Differential Expression of Myosin in Developing Muscles of Urodelan Amphibians.

Assia Saadi, Claude L. Gallien and Christophe Chanoine

Laboratoire de Biologie du Développement, URA CNRS 1188, Université René Descartes, Paris, France

Abstract

In *Pleurodeles waltlii*, an urodelan amphibian which undergoes spontaneously anatomical metamorphosis, analysis of native myosin by pyrophosphate gel electrophoresis revealed that the transition from larval to fast myosin isoforms occurred more precociously in developing forelimb muscles than in dorsal and ventral muscles. In the neotenic *Ambystoma mexicanum*, an hypothyroidian species where metamorphosis does not occur in natural conditions, forelimb muscle development was characterized by a complete myosin isoform transition which is in contrast with the partial myosin isoform transition observed in the dorsal muscle. This work demonstrated that in urodelan amphibians, the transition from larval to fast myosin isoforms was strictly dependent on both the species- and the muscle type.

Key words: myosin isoforms, urodelan amphibians, muscle development.

BAM 2 (3): 175 - 181, 1992

It is now well established that different myosin isoforms are sequentially expressed during muscle development of mammals [3, 32], birds [1, 18] and amphibians (Rev. in 8) and are regulated by either physiological activity [27] or neural [2, 17, 25] and thyroid hormone control [4, 17). In mammals and urodelan amphibians, the transitional increase in serum of thyroid hormone levels observed during the first postnatal weeks and during anatomical metamorphosis respectively, regulates a transition from neonatal/larval to fast myosin heavy chain [5, 12].

More recently, in different developing rat muscles, D'Albis *et al* [13, 14] indicated that the timing of this sequential transition from neonatal to adult fast isomyosins was highly dependent on the muscle type, each muscle being subjected to a specific program of myosin isoform transition which combines the influences of both genetic and epigenetic factors.

The aim of this report was to demonstrate, in different urodelan amphibian muscles, if the myosin isoform transition was dependent only on a specific thyroid hormone control always exerted during the overall metamorphosis process or if each muscle was also subjected to a specific intrinsic program of myosin expression. To do this, we have analysed quantitatively the myosin isoform transition in three developing muscles of *Pleurodeles waltlii*, a species which undergoes spontaneously anatomical metamorphosis, and in two developing muscles of the neotenic *Ambystoma mexicanum*, an hypothyroidian species where metamorphosis does not occur in natural conditions.

Materials and Methods

Animals

Animals of the species *Pleurodeles waltlii* (Michahelles) originate from a stock introduced into the laboratory in 1950. *Ambystoma mexicanum* (Shaw) individuals were obtained from a white mutant line maintained in the laboratory since 1970. All animals were maintained individually at 18°C in tap water in 1 liter plastic boxes and fed with *Chironomus* larvae three times a week.

Staging

For development, the nomenclature established for *P. waltlii* by Gallien and Durocher [16] was used for both species in order to make easier comparisons. Thus the climax of larval life corresponds to stage 54. Anatomical metamorphosis started spontaneously in *P. waltlii* larvae aged 90-100 days at late stage 54 and was generally completed within 3 weeks at stage 56. The general forelimb pattern was established at stage 45 (larvae aged 40-45 days). Larvae of the species *A. mexicanum* reached stage 54 at the age of 100-120 days. In this neotenic species no spontaneous external metamorphosis was subsequently observed.

Experimental procedures

Sampling

Animals were anaesthetized with tricaine methane sulfate (MS222) and samples stocked in cryotubes were immediately frozen and stored in liquid nitrogen.

Myosin extraction

Preparation of crude myosin was performed on ice according to [28]. The muscles were cut into small pieces and washed with 4 vols of 600mM KCl, 4O mM $NaHCO_3$, 10 mM Na_2CO_3, 1mM $MgCl_2$, 10mM $Na_4P_2O_7$ pH 8,8 (buffer A). Myosin was then extracted with 4 vols of buffer A; after 90mn of gentle shaking, the mixtures were centrifuged at 10,000 x g and the supernatants containing myosin were diluted twice with glycerol for preservation and study of native myosin.

