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Contractile differentiation of foetal cattle muscles: intermuscular variability

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Abstract — Contractile differentiation was studied in six foetal muscles exhibiting different contractile characteristics in adult cattle: the *Masseter, Diaphragma, Biceps femoris, Longissimus thoracis, Semitendinosus* and *Cutaneus trunci*. These muscles were excised from foetuses aged 60–260 days. Fibre types were identified by immunohistochemistry using three monoclonal antibodies raised against types 1, 2a, 2b (or 2x) and foetal myosin heavy chains. The different myosin isoforms were also separated by electrophoresis, identified by immunoblotting and quantified by ELISA. At least two generations of cells were observed in all the muscles studied. The primary, early differentiated one, gave rise to type II fibres in *Cutaneus trunci* and type I fibres in all remaining muscles. The secondary generation of cells differentiated later than the first generation of cells. Its pattern of differentiation was more complex in particular from 150 to 210 days. It formed slow fibres in slow adult muscles, fast fibres in fast adult muscles and both types in mixed muscles. Precocity of differentiation was muscle-type dependent and related to muscle function at birth. © Inra/Elsevier, Paris

muscle / contractile differentiation / bovine / foetus

Résumé — Différenciation contractile chez le fœtus bovin : variabilité entre muscles. La différenciation contractile durant le stade fœtal (de 60 à 260 j) est étudiée dans six muscles différant par leurs caractéristiques chez l'adulte : le *Masseter, Diaphragma, Biceps femoris, Longissimus thoracis, Semitendinosus* et *Cutaneus trunci*. Le type de fibre est identifié par immunohistochimie à l'aide d'anticorps spécifique des isoformes lente (type 1), rapides (2a et 2b (ou 2x)) et fœtale de chaînes lourdes de myosine. Celles-ci sont également séparées par électrophorèse, identifiées par *immunoblotting* et quantifiées par Elisa. Au moins deux générations de cellules sont observées dans tous les muscles. La première qui a une différenciation précoce donne naissance à des fibres rapides dans le *Cutaneus trunci* et à des fibres lentes de type I dans tous les autres muscles. La différenciation de la seconde génération est plus complexe, particulièrement entre 150 et 210 j. Elle donne naissance soit

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à des fibres lentes dans les muscles lents, soit à des fibres rapides dans les muscles rapides, soit aux deux types de fibres dans les muscles mixtes. La différenciation contractile est plus ou moins précoce selon la fonction du muscle à la naissance. © Inra/Elsevier, Paris

muscle / différenciation contractile / bovin / foetus

1. INTRODUCTION

In adult animals, skeletal muscle is composed of four principal fibre types (I, IIA, IIB and IIX) which have different contractile and metabolic properties [17]. The last type IIX has been elucidated in some species. In bovine muscle, however, only two types of fast fibres are observed [22]. Recently, Tanabe et al. [33] have sequenced 2a, 2x and slow myosin heavy chain isoforms in this species. Therefore, we could speculate that fibres classified as IIB in this species correspond in fact to IIX fibres.

Muscle fibres are derived from two generations of cells appearing sequentially during foetal life. Myogenesis has been widely studied in birds, rodents and mammals [1, 6, 18, 23, 27, 30]. During the early stages of differentiation, primary myotubes appear after fusion of embryonic myoblasts. They give rise to the first generation of muscle cells. Later, foetal myoblasts merge into secondary myotubes using primary myotubes as a scaffold. These secondary myotubes give rise to the secondary generation of muscle cells (see [29] for a review). A third generation of fibres has been previously suggested in human, sheep, pig and cattle muscles [6, 15, 18, 36].

Different myosin isoforms appear sequentially during muscle development. Developmental myosin isoforms, termed as embryonic, foetal or neonatal depending on their stage of expression, are transitorily expressed during development. They progressively disappear and are replaced by adult myosin isoforms [1]. Adult myosin isoforms are, however, detected earlier during foetal life in several species. MHC1 is expressed in primary myotubes from their formation onwards and in some secondary generation of muscle cells at the end of foetal life. In contrast, MHC2 is transitorily expressed during early stages in primary muscle cells, whereas it is present in secondary myotubes from their formation onwards [6, 23, 27, 30]. A previous study carried out in four cattle muscles showed a similar pattern of differentiation involving two populations of cells [18].

