0014-4754/96/020184-04$1.50 + 0.20

Seasonal variation in the phenotype of adult ferret (*Mustela putorius furo*) cremaster muscle

T. Rouaud, J. Fontaine-Perus and M. F. Gardahaut*

CNRS URA 1340, Faculté des Sciences et des Techniques, 2 rue de la Houssinière, F-44072 Nantes Cedex 03 (France), Fax +33 1 40 37 31 83
Received 18 January 1995; received after revision 16 May 1995; accepted 19 June 1995

Abstract. Using immunocytochemistry, electrophoresis and immunoblotting, we studied the expression of fast and slow myosin heavy chain isoforms in adult ferret muscles during quiescent and breeding periods. Adult cremaster muscle expressed slow and fast myosin heavy chain in relatively similar amounts during the quiescent period. During the breeding period, the expression of slow myosin heavy chain I, significantly decreased, and fast myosin heavy chain II, was predominant. No alteration of the MHC pattern in EDL and soleus muscles was detected between the quiescent and breeding periods. The possible involvement of androgens and mechanical factors in the regulation of myosin heavy chain expression in adult cremaster muscle is discussed.
Key words. Cremaster muscle; seasonal variation; myosin heavy chain isoforms.

In adult skeletal muscle, fibers are broadly classified into two main categories, fast type II and slow type I, characteristic of rapidly and slowly contracting mucles respectively. These different fiber types contain different isoforms of myosin heavy chain (MHC) which are in large part responsible for the different intrinsic rates of fiber shortening[1,2]. Even after terminal differentiation, skeletal muscle fibers are able to modulate the properties of the contractile apparatus reversibly in response to altered physiological stimuli such as innervation (for review, see ref 3), exercise[4,5] and thyroid hormone[6,7,8]. In male mammals some muscles, such as neck and masticatory muscles and the perineal muscle complex[9,10,11], are highly dependent on androgens for their development and maintenance. In species with seasonal sexual activity, it has been assumed that the cremaster (Crem) muscle surrounding the testes is testosterone-sensitive. In this study we compared myosin expression in adult ferret Crem during the breeding period (March to September) with that of adult muscle during the period of sexual inactivity (October to February). The myosins in Crem muscle were also compared with those found in fast-twitch *Extensor digitorum longus* (EDL) and slow-twitch Soleus (Sol) muscles.

Materials and methods

Male adult ferrets (*Mustela putorius furo*) weighing 1.3/–1.8 kg, purchased from a breeding farm, were killed using an ether vapor flow. After respiratory arrest, Sol, EDL and Crem skeletal muscles were excised and quickly frozen in liquid nitrogen for histochemical, immunocytochemical and electrophoretic analysis. Fourteen adult ferrets were used in this study. Eight were in breeding condition (March to September) at the time of euthanasia, as attested by testicular size at palpation and observation of copulative activity a few days before sacrifice. Six ferrets killed during the quiescent period had regressed testicles at the time of sacrifiice.

Histoenzymology and immunocytochemistry. Transverse serial sections were cut in a cryostat, collected on gelatine-coated slides and air-dried for 1 hour at room temperature. Alternate slices were used either for MHC immunostaining or histochemical ATPase staining. For immunochemistry, sections (6 µm) were incubated overnight at 4 °C with monoclonal antibody (mAb) raised against fast (type II) myosin MHC (Sigma Chimie France, MY 32) and with anti-slow MHC NA8 mAb prepared against the MHC of slow anterior latissimus dorsi muscle of the chicken (Bandman, personal communication). To characterize the mAbs better, they were tested against ferret Sol (slow-twitch) and EDL (fast-twitch) muscles[12]. The mAbs were appropriately diluted with phosphate-buffered saline (PBS). Sections were washed with PBS and incubated for 1 hour at room temperature with the second antibody, a fluorescein isothiocyanate-labeled goat anti-mouse IgG1 antibody diluted 1:100 in PBS. To reveal myofibrillar ATPase activity[13], 12 µm-thick unfixed sections were first pre-incubated, at room temperature, either in acid buffer (pH 4.2) for 5 min or in alkaline buffer (pH 10.4) for 10 min. The sections were then incubated in a buffer (pH 0.4) with 4mM ATP at 37 °C for 20 min.