Electrophoretic analysis of native myosin

Non-dissociating conditions were used [11] but with some modifications [9]. Running buffer was 20mM $Na_4P_2O_7$ (pH 8,5), 10% glycerol, 0,01% 2-mercaptoethanol, 2mM $MgCl_2$ and 2mM ATP. Cylindrical gels (6 x 0,5 cm) contained 4% polyacrylamide; about 1µg of myosin per band was loaded. Electrophoresis was carried out at 80 V for 16h30 at -2°C/-3°C. Gels were stained with Coomassie blue R-250 and the relative amounts of the different myosin isoforms quantified with a densitometer equipped with an integrator.

Myosin ATPase profile

The histochemical reaction for myosin ATPase activity on frozen sections (8 µ) was carried out as described previously [5]. Staining for myosin ATPase reaction was performed at alkaline pH (9.4). Preincubation at room temperature was performed for 2 min at alkaline pH (10.4) or for 10 min at acidic pH (4.63).

Results

In muscles from *P. waltlii*, by employing a modified pyrophosphate gel electrophoresis technique adaptated to study myosin from lower vertebrates [9], we have previously demonstrated the existence of differents kinds of myosin isoforms. Larval muscles were characterized by three larval isoforms composed by one specific heavy chain (HCl) whereas adult muscles displayed three fast (FM3-FM1 with respect to their increasing mobilities), one intermediate and one slow isomyosins which were characterized by one specific heavy chain (HCf, HCi and HCs respectively) [6, 10].

Analysis of the myosin isoform pattern during forelimb muscle development from stage 39 to metamorphosed adult showed a myosin isoform transition characterized by the progressive replacement of the three larval isomyosins by the adult isomyosins of lower electrophoretic mobilities (Fig. 1). This transition occured more precociously in forelimb muscles than in dorsal and ventral skeletal muscles (Fig. 1 and Fig. 2). Indeed at stage 45, forelimb muscles already contained adult intermediate/fast myosin isoforms in contrast to what occurs in dorsal and ventral muscles which, at this same stage, express only larval isomyosins. At the time of anatomical metamorphosis (stage 55), forelimb muscles were essentially composed of adult myosin isoforms. These isoforms only began to appear in dorsal and ventral muscles at this stage. Moreover, the slow myosin isoform appeared in forelimb muscles as early as stage 55c whereas it was never detected in

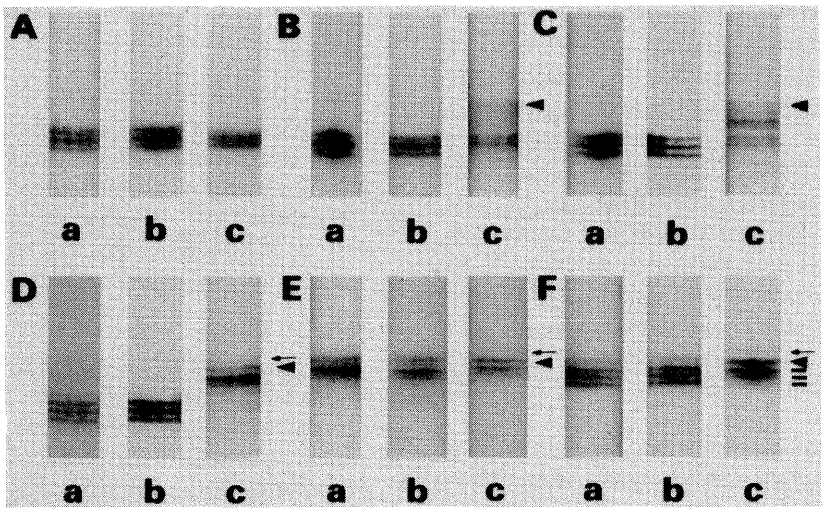

Figure 1: Electrophoretic analysis under nondissociating conditions of native myosin extracted from P. waltlii *muscles at differents stages of development: stage 39 (A), stage 45 (B), stage 53 (C), stage 55c (D), stage 56c (E) and metamorphosed adult (F).*
(a): ventral muscle
(b): dorsal muscle
(c): forelimb muscles
←: slow isomyosin
◄ : intermediate isomyosin

