***, stage 53;* **F***, stage 54;* **G** *and* **H***, stage 55a). (Large arrow) Potential type IIA muscle fibers.*

TABLE 1

IMMUNOHISTOCHEMICAL AND ENZYMEHISTOCHEMICAL IIA FIBER TYPES IN THE *DORSALIS TRUNCI* AT VARIOUS STAGES OF DEVELOPMENT

Stages	Total number of fibers	Numbers of fibers containing MHC-IIA	Percents (%)
41[a]	61	0	0%
44[a]	148	9	6%
52[a]	693	224	32%
53[a]	855	285	33,5%
54[a]	1005	360	36%
55[a]	1710	1008	59%
adult metamorphosed animal[b]	5680	805	67%

[a]determined as the fibers reacting strongly positive in the immunological reaction after incubation with anti-MHC-IIA; [b]determined as the fibers reacting strongly positive in the histochemical ATPase reaction after acid preincubation at pH 4.63.

Expression of the fast IIA myosin heavy chain from hatching until metamorphosis

At stages 34, 39 and 41 all of the myotubes reacted strongly with the anti-MHC-La (Fig. 2A) and did not react with the adult fast anti-MHC-IIA (Fig. 2B).

At stage 44 (Fig. 3A), the first fibers expressing IIA myosin were detected in a population of very small diameter fibers situated at the periphery of the muscle, just under the pigmented skin cells in the lateral region of the cord at the level of the transverse myoseptum. This septum divides the epiaxial and hypoaxial musculature of the larvae (Watanabe *et al.*, 1980).

At stage 49 (Fig. 3B), there are only a few remaining myotubes and the muscle has a more homogeneous appearance. The population of very small diameter fibers labelled with the fast IIA antibody are still present and are distributed in a similar manner to stage 44. At this stage, however, there is a slight inversion of the ATPase reaction at pH 4.63 at the periphery of the section.

At larval stage 50 (Fig. 3C and 3D), the large fibers which react with the anti-MHC-La have a more elongated aspect in transverse sections. The small fibers at the periphery which coexpress both the larval and fast IIA myosin are distributed in two more or less even layers in the hypoaxial and epiaxial peripheral muscle layers and spread in between the large fibers at the level of the transverse septum. The majority of these fibers are larval type IIC. Apart from these areas of small diameter fibers, the remaining muscle is made up of large diameter muscle fibers.

During the following stages of development in those regions of the muscle lateral to the vertebral axis there is a large increase in the number of small and medium diameter fibers which react with the anti-MHC-IIA (Table 1). Newly formed small diameter fibers appear among the large diameter fibers in the dorsal and ventral regions, giving the muscle a very heterogeneous appearance. There is also an increase in the number of small diameter fibers in the peripheral subcutaneous region (Fig. 3E,3F,3G and 3H).

At stage 55b/55c an irregular peripheral crown, two or three fibers in depth, reacts exclusively with the anti-MHC-IIA antibody showing that the maturation as indicated by the appearance of the IIA fibers begins in the lateral and peripheral muscular layers at the level of the transverse septum. The larval antibody reacted with all fibers except those with the smallest diameter at the periphery. (Fig. 4A and 4B).

At stage 56a (approximately 3 weeks after metamorphosis) the diameter of the muscle fibers was variable. In general the IIA fibers have the largest diameter and the type I fibers the smallest. Following preincubation at pH 4.63 it was possible to identify 10% type I fibers and 20% type IIA. The remaining 70% of the fibers, poorly differentiated by enzymehistochemistry, were predominantly classed as type IIC (Fig. 5A). The phenomenon of regionalization, previously described in our laboratory, was clearly seen in these samples. The type I fibers were preferentially localized in the subcutaneous and para-axial zones. The anti-MHC-IIA reacted with all of the muscle fibers that could be