The kinetics of expression of myosin heavy chain (MHC) also differ according to the muscle studied. The programme of myosin expression was analysed in several rat muscles during the month following birth [5]. Each rat muscle is subjected to its own specific programme of myosin transition in relation to the origin and embryonic stage of muscle formation. Thus, muscles are not all functional to the same extent at birth. Respiratory muscles such as intercostals and *Diaphragma* are among the first muscles to function very actively and regularly at birth and they display the most precocious MHC transition.

To investigate the kinetics of differentiation and to specify the origin of bovine muscle fibre types and its variability between muscles, we studied the differentiation of muscle fibres in six muscles from 60 days of foetal life up to just before birth. Muscles were chosen according to their contractile and metabolic characteristics in the adult: the *Masseter* (Ma), which contains only type I fibres in adult cattle [21] and the *Diaphragma* (Di), a predominantly slow oxidative muscle (80 % type I fibres) which differentiate early [18]. Three mixed muscles displaying an intermediate precocity in cattle

were also chosen [18]: the *Biceps femoris* (BF) in which 20 % of the fibres are type I [34]; the *Semitendinosus* (ST), in which more than 50 % of the fibres are type IIB [11, 34]; and the *Longissimus thoracis* (LT), a predominantly fast glycolytic muscle (30 % I, 40 % IIA, 30 % IIB) [34]. Finally, the *Cutaneus trunci* (CT), made up of fast type IIA and IIB fibres [19] was also included. Myogenesis in the six muscles was studied by comparing the differentiation of MHC by several complementary methods: immunohistochemistry, electrophoresis, immunoblotting and ELISA.

2. MATERIALS AND METHODS

2.1. Muscle samples

Thirty-one foetuses aged 60, 110, 180, 210 and 260 days were obtained by artificial insemination of Charolais heifers with Charolais sperm. After slaughter of pregnant heifers, six muscles with differing contractile and metabolic properties in adult cattle were excised from four foetuses at each stage: Ma, Di, ST, LT, BF and CT. This last muscle was not sampled at 60 days because its size was too small. Muscle samples used for immunohistochemistry were frozen in isopentane, cooled by liquid nitrogen. Samples used for electrophoresis and ELISA were directly frozen in liquid nitrogen. Then, all samples were stored at –80 °C until analysis.

2.2. Monoclonal antibodies

Three monoclonal antibodies were used. The conditions under which they were produced, purified and characterised were described by Léger et al. [14]. Their specificity was studied by Pons et al. [23] in human muscle and their reactions with myosin from foetal and adult cattle were analysed by Robelin et al. [25]. The F88 8H8 antibody, called S, was prepared from adult human auricle. It was specific to MHC1. The F113 15F4 antibody, specific for fast MHC (2a and 2b or 2x), was obtained from adult myosin of rabbit *Tibialis* muscle. It was called R. The F158 4C10, called F (foetal MHC), was obtained from myosin of a 22-week-old cattle foetus.

2.3. Immunohistochemistry

Myosin heavy chains present at different stages were revealed by immunohistochemical staining using the three monoclonal antibodies previously described. Transverse serial cross-sections of 10 µm in thickness were obtained on a cryostat microtome at –25 °C. They were incubated directly with the first monoclonal antibody without dilution for 30 min at 37 °C. Sections were washed in PBS and incubated with the second antibody (rabbit anti-mouse IgG labelled with dichlorotriazinylaminofluorescein, Interchim) diluted 1/30 in PBS for 30 min at 37 °C. After washing in PBS, the sections were mounted with mowiol (Calbiochem).

2.4. Protein preparation

Frozen muscle (200 mg) was ground (polytron) for 20 s at speed 6, in 5 mL of a buffer solution: 0.5 M NaCl, 20 mM sodium pyrophosphate, 50 mM Tris, 1 mM EDTA, 1 mM dithiothreitol. After 10 min at 4 °C, the samples were centrifuged for 10 min at 2 500 *g*. The supernatant was mixed with glycerol at 50 % (v/v) and stored at –20 °C for several months. Protein concentration was determined according to the method of Bradford [2], with bovine serum albumin as a standard. These protein preparations were used for electrophoresis, immunoblotting and ELISA methods.