Myosin preparation and MHC analysis. The MHC composition of muscles from each animal was determined as follows: Preparation of crude myosin was carried out as

* Corresponding author.

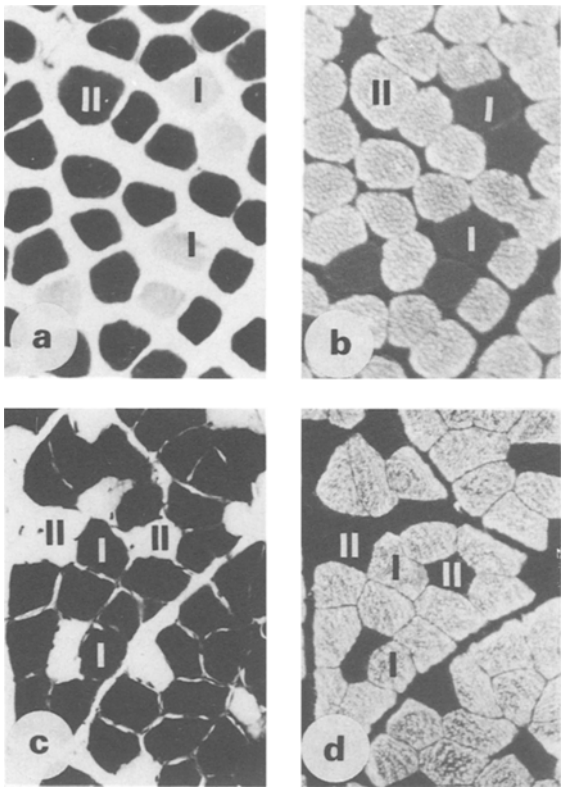


Figure 1. Antibody specificity. Serial transverse sections of adult ferret EDL (*a*, *b*) and Sol (*c*, *d*) muscles were treated either for myofibrillar ATPase activity after alkaline preincubation (pH 10.4) (*a*) or acid preincubation (pH 4.2) (*c*), or for immunochemical reactivity with anti-fast MHC MY32 mAb (*b*) and anti-slow MHC NA8 mAb (*d*). Anti-fast MHC mAb stained type II fibers, while anti-slow mAb stained type I fibers (magnification × 140).

previously described[14]. Extracts were diluted in 20% glycerol, 25 mM Tris-HCl, 5% SDS, 5 mM dithiothreitol, 1 mM EGTA, pH 6.8. MHC were separated by SDS polyacrylamide gel electrophoresis using a 6% acrylamide-bisacrylamide separation slab gel in the presence of 0.1% SDS and 37.5% glycerol. Electrophoresis was carried out at 20 mA/gel for 4 hours. Gels were stained with Coomassie blue, and the percentage distributions of MHC isoforms were evaluated densitometrically. For Western blot analysis, proteins were electrophoretically transferred onto a nitrocellulos sheet at about 200 mA for 4 hours in a Trans-Blot cell. After washing in 0.2% Tween, the sheet was incubated overnight at 4 °C for 1 hour at room temperature with anti-fast or anti-slow MHC mAb diluted 1:100. Bound antibody was detected with diaminobenzidine following incubation with peroxidase-conjugated goat anti-mouse secondary antibody IgG1. Tests for immunochemical specificity were performed by omission of primary mAbs and the use of preabsorbed controls.

Statistical analysis. Results are expressed as mean ± SEM. Statistical analysis was performed by Student's t-test. Statistical significance was accepted at the 0.05 level.