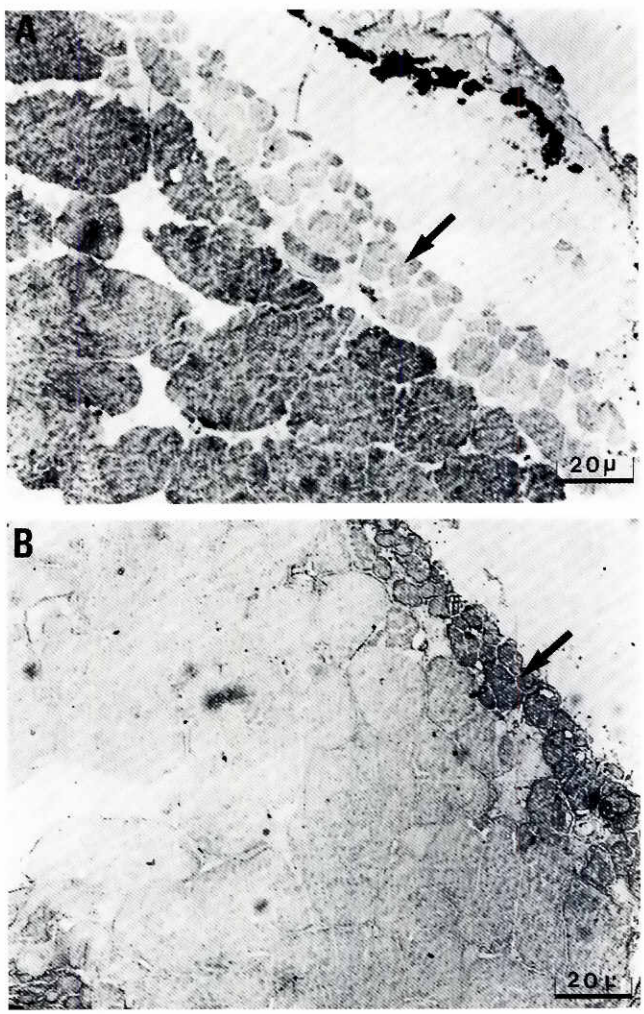


Fig. 4. Immunohistochemical analysis of transverse sections of the *dorsalis trunci* at stages 55b/55c: (A) *reaction with the anti-MHC-Larval. Only the peripheral muscles fibers were unreactive.* **(B)** *Reaction with the anti-MHC-IIA. In this case only the peripheral fibers were positive. (large arrow) Potential type IIA muscle fibers.*

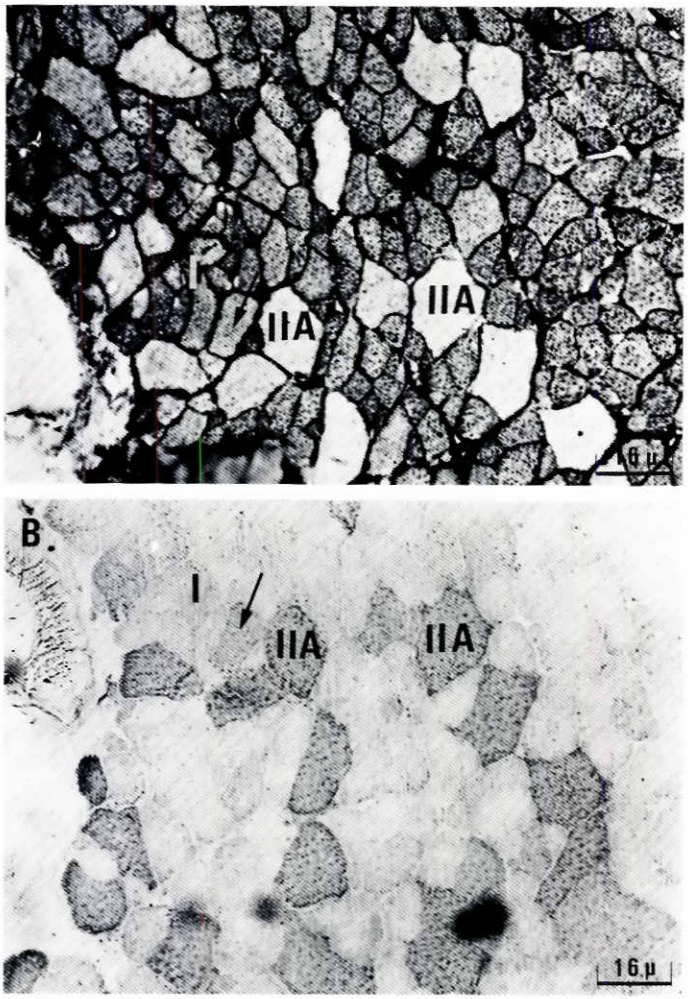


Fig. 5. Transverse sections of the *dorsalis trunci* at stage 56a. (A) *ATPase reaction at pH 4.63 (type IIA-pale; type I-dark; type IIB and IIC intermediate staining).* **(B)** *Positive reaction with the anti-MHC-IIA on type all IIA fibers and on certain type IIC immature transition fibers (medium arrow).*

classified as type IIA by enzymehistochemistry and with some of the type IIC fibers (Fig. 5B).