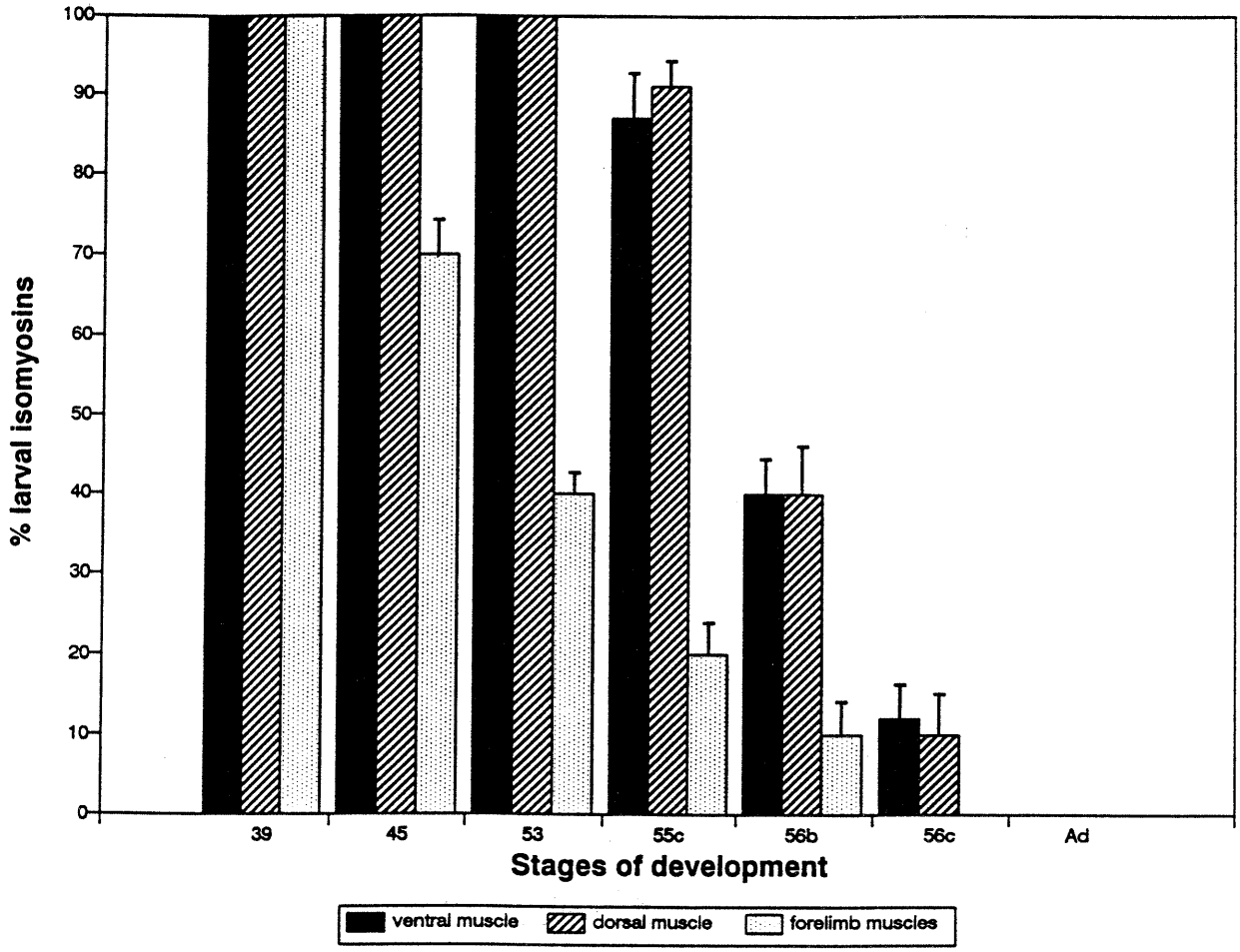

Figure 2: Proportions of larval isomyosins in relation to total isomyosins as a function of the stage of development in P. waltlii.

dorsal and ventral muscles from metamorphosed adults. The intermediate isomyosin which was detected as early as stage 45 in forelimb muscles, appeared only at the end of anatomical metamorphosis in dorsal and ventral muscles (stage 56c). In adult muscles, the intermediate/fast myosin isoforms pattern was clearly different between forelimb and dorsal/ventral muscles. Intermediate myosin was the predominant isoform in forelimb muscles while FM1, the LC3 homodimer, was present in small amounts. In dorsal and ventral muscles, fast myosin was predominant and the three fast isomyosins FM1-FM3 were present in equal amounts (Fig. 1).