2.5. Electrophoresis

Protein preparations stored at –20 °C were diluted 50 % (v/v) in a solution containing 1.4 % (w/v) SDS, 10 % (v/v) 1 M Tris pH 6.8, 17 % (v/v) glycerol, 0.6 % (w/v) pyronin Y and 7.5 % (v/v) mercaptoethanol. After 10 min at room temperature, the samples were heated at 90 °C for 10 min.

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was performed on plates of 160 × 160 × 1.5 mm by the method of Laemmli [12]. The separating gel was a 3.5–10 % gradient SDS-polyacrylamide gel and the stacking gel was at 3.5 %. To improve separation, the separating gel also contained 30–40 % (v/v) glycerol gradient according to Suguira and Murakami [31]. Wells were loaded with 5 µg of protein when electrophoresis alone was performed, or with 0.5 µg when electrophoresis was followed by immunoblotting. Migration was performed

at 110 V overnight at 4 °C. The gels were stained with Coomassie blue R250 or used for immunoblotting.

2.6. Immunoblotting

Proteins were electrotransferred from the SDS-PAGE gel onto a membrane of polyvinyl (Immobilon P., Millipore) according to the method of Towbin et al. [35]. The membrane was saturated in TBS: 100 mM Tris, 150 mM NaCl, 0.05 % Tween 20, pH 8 with 3 % (w/v) powdered skim milk and shaken for 4 h at room temperature. Then it was incubated overnight with the first antibody, either S diluted 2 000-fold (v/v), or R or F diluted 1 000-fold (v/v), in TBS containing 3 % (w/v) powdered skim milk. The second antibody (rabbit anti-mouse IgG labelled with alkaline-phosphatase, Jackson Interchim) was diluted 5 000-fold (v/v) in TBS containing 3 % powdered skim milk and applied for 1 h at room temperature. After washing in a buffer (pH 9.5) containing 100 mM Tris, 100 mM NaCl, 50 mM $MgCl_2$, development was carried out in a solution of nitro blue tetrazolium (NBT) and 5-bromo-4-chloro-3-indolyl phosphate (BCIP). The reaction was stopped in a solution of pH 8, 20 mM Tris-HCl, 10 mM EDTA.

2.7. ELISA

This technique was used to quantify either MHC1, MHC2 (2a and 2b, or 2x) or foetal MHC. The method used was that of Winkelman et al. [37] modified by Picard et al. [19]. Briefly, 2.4 µg/mL of myofibrillar proteins were loaded on each well of a 96-well plate (Nunc) and incubated overnight at 4 °C. The following day, non-specific sites were saturated with PBS: 138 mM NaCl, 2 mM KCl, 9 mM Na_2HPO_4, 1 mM KH_2PO_4, pH 7.4 containing 10 % (w/v) powdered skim milk for 30 min at room temperature. First monoclonal antibodies were diluted either 400-fold (v/v) for S or 200-fold (v/v) for R and F in PBS containing 10 % (w/v) powdered skim milk. The second antibody (rabbit anti-mouse IgG labelled with alkaline phosphatase, Jackson Interchim) was diluted in PBS containing 10 % (w/v) powdered skim milk either 700-fold (v/v) to determine MHC1 and foetal MHC percentages or 1 400-fold (v/v) for MHC2 percentage. The substrate of the enzyme was paranitrophenylphosphate (Sigma). Absorbance was red at 405 nm with a microtitre plate reader.

MHC concentration was proportional to OD. A standard curve was obtained with a mixture of two muscles whose fibre compositions were opposite. To quantify MHC1 and MHC2 proportions, adult bovine Ma (100 % MHC1) and median part of adult bovine CT (100 % MHC2) were chosen. To determine foetal MHC percentages, LT of a 180-day-old bovine foetus for which the foetal MHC was the highest throughout gestation (100 % foetal MHC), and adult bovine CT (0 % foetal MHC) were chosen.

2.8. Statistical analysis

Variance analysis using SAS software [28] was performed to study the effect of age and muscle type.

3. RESULTS

3.1. Different types of fibres during myogenesis

3.1.1. Sixty days

Cells were organised in bundles which were less clear in Ma than in other muscles. Only one population of fibres was observed in all muscles. It was shaped like large myotubes and was recognised by S, R and F antibodies (data not shown).