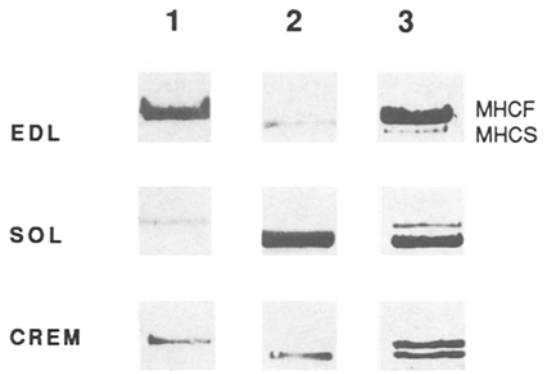


Figure 2. Immunoblots of MHC from EDL, Sol and Crem muscles. MHC were stained with MY 32 mAb for fast MHC (lane 1), NA8 mAb for slow MHC (lane 2) and amino black (lane 3).

Results

The immunoreactivity of each mAb used in this study was tested first on adult ferret EDL and Sol muscles during the period of sexual inactivity. Immunocytochemical staining of fibers was compared with standard myosin ATPase histochemistry in serial sections, as illustrated in figure 1. Anti-fast MHC mAb (MY 32) stained only fibers classified as type II on the basis of myosin ATPase activity, whereas mAb (NA8) stained only fibers classified as type I.

For these two muscles as well as Crem muscle, immunoblotting analysis demonstrated that mAb MY32 recognized the slowest migrating band and mAb NA8 the fastest migrating band, corresponding to type II and I MHC respectively (fig 2).

During periods of sexual inactivity and breeding, EDL contained about 90% of fast fibers and 10% of slow fibers, whereas in Sol about 90% of fibers were labeled with anti-slow mAb, and 10% with anti-fast MHC mAb (table 1). During the period of sexual inactivity, Crem displayed a mosaic pattern of slow (50%) and fast (32%) fibers and contained some fibers (18%) which reacted positively with both anti-fast (MY 32) and anti-slow (NA8) mAbs. During the breeding period, the

Table 1. Relative proportions of the number of fibers stained with anti-fast myosin (MY32) and anti-slow myosin (NA8) in ferret muscles during quiescent and breeding periods.

Muscle	mAbs	Reproductive state			
		quiescent	(n)	breeding	(n)
EDL	NA8	10 ± 7	(6)	12 ± 5	(8)
	MY32	90 ± 7	(6)	88 ± 5	(8)
Sol	NA8	87 ± 5	(6)	91 ± 6	(8)
	My32	13 ± 4	(6)	9 ± 6	(8)
Crem	NA8	50 ± 10	(6)	2 ± 1*	(8)
	MY32	32 ± 15	(6)	87 ± 5	(8)
	NA8/MY32	18 ± 9	(6)	11 ± 10	(8)

Values are means ± SEM. (n) muscles and at least 300 fibers per muscle were examined. *Statistically different from quiescent period.