In the neotenic *A. mexicanum*, forelimb muscle development was characterized by a complete myosin isoform transition which seems to be in contrast with the partial myosin isoform transition observed in the dorsal muscle [5] (Fig. 3 and Fig. 4). In forelimb and dorsal muscles of animals aged 1 month, only larval isomyosins were detected. In animals aged 4 months, forelimb muscles presented larval and adult myosin isoforms while only

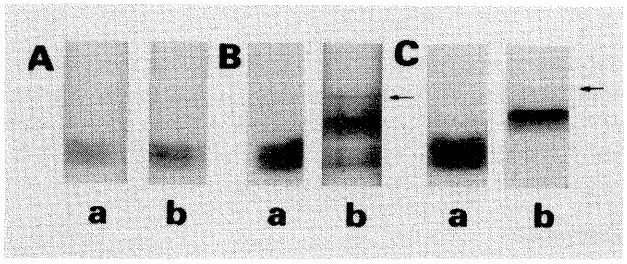

Figure 3: Electrophoretic analysis under nondissociating conditions of native myosin extracted from A. mexicanum *dorsal (a) and forelimb (b) muscles. Animals aged 1 month (A), 4 months (B) and 1 year (C).*
←: slow isomyosin

larval isomyosins were observed in dorsal muscle. Animals aged 1 year displayed only adult myosins in forelimb muscles while they began just to appear in dorsal muscle. Moreover slow myosin was detectable in forelimb muscles but not in dorsal muscle as observed in *P. waltlii* muscles (Fig. 3). Analysis of myosin ATPase profile in forelimb