3.1.2. One hundred and ten days

All muscles were composed of two generations of cells (*table I*). The first one represented 30 % on average and the second 70 % in all muscles except Ma. In this muscle, the first generation represented 43 % and the secondary one 57 %. This indicated a delay in the appearance of the secondary generation in this muscle. This secondary generation of cells was located around primary myotubes, using them as a scaffold (*figure 1*).

The primary generation of cells, shaped like large myotubes, was composed of one category of cells (S–F) only in LT. It was divided into two categories in the other mus-

Table I. Proportions of the different categories of cells in *Cutaneus trunci* (CT), *Longissimus thoracis* (LT), *Semitendinosus* (ST), *Biceps femoris* (BF), *Masseter* (Ma) and *Diaphragma* (Di) muscles at 110, 180, 210 and 260 days of foetal life.

Age day	Generation of cells	Labelling with antibodies	*Cutaneus trunci* (%)		*Diaphragma* (%)		*Masseter* (%)		*Semitendinosus* (%)		*Longissimus* (%)		*Biceps fermoris* (%)	
			a	b	a	b	a	b	a	b	a	b	a	b
110	primary myotubes	SRF	21	19	27	7	43	41	29	26	34		35	23
		S–F		2		20		2		3		34		12
	secondary myotubes	SRF		33		19		6		28		21		20
		–RF	79	14	71	18	57	22	71	11	66	19	65	9
	small cells	– – F		32		34		29		32		26		36
180	primary generation	S – –		–		4.5		–		3.9		5.0		6.2
		S–F		1.4		–		1.7		–		–		–
	secondary generation	–RF		19		44		40		54		37		33
		–rF		20		24		20		29		17		11
		SRF		0.7		3.5		0.7		1.4		0.5		–
		– – F		46		24		30		12		32		28
	small cells	– – F		13		–		7		–		7.8		21
210	primary generation	S – –		3.4		7.9		5.8		5.4		4.8		9
	secondary generation	–RF		52		69		69		58		51		59
		–R–		6		–		–		20		24		14
		SRF		5.4		15		25		11		13		12
		– –F		11		5.1		–		2.3		7.2		–
		– – –		19		–		–		–		–		–
	small cells	– –F		3.1		–		–		–		–		3.6
		–RF		–		–		–		2.3		–		2.5
260	low fibres (from the primary and secondary generations)	S – –		–		23		3.9		7.9		13		12
	secondary generation	–RF		27		13		18		20		17		18
		–R–		62		22		21		47		50		49
		SRF		11		32		35		14		20		15
		–SF		–		9		14		–		–		–
		– –F		–		0.6		–		–		–		–
	small cells	– RF		–		–		–		5.3		–		5.2

S: anti MHC1 antibody; R: anti MHC2 (2a and 2b) antibody; F: anti MHC foetal antibody.
a: Percentage of the total primary and secondary generations. b: Percentage of each category of primary and secondary generations.

cles. The main group was composed of cells recognised by three antibodies (SRF). Other myotubes no longer expressing fast myosin (S–F) were more numerous in Di (*table I*).

The secondary generation was composed of three categories of cells in all muscles (*figure 1*) but they were present in different proportions. All cells were recognised by F antibody in all muscles. The proportion of secondary myotubes labelled with the three antibodies (SRF) was the highest in CT and the lowest in Ma (*table I*). Secondary myotubes no longer expressing slow myosin (–RF) represented between 9 % (BF) and 22 % (Ma) of the total number of fibres depending on muscle type (*table I*)

3.1.3. One hundred and eighty days

The primary generation of cells did not express foetal MHC in any muscles except Ma and CT (*table I*).

The secondary generation represented the greatest population. It was composed of five categories of cells. The response to the R antibody showed two types of positive cell (*figure 2*). Some (–RF) reacted intensively with it, whereas others (–rF) reacted weakly. These two categories represented the majority of secondary cells in all the muscles. The first ones, middle-sized cells, were the most numerous in all muscles except in CT. This muscle was composed of a greater proportion of cells (—F) recognised only by F antibody. In all the muscles except in BF, few secondary cells were labelled with S, R, F (SRF) (called IIC fibres) (*figure 2*).

3.1.4. Two hundred and ten days

The primary generation of cells was only recognised by antibody S in all muscles (*table I*). These cells look like adult type I fibres.