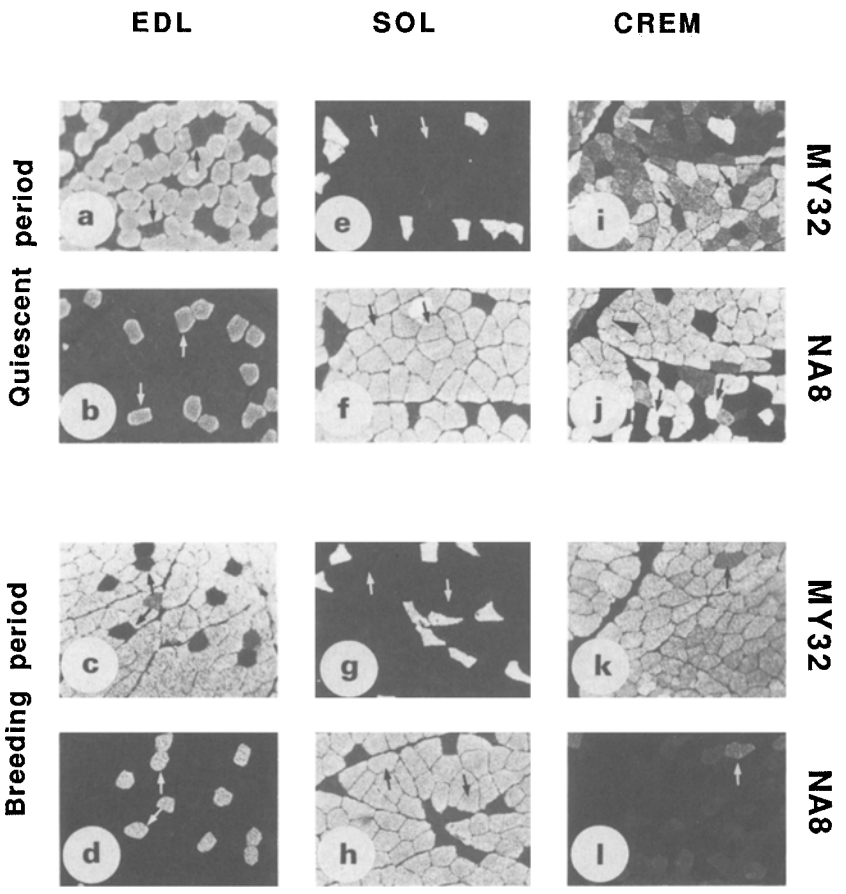


Figure 3. MHC expression in adult ferret muscles during quiescent and breeding periods. Serial transverse sections from EDL (*a–d*), Sol (*e–h*) and Crem (*i–l*) were treated with anti-fast MHC MY32 mAb and anti-slow MHC NA8 mAb. Arrows indicate slow-type fibers, and arrowheads (*i, j*) fibers labeled with anti-fast and anti-slow MCH mAb (magnification × 75).

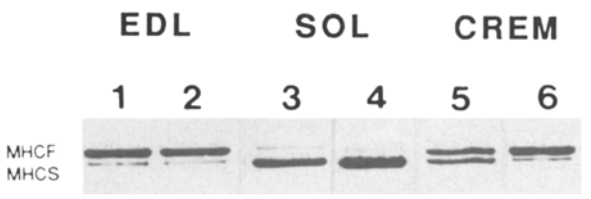
overwhelming majoriy of fibers were labeled with anti-fast (MY 32) mAb, and the proportion of fibers containing slow MHC (NA8 mAb) was markedly decreased (table 1). The immunocytochemical analysis is illustrated in fig 3. Densitometric evaluations of MHC isoforms separated by SDS-PAGE electrophoresis were consistent with these results. No modifications in MHC composition were observed during the season in Sol or EDL muscle, whereas a switch from slow to fast expression occurred in Crem muscle during the period of sexual activity (fig 4).

Discussion

We examined the expression of MHC isoforms in adult ferret EDL, Soleus and Crem muscles by combining immunocytochemistry, SDS-polyacrylamide gel electrophoresis and immunoblotting. We demonstrated that annual modifications in fiber phenotype occurred in ferret Crem muscle that may be correlated with seasonal variations of sexual activity. Indeed, during the period of sexual activity, expression of slow MHC I decreased and that of fast MHC II increased in Crem, as compared to the quiescent period. No alteration in the pattern of MHC expression was noted in Sol and EDL between quiescent and breeding periods.