At stage 56b (approximately two and a half months after external metamorphosis), the IIB fibers can also be clearly identified enzymehistochemically. They were clearly distinguished from the IIC fibers by their lability at pH 4, 35 whereas the IIC fibers were still reactive at this pH. (Fig. 6A). As at stage 56a, all IIA fibers reacted strongly and some of the type IIC fibers reacted weakly with the anti-MHC-IIA (Fig. 6B). The larval antibody stained all the type IIC fibers, some of the type I but none of the IIA fibers, which could therefore be considered to be mature at this stage (Fig. 6C). Thus the larval MHC was eliminated from the IIA fibers as early as stage 56a whereas it persisted in the type IIB and I fibers until stage 56b.

Fig. 6. Enzymehistochemical and immunohistochemical analysis of transverse serial sections of the *dorsalis trunci* at stage 56b. (A) *Myosin ATPase at pH 4.63.* **(B)** *Reaction with the anti-MHC-IIA: all IIA and some IIC fibers (medium arrow) are positive.* **(C)** *Reaction with the anti-MHC-Larval: all IIA fibers are negative.*

The maturation of the different fiber types by the elimination of the larval MHC has been summarized in Table 2.

The adult muscle was characterized by the absence of type IIC fibers: it was now made up of 67% type IIA, 20% IIB and 13% type I muscle fibers. The type IIA fibers now express exclusively the IIA myosin heavy chain.

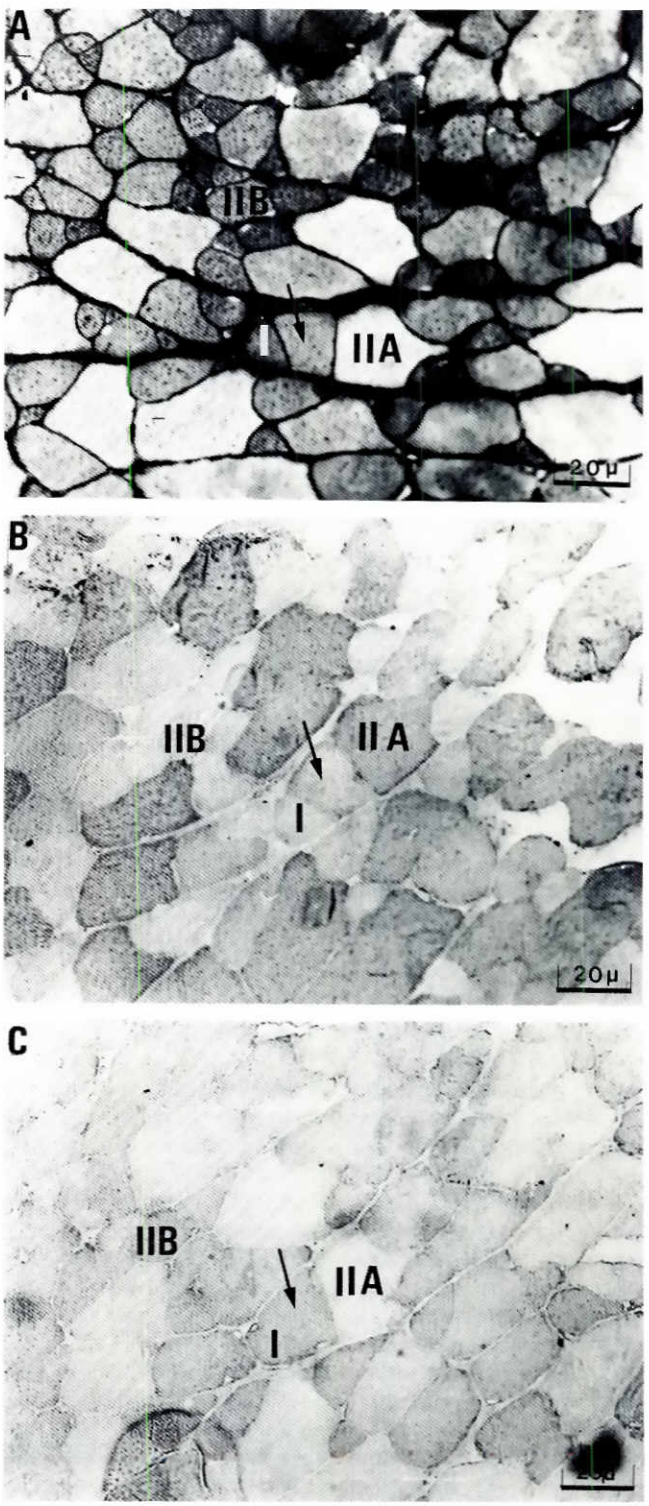
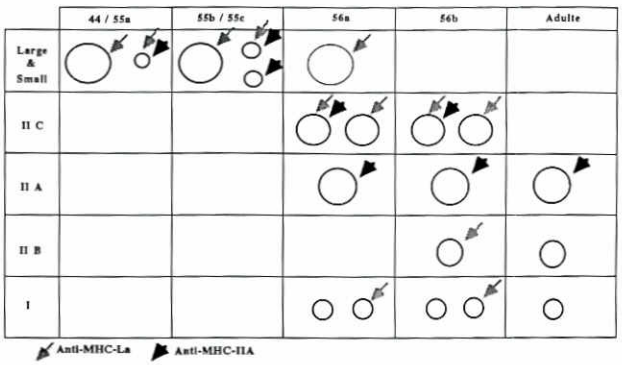