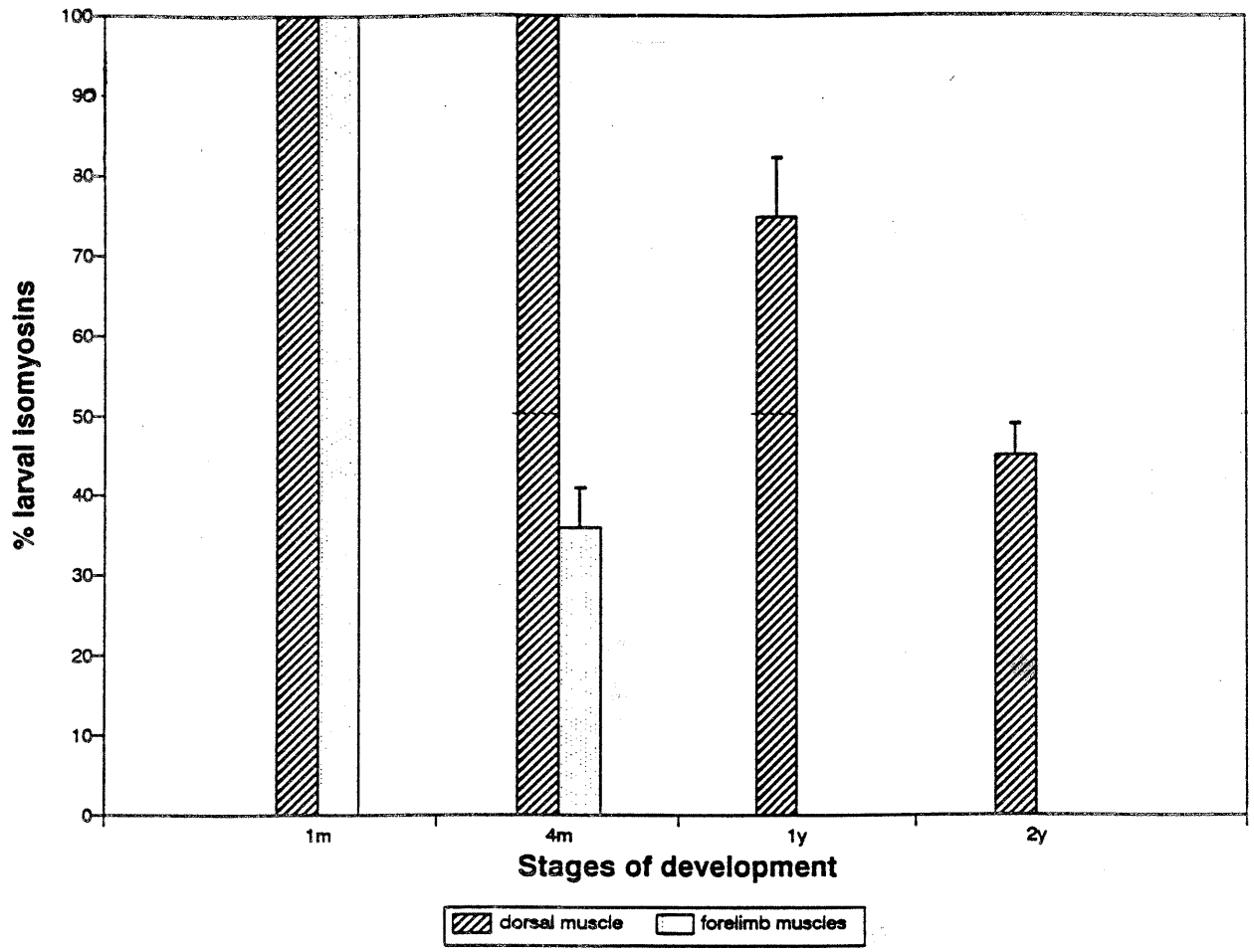

Figure 4: Proportions of larval isomyosins in relation to total isomyosins as a function of the stage of development in A.mexicanum.

muscles of animals aged 1 year showed the presence of adult type IIA, IIB and I fibers (Fig. 5). The dorsal muscle of the same animals presented not only these adult fibers but also transitional type IIC fibers which represented 75% of the total fibers as demonstrated previously [5] (Fig. 5).

Discussion

In *P. waltlii*, we have observed that the transition from larval to fast myosin isoforms characteristic of normal development occurs earlier in forelimb muscles than in dorsal and ventral muscles. Indeed, as early as stage 45, i.e. when the general forelimb pattern was established, fast isomyosins were observed. This is in contrast to what happened in dorsal and ventral muscles where the fast myosin isoforms only began to appear at stage 55 during the process of anatomical metamorphosis. In the neotenic *A. mexicanum*, forelimb development was characterized by a complete myosin isoform transition in opposition to the partial myosin isoform transition observed in dorsal muscle. The presence (in dorsal muscle) or the absence (in forelimb muscles) of the transitional type IIC fibers containing larval isomyosins [5] account for the partial or complete myosin isoform transition respectively. It has now been clearly demonstrated in mammals [4, 12, 29] and in amphibians [5, 6] that thyroid hormone regulates the transition from larval/neonatal to fast myosin heavy chain. In urodelan amphibian muscles, we had previously shown that the appearance of fast isomyosins did not require thyroid hormone (even though experimental T3 treatement increased their level): however, the presence of thyroid hormone seemed to be essential for the disappearance of the larval isoforms [8]. The characterisation of both thyroid hormone receptors as members of the nuclear receptor family [15, 21] and transcription factors [30, 31] which regulate gene expression in the presence of thyroid hormone suggests that the action of thyroid hormone on the transition from neonatal to fast myosin heavy chain may be direct [33] and not nerve-mediated [29]. In different developing rat muscles, d'Albis *et al* [13] have previously shown that each muscle had a specific program of myosin

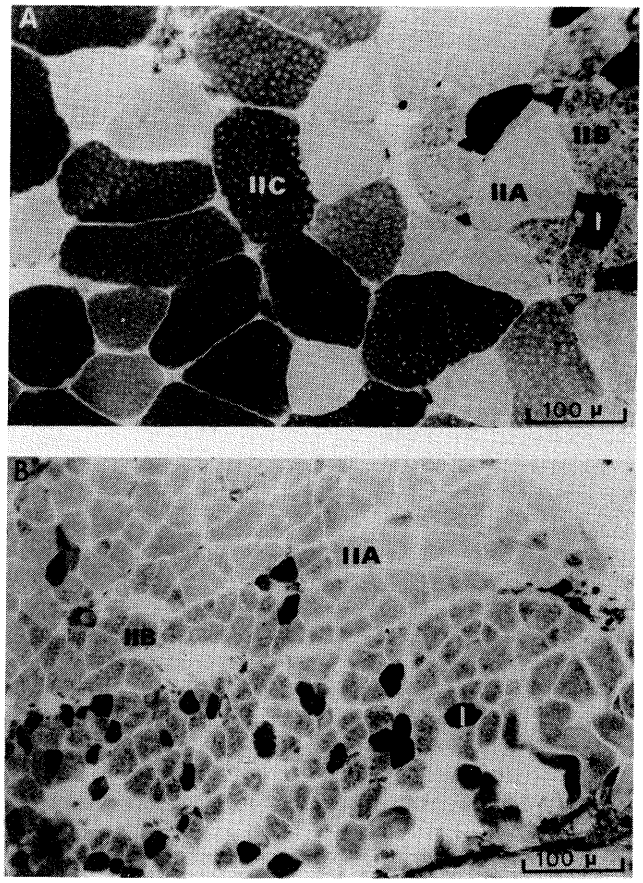

Figure 5: transversal cryostat sections stained for myosin ATPase at pH 4.63 in dorsal muscle (A) and forelimb muscles (B) of A.mexicanum *aged 1 year. This acidic preincubation was suitable to discriminate all different fiber types. Type IIC fibers were observed in dorsal muscle only.*