Seven categories of secondary cells were observed in the studied muscles. They were present in different proportions depending on muscle type (*table I*):

– large- and medium-sized cells which expressed fast and developmental MHC (–RF) were observed in all muscles and represented more than 50 % of the secondary generation of cells;

– cells recognised only by antibody R were present in all muscles except in Di and Ma;

– type IIC fibres (SRF) were present in all muscles; however, they were more numerous in Ma and Di than in other muscles;

– large cells recognised by antibody F were observed in all muscles except in Ma and BF;

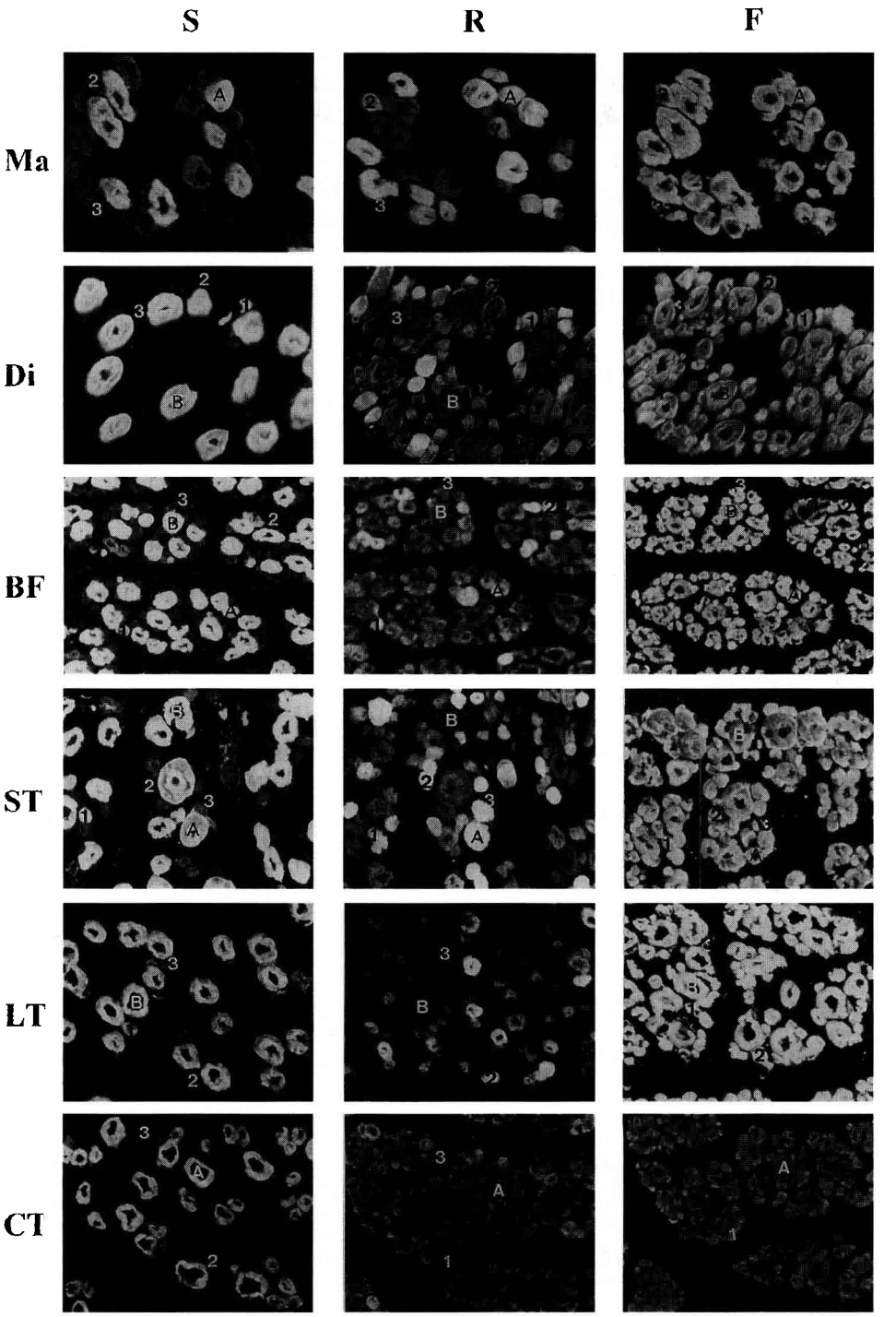
– small cells recognised either by antibodies R, F (–RF) or by F (– – F) were observed in low proportions only in CT, ST and BF;

– large secondary cells not recognised by any of the used antibodies (– – –), were only observed in CT (19 %).