TABLE 2

SCHEMATIC REPRESENTATION OF THE MATURATION OF FIBERS IN THE *DORSALIS TRUNCI* MUSCLE



Stage 44/55a: only small diameter fibers at the periphery of the muscle reacted with both the larval and the IIA antibodies; stage 55b/55c: the peripheral small diameter fibers now reacted exclusively with the IIA antibody whereas the more central ones reacted with both antibodies; stage 56a: type IIA fibers react exclusively with the IIA antibody; the type IIC fibers are made up of two populations: those which react with both antibodies are probably the immature type IIA fibers; stage 56b: the IIB fibers still react with the larval antibody whereas certain type I fibers no longer react with any of these antibodies. Adult: The IIC transition fibers have now disappeared. No fibers react with the larval antibody.

Discussion

As demonstrated by the myosin ATPase reaction it is during the anatomical metamorphosis of *P. waltlii* (stage 55a-55b) that the mature muscle phenotype is established and the muscle fibers attain their definitive metabolic properties. This maturation is correlated with an increase in the level of circulating thyroid hormones (Etkin, 1968; Chanoine *et al.*, 1990).

In a previous study we had already demonstrated the possible importance of thyroid hormone on the maturation of type IIA muscle fibers, since in the absence of pituitary TSH, the immature type IIC fibers which give rise to type IIA fibers persist and do not form metabolically mature muscle fibers. The persistence of the MHC-La in these type IIC fibers was confirmed by electrophoresis (unpublished data).

The aim of the present study was to correlate the action of thyroid hormone with the expression of the MHC-IIA in the urodelan amphibian *P. waltlii*.

Using polyclonal antibodies against the larval MHC, we have been able to confirm that the type IIC transition fibers all express the MHC-La. During development in both amphibians and mammals (Pierobon-Bormioli *et al.*, 1981) these IIC fibers coexpress both larval and adult MHC isoforms. It is not known however if this antibody reacted exclusively with the larval isoform or if it could also recognize a hypothetical embryonic isoform. Since a slow MHC antibody was not used in this study it was not possible to confirm whether the fibers which reacted with the larval antibody also contained small amounts of slow MHC.

It seems evident however that the IIC fibers are in fact a heterogeneous population. Those fibers which react with the anti-MHC-IIA will become the mature IIA fibers in the adult whereas the remaining fibers which reacted only with the larval antibody in these experiments would gradually eliminate the larval isoform to express either MHC-I or MHC-IIB in the mature animal.

The small diameter muscle fibers which were found in the muscle at different stages of larval development reacted with the IIA antibody and were evidently the precursors of the type IIA fibers found in the adult. These fibers progressively increased both in size and in number during development in a similar manner to that which has been described previously in fish (Scapolo *et al.*, 1984), birds (Mouly *et al.*, 1987) and man (Gros *et al.*, 1990; Barbet *et al.*, 1991).

The differentiation of the IIA fibers begins with the appearance of the first fibers in the lateral peripheral region at the level of the transverse myoseptum in the pleurodelan larvae at stage 44 and finishes with the disappearance of the last IIC fibers.

The fast MHC-IIA is accumulated in the muscle fibers very early during development completely in the absence of thyroid hormone and one can therefore conclude that the expression of the fast MHC-IIA is independent of any influence from the thyroid hormones.

At stage 56a, the IIA fibers which can be identified by their ATPase reaction, have now become the predominant fiber type and are also the largest. They no longer express the larval isoform, in contrast to the IIB fibers which express the larval MHC up until stage 56b (i.e. 7 weeks later).