isoform transition which combines the influences of both genetic and epigenetic factors, since the timing of the sequential expression from neonatal to fast-type II isomyosins was highly dependent on the muscle type. These authors suggested that this diversity in myosin expression could closely correlated to the own story of each muscle. For example, it was observed that in the plantar muscles of the foot, which are formed later than leg muscles, the isomyosin transition occured later than in leg muscles. Furthermore, the different muscles are not all functional to the same extent at birth; respiratory muscles as well as the intercostals and the diaphragm, which are among the first muscles able to function very actively and regularly, just display the most precocious myosin isoform transition.

The condition of urodelan muscles was quite different, since in the dorsal and ventral muscles which are formed before the forelimb muscles, the adult myosin heavy chains are expressed later than in the forelimb muscles. In fact, the dorsal muscle of urodelan amphibians differentiates very early during the development and turns out to be very active during the aquatic larval phase; the differentiation of the forelimb muscles and their complete employment in locomotion occur only later during the development. The slow type I fibers which contain slow myosin are responsible for maintaining posture [20]: this can explain the presence of the slow myosin isoform in forelimb muscles. The different distribution of the intermediate/fast isoforms observed between forelimb and dorsal/ventral muscles can be probably correlated to the different types of movements.

In the rat, it was shown that the various muscles did not display the same sensitivity to the thyroid hormone but were regulated by it in the same way since experimentally induced hypo- and hyperthyroidism respectively delayed or accelerated the myosin isoform transition in all the muscles examined [14]. This suggested that the differents muscles investigated did not contain the same amount of thyroid hormone receptors and for this reason required different concentrations of thyroid hormone to carry out their isomyosin transition. Izumo *et al* [24] and Gustafson *et al* [22] clearly demonstrated indeed that all members of the myosin heavy chain family respond to thyroid hormone in a highly muscle-specific way; different sensitivities to thyroid hormones were also demonstrated in cardiac and

skeletal muscles [33].

Amphibian metamorphosis is a complex biological event which totally depends on the presence of thyroid hormone [19, 23]. It should be emphasized that we had previously demonstrated a partial myosin isoform transition in the dorsal muscle of the perennibranchiate species *Proteus anguinus*, which is characterized by the persistence of larval isoforms in adult muscles comparable to that observed in the neotenic *A. mexicanum* [7]. Nevertheless, experimental hyperthyroidism, which implied a complete transition from larval to fast isomyosins in *A. mexicanum* [5], never permits a complete transition in *P. anguinus* even after long term T3 treatment [7]. This low receptiveness of the perennibranchiate tissues to thyroid hormone has recently been explained at the molecular level [34]. The employment of cDNA probes of *Xenopus laevis* thyroid hormone receptor [34] demonstrated that no transcripts encoding for thyroid hormone receptors were present in *P. anguinus* gills in contrast to what happened in *A. mexicanum* whose gills did contain these transcripts.

In urodelan amphibians, the differential expression of myosin isoforms that was observed during forelimb and ventral/dorsal muscles development can be correlated to the differences in the type or amount of thyroid hormone receptors present in each of the muscles. In fact, in the anuran amphibian *Xenopus laevis*, two thyroid hormone receptor families (TR α and TR β) and their respective genes have been characterized [35]. These receptors display different patterns of expression during development [34]. During the prometamorphosis of *Xenopus* tadpoles, Kawahara *et al* [26], using thyroid hormone receptor α cRNA as probe for in situ hybridization, demonstrated a strong hydridization signal in limb buds, if compared to that of other muscles. The levels of thyroid hormone receptors in urodelan forelimb muscles is very high in comparison to that of other muscles: this could explain the reason why, in *P. waltlii*, the myosin isoform transition is more precocious in forelimb muscles than in dorsal/ventral muscles. This hypothesis could also explain the complete myosin isoform transition which occurs in *A. mexicanum* forelimb muscles in contrast with the partial transition observed in dorsal muscle although the level of serum thyroid hormone is maintained low in this species all through its life [5]. Our next step will be the attempt to characterise the thyroid hormone receptors in urodelan amphibians and to study their expression during muscle development.