3.1.5. Two hundred and sixty days

Just before birth, type I fibres expressing only slow MHC were observed in all muscles except in CT in which all cells were also recognised by the R antibody (*figure 3*).

Figure 1. Immunohistochemical analysis of transverse serial sections of *Masseter* (Ma), *Diaphragma* (Di), *Biceps femoris* (BF), *Semitendinosus* (ST), *Longissimus thoracis* (LT) and *Cutaneus trunci* (CT) muscles of 110-day-old foetuses with monoclonal antibodies directed against slow MHC (S), fast MHC (R) and foetal MHC (F). Large myotubes called A were recognised by S, R, F and others called B were recognised by S, F. They corresponded to the primary generation of cells. The secondary generation of cells was composed of smaller myotubes called 1 when recognised by S, R, F; 2 when recognised by R, F; and 3 when recognised by F.


S
R
F
Ma
Di
BF
ST
LT
CT

The secondary generation was composed of six categories of cells:

– fast fibres only recognised by the R antibody were present in all muscles. They were more numerous in fast muscles such as CT than in slow ones such as Di and Ma (*table I*);

– cells labelled with R, F antibodies were also observed in all muscles. They were also more numerous in CT (*table I*);

– type IIC fibres (SRF) which still expressed developmental MHC were present in all muscles (*table I*). They were twice as numerous in slow muscles (Di and Ma) than in others;

– a few small cells recognised either by antibodies R, F or only by F were present only in ST, BF and Di muscles (*table I*);

– cells labelled with S and F were only present in Di and Ma (*table I*).

On the basis of the results obtained in this study we can propose a schematic evolution of the different generations of the cells (*figure 4*). The primary generation observed at 60 days of foetal life would give rise to type I fibres from two-thirds of gestation onwards in Ma, Di, BF, ST and LT muscles and to IIC fibres in CT. In this last muscle, primary myotubes would be transformed into fast fibres after birth. The secondary generation of cells, observed later, was initially recognised by all antibodies (S, R, F). It would stop expressing slow and developmental isoforms and would give rise at birth to type II fibres in all muscles. A third generation of cells was composed of small cells only expressing developmental isoforms at 110 days. It would form type IIC fibres (SRF) from the last third of foetal life and give rise to specific fibres in specialised muscles: cells labelled with S, F only observed in Di and Ma would transform into slow fibres just before birth or at weaning [21], respectively. In mixed muscles, this cell type IIC would give rise either to type I or type II fibres depending on regulatory factors such as innervation, exercise, hormonal status and nutrition. We could speculate that cells not recognised by any of the antibodies in CT at 210 days belong to the third generation of cells and mature to type II fibres at ulterior stages.