It appears from this study that the larval isoform is repressed later in the presumptive IIB fibers than in the presumptive IIA fibers. These results show that type II fibers within the same muscle do not attain their mature phenotype at the same stage of development. The IIA fibers as in mammalian (Close, 1972; Haltia *et al.*, 1978; Ho *et al.*, 1983) muscles mature before the IIB fibers.

In the *dorsalis trunci* of the adult, none of the IIB fibers react with the anti-MHC-IIA whereas in the forelimbs some of the IIB fibers do react with this antibody (unpublished data). These results confirm that the expression of the different myosin isoforms is determined by the type of muscle in which they are expressed (Mahdavi *et al.*, 1987; d'Albis *et al.*, 1989, 1990; Saadi *et al.*, 1992).

From this study we conclude that in the *dorsalis trunci* of *P. waltlii* the appearance and the persistence of the adult fast MHC-IIA seems to be completely independent of the thyroid hormone status of the animal. However, the repression of the expression of the MHC-La may be controlled by thyroid hormone.

It is possible that factors other than thyroid hormone — either circulating factors or innervation — may be implicated in the regulation of the expression of the myosin fast heavy chains.

Materials and Methods

Animals

The larval and adult specimens of *Pleurodeles waltlii* used in this study were obtained from a breeding colony which was established in our laboratory in 1950. The different stages of larval development were determined by referring to the data previously obtained by Gallien and Durocher (1957).

Myosin extraction

Crude myosin was extracted according to the technique of Plizska *et al.* (1981).

Fractionation of myosin heavy chains by preparative electrophoresis (quantitative PAGE) and electroelution

Crude myosin extracts were loaded into the well (1.8 ml) of a 2 mm thick 5% polyacrylamide and 0.1% SDS gel. It was run for 6 h at 400 V and at 15°C

in a standard horizontal gel system. At the end of the migration the gels were stained in a solution containing 0.3 M $CuCl_2$ $2H_20$. The uncolored band corresponding to the MHCs was cut out and electroeluted in a Biotrap chamber (Schleider and Schull, Ceralabo) filled with Tris-glycine-SDS. Electroelution was carried out for 8 h at 150V and at 15°C (Jacobs and Clad, 1986).

Preparation and purification of the antibodies

Purified and denatured myosin heavy chains were injected into New Zealand rabbits weighing 2.5 kg (Whalen *et al.*, 1979) Immunization was carried out by three successive subcutaneous injections of an emulsion of myosin (2 mg/ml) in Freund's adjuvant. This was followed by two or three subcutaneous injections of 500 μg of myosin at one month intervals.

The antibodies were affinity purified on Sepharose 4B columns activated by CNBr (Butler-Browne and Whalen, 1984).

The titer of the antibodies was determined by dot blot analysis following the technique described by Towbin and Gordon (1984).

Immunoabsorption

Antibodies were made specific by absorbing the affinity purified serum with the tissue which had not been used for the immunization (Butler-Browne *et al.*, 1982).

Electrophoretic analysis of myosin heavy chains in the presence of SDS

Electrophoretic analysis of myosin heavy chains in the presence of SDS was carried out following the procedure originally described by Carraro and Catani (1983) The heavy chains were separated on vertical slab gels containing 5% polyacrylamide, 0.1% SDS and 37.5% glycerol at 95 V for 18 h. The gels were then silver stained (Blum *et al.*, 1987).

Verification of antibody specificity by immunoblot analysis

Electrotransfer was carried out in the presence of SDS which favors the transfer of high molecular weight proteins such as myosin. Immunodetection was carried out using the BRAB avidine-biotin system (Guesdon *et al.*, 1979; Towbin *et al.*, 1979; Hsu *et al.*, 1981; Ternynck and Avrameas, 1990) (Kit Vecta ABC).

Histochemical ATPase reaction and Immunohistochemistry

Muscle samples were frozen in deep cooled isopentane. Histochemistry and immunohistochemistry was carried out on 8 μm serial frozen sections. Myosin ATPase was carried out according to Padykula and Herman (1955) as modified by Brooke and Kaiser (1969, 1970). Sections for immunohistochemistry were incubated with the myosin antibody diluted either at 1/500 (fast IIA) or 1/200 (larval myosin) for 1 h at 37°C. Specific antibody binding was revealed using the peroxidase-anti-peroxidase technique (Vecta ABC). Sections were dehydrated and mounted in euparal.

Acknowledgments

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