Address correspondence to:

Christophe Chanoine, Laboratoire de Biologie du Développement, URA 1188 CNRS, Université René Descartes, 45 rue des Saints-Pères, 75270 Paris cedex 06, France.

Acknowledgments

The authors would like to thank Dr. Gillian Butler-Browne for helpful comments on this paper.

A. Saadi held a fellowship from the Association Française contre les Myopathies.

This work was supported by grants from the Centre National de la Recherche Scientifique.

References

[1] Bandman E, Bennet T: Diversity of fast myosin heavy chain expression during development of gastrocnemius, biceps brachii and posterior latissimus dorsi muscles in normal and dystrophic chickens. *Dev Biol* 1988; 130: 220-231.

[2] Butler-Browne GS, Bugaisky LB, Cuenoud S, Schwartz K, Whalen RG: Denervation of newborn rat muscle does not block the appearance of adult fast myosin. *Nature (London)* 1982; 299: 830-833.

[3] Butler-Browne GS, Whalen RG: Myosin isozyme transitions occuring during the post-natal development of the rat soleus muscle. *Dev Biol* 1984; 102: 324-334.

[4] Butler-Browne GS, Herlicoviez D, Whalen RG: Effects of hypothyroidism on myosin isozyme transitions in developing rat muscle. *FEBS Lett* 1984; 166: 71-75.

[5] Chanoine C, d'Albis A, Lenfant-Guyot M, Janmot C, Gallien CLL: Regulation by thyroid hormones of terminal differentiation in the skeletal dorsal muscle. II. Urodelan amphibians. *Dev Biol* 1987; 123: 33-42.

[6] Chanoine C, Gallien CLL: Myosin isoenzymes and their subunits in urodelan amphibian fast skeletal muscle - Coexistence of larval and adult heavy chains in neotenic individuals. *Eur J Biochem* 1989; 181: 125-128.

[7] Chanoine C, Guyot-Lenfant M, d'Albis A, Durand JP, Perasso F, Salles-Mourlan AM, Janmot C, Gallien CLL: Thyroidal status and isoenzymic pattern in the skeletal dorsal muscle of urodelan amphibians - the perennibranchiate *Proteus anguinus*. *Cell Diff* 1989; 28: 135-144.

[8] Chanoine C, Guyot-Lenfant M, Saadi A, Perasso F, Salles-Mourlan AM, Gallien CLL: Myosin structure and thyroidian control of myosin synthesis in urodelan amphibian skeletal muscle. *Int J Dev Biol* 1990a; 34: 163-170.

[9] Chanoine C, Saadi A, Guyot-Lenfant M, Hebbrecht C, Gallien CLL : Myosin structure in the eel (*Anguilla anguilla* L.). Demonstration of three heavy chains in adult lateral muscle. *FEBS Lett* 1990b; 277: 200-204.

[10] Chanoine C, Saadi A, Guyot-Lenfant M, Gallien CLL: Myosin polymorphism in urodelan amphibians dorsal skeletal muscle. *Comp Biochem Physiol* 1991; 100B: 153-159.

[11] D'Albis A, Janmot C, Bechet JJ: Myosin switches in skeletal muscle development of an urodelan amphibian, *Pleurodeles waltlii.* Comparison with a mammalian, *Mus musculus. Biochem Biophys Res Com* 1985; 128: 94-100.

[12] D'Albis A, Lenfant-Guyot M, Janmot C, Chanoine C, Weinman J, Gallien CLL: Regulation by thyroid hormones of terminal differentiation in the skeletal dorsal muscle. I. Neonate mouse. *Dev Biol* 1987; 123: 25-32.

[13] D'Albis A, Couteaux R, Janmot C, Roulet A: Specific programs of myosin expression in the postnatal development of rat muscles. *Eur J Biochem* 1989; 183: 583-590.

[14] D'Albis A, Chanoine C, Janmot C, Mira JC, Couteaux R: Muscle-specific response to thyroid hormone of myosin isoform transitions during rat postnatal development. *Eur J Biochem* 1990; 193: 155-161.