It is well known that the intrinsic rate of muscle fiber shortening is correlated with actomyosin ATPase activity and that this enzymatic activity is essentially determined by the type of MHC. Therefore, it may be considered that the redistribution of MHC expression to the highly active fast MHC II isoform in Crem accounted for the increased rate of shortening of this muscle during the breeding period. The prevailing notion is that the level of expression of each MHC isoform is determined by how efficiently it meets the functional demands of the muscle. It is well established that MHC isoforms determine the ATP consumption and the isometric tension cost (for review see ref 15). Previous studies have demonstrated that the testes themselves play a very important role in the maintenance of muscle tension and the weight of rat Crem[16]. In the ferret, the testes are in the abdominal cavity during quiescence and migrate into the scrotum during the breeding period[17]. As previously demonstrated[18], the presence in the cremaster muscle of slow fibers which can contract with a maintained tension for prolonged periods of time suggests that these fibers have an important role in maintaining the testis in the abdominal cavity during quiescent period. The fact that Crem expressed more slow MHC during the quiescent period can be considered as an adaptation to maintain isometric force, since slow MHC has a lower specific ATPase activity and is therefore more economical in developing such force.

A



B

Muscle	MHC	Reproductive State Quiescent	(n)	Breeding	(n)
EDL	Slow type I	13 ± 1	(6)	12 ± 1	(8)
	Fast type II	87 ± 1	(6)	88 ± 1	(8)
Sol	Slow type I	89 ± 1	(6)	89 ± 3	(8)
	Fast type II	11 ± 1	(6)	11 ± 3	(8)
Crem	Slow type I	40 ± 4	(6)	14 ± 3 *	(8)
	Fast type II	60 ± 4	(6)	86 ± 3 *	(8)

Figure 4. A. Electrophoretic separation by SDS-PAGE of MHC from EDL (lanes 1, 2), Sol (lanes 3, 4) and Crem (lanes 5, 6) muscles during periods of sexual inactivity (lanes 1, 3, 5) and breeding (lanes 2, 4, 6). Gels were stained with Coomassie blue. B. Relative proportions of MHC isoforms separated on SDS-PAGE in ferret muscles during quiescent and breeding periods. The MHC proportions were determined by densitometric evaluation of the intensity of staining with Coomassie Blue of the electrophoretic bands. (n) muscles were examined. Values are means ± SEM. *Statistically different from quiescent period.

The energy demands required to maintain the testes in a particular position during quiescence would no longer exist after the migration of the testes into the scrotum, so that the energetically less efficient fast MHC could be reinstated under conditions in which overall ATP consumption was reduced. Interestingly, in response to a sustained increase in contractile activity, such as that resulting from chronic electrical stimulation of the motor nerve, fast-twitch mammalian skeletal muscle undergoes a transistion from the fast to the slow myosin isoform[3,19]. During the recovery period that follows cessation of stimulation, the decrease in contractile activity induces a slow-to-fast MHC transition[20].

Factors other than mechanical ones could be involved in the changes in MHC expression observed during the breeding season. It is noteworthy that in adult male ferrets the circulating concentration of androgens needed to sustain spermatogenesis and activate sexual behavior is considerably higher than that measured in adult males whose testes have undergone seasonal regression[21]. These seasonal modifications in gonadal steroids could be another signal inducing the switch in Crem MHC toward the fast phenotype during the sexual activity period. Some studies indicate that testicular androgens can influence muscle phenotype (for review see refs 22, 23). Moreover, among sexually dimorphic muscles, guinea pig temporalis has been shown to exhibit sexual dimorphism in contractile protein isoforms. The transition from a fast-red MHC isoform to a fast-white isoform at puberty is influenced by testosterone[24]. However, it is noteworthy that a high androgen level during the breeding period has no effect on MHC expression in EDL and Sol muscles. This may be due to differences in androgen receptor numbers between Crem and hind-limb muscles. Interestingly, studies have demonstrated the presence of a larger number of androgen receptors in the levator ani, a muscle highly sensitive to androgens, as compared to the quadriceps[25]. Further investigation is required to determine whether phenotypic changes in Crem during the breeding season are mediated by mechanical factors and/or androgen level. The ferret Crem appears to be a convenient model for the study of hormonal and mechanical influences on adult phenotype plasticity.

Acknowledgments. The authors are grateful to Professor C. Léoty and Dr C. Huchet for providing the ferret muscles and to Dr E. Bandman for gifts of NA8 monoclonal antibody.

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