[15] Evans RM: The steroid and thyroid hormone receptor superfamily. *Science* 1988; 240: 889-895.

[16] Gallien L, Durocher M: Table chronologique du développement chez *Pleurodeles waltlii. Michah Bull Biol Fr Belg* 1957; 19: 97-114.

[17] Gambke B, Lyons GE, Haselgrove J, Kelly AM, Rubinstein NA: Thyroidal and neural control of myosin transitions during development of rat fast and slow muscles. *FEBS Lett* 1983; 156: 335-339.

[18] Gardahaut MF, Rouaud T, Renaud D, Le Douarin G: Developmental changes in myosin isoforms from slow and fast latissimus dorsi muscles in the chick. *Differentiation* 1988; 37: 81-85.

[19] Gilbert LI, Frieden E (eds): Metamorphosis. A problem in developmental biology. New York, Plenum Press, 1981.

[20] Goldspink G: Alterations in myofibril size and structure during growth, exercise, and changes in environmental temperature, in Peachey, LD, Adrian, RH, Geiger, SR (eds): Handbook of physiology. Section 10 : Skeletal Muscle. American Physiological Society. Bethesda, Maryland, 1983, pp 539-554.

[21] Green SD, Chambon P: Nuclear receptors enhance our understanding of transcription regulation. *Trends Genet* 1988; 4: 309-314.

[22] Gustafson TA, Markham BE, Morkin E: Effects of thyroid hormone on alpha-actin and myosin heavy chain gene expression in cardiac and skeletal muscles of the rat. Measurement of messenger-RNA content using synthetic oligonucleotide probes. *Circ Res* 1986; 59: 194-201.

[23] Hourdry J: Quelques aspects biochimiques de la métamorphose des amphibiens. *Ann Biol* 1980; 19: 250-287.

[24] Izumo S, Nadal-Ginard B, Mahdavi V: All members of the MHC multigene family respond to thyroid hormone in a highly tissue-specific manner. *Science* 1986; 231: 597-600.

[25] Jolesz F, Sreter, FA: Development, innervation, and activity induced changes in skeletal muscle. *Ann Rev Physiol* 1981; 43: 531-552.

[26] Kawahara A, Baker BS, Tata JR: Developmental and regional expression of thyroid hormone receptor genes during *Xenopus* metamorphosis. *Development* 1991; 112: 933-943.

[27] Lomo T, Westegaard RH, Dahl HA: Contractile properties of muscle: control by pattern of muscle activity in the rat. *Proc R Soc Lond B* 1974; 187: 99-103.

[28] Pliszka B, Strzelecka-Golaszewska H, Pantaloni C, D'Albis A: Comparison of myosin isoenzymes from slow-tonic and fast-twitch fibers of frog muscle. *Eur J Cell Biol* 1981; 25: 144-149.

[29] Russell SD, Cambon N, Nadal-Ginard B, Whalen RG: Thyroid hormone induces a nerve-independent precocious expression of fast myosin heavy chain mRNA in rat hindlimb system. *J Biol Chem* 1988; 263: 6370-6374.

[30] Sap J, Munoz A, Damm K, Goldberg Y, Ghysdael J, Leutz A, Beug H, Vennstrom B: The c-erb-A protein is a high-affinity receptor for thyroid hormone. *Nature (London)* 1986; 324: 635-640.

[31] Weinbergerg C, Thompson CC, Ong ES, Lebo R, Gruol DJ, Evans RM: The c-erb-A gene encodes a thyroid hormone receptor. *Nature (London)* 1986; 324: 641-646.

[32] Whalen RG, Sell SM, Butler-Browne GS, Schwartz K, Bouveret P, Pinset-Harstrom I: Three myosin heavy chains isozymes appear sequentially in rat muscle development. *Nature (Lond)* 1981; 292: 805-809.

[33] Whalen RG, Toutant M, Butler-Browne GS, Watkins SC: Hereditary pituitary dwarfism in mice affects skeletal and cardiac myosin isozyme transitions differently. *J Cell Biol* 1985; 101: 603-609.

[34] Yaoita Y, Brown DD: A correlation of thyroid hormone gene expression with amphibian metamorphosis. *Genes Dev* 1990; 4: 1917-1924.

[35] Yaoita Y, Shi YB, Brown DD: *Xenopus laevis* et thyroid hormone receptors. *Proc Nat Acad Sci USA* 1990; 87: 7090-7094.