3.2. Identification of myosin heavy chains by electrophoresis and immunoblotting – quantification by the ELISA method

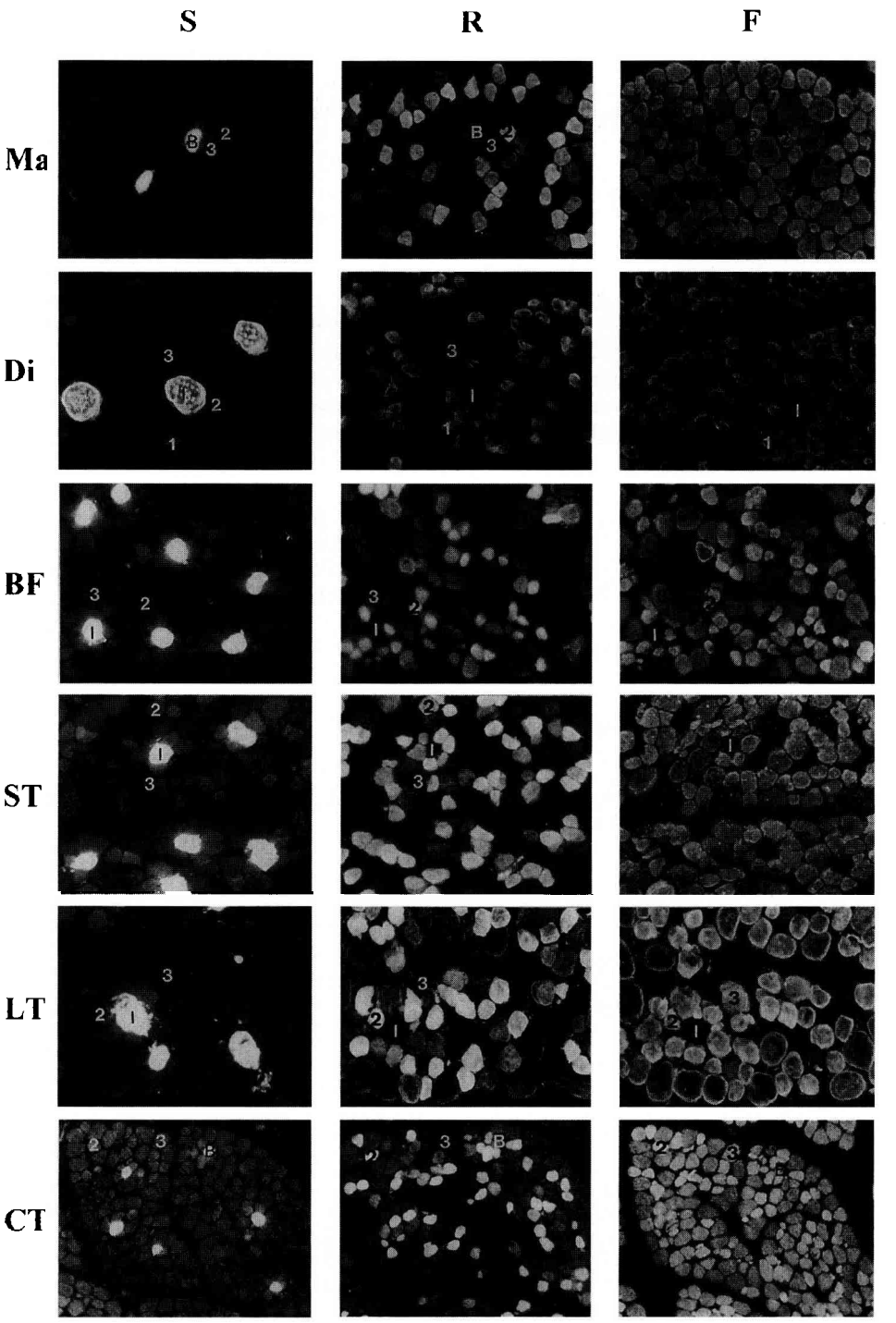
The different MHC isoforms were separated by SDS-PAGE and identified by immunoblotting. The MHC1, MHC2 (2a and 2b, or 2x) and foetal MHC were quantified by the ELISA method.

Electrophoretic analysis showed the existence of five MHC isoforms in all studied muscles. Four isoforms were identified by immunoblotting as adult forms MHC 1, 2a and 2b (or 2x), and foetal form. The remaining form could not be identified with the antibodies used (data not shown).

3.2.1. MHC1

MHC1 was found by electrophoresis and immunoblotting in BF, Di, LT, Ma and ST throughout foetal life. In CT it was never detected by electrophoresis; however it was revealed only at 110 days by immunoblot-

Figure 2. Immunohistochemical analysis of transverse serial sections of *Masseter* (Ma), *Diaphragma* (Di), *Biceps femoris* (BF), *Semitendinosus* (ST), *Longissimus thoracis* (LT) and *Cutaneus trunci* (CT) muscles of 180-day-old foetuses with monoclonal antibodies directed against slow MHC (S), fast MHC (R) and foetal MHC (F). The primary generation of cells was composed of fibres called I, only recognised by S in Di, ST, LT and BF. Fibres called B labelled with S, F were only present in Ma and CT. The secondary generation of cells was composed of cells recognised by S, R, F (1) corresponding to type IIC fibres; cells labelled with R, F (2) and cells only recognised by F (3).


S
R
F
Ma
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CT

ting (*figure 5*). The MHC1 percentage determined by the ELISA method was weak and stable until the last third of foetal life in most muscles (*figure 6*). It significantly increased after 210 days in Di ($P < 0.05$) and BF ($P < 0.01$), and 230 days in Ma, LT and ST ($P < 0.001$). In contrast, the MHC1 proportion progressively disappeared in CT from 110 to 260 days. The MHC1 percentage was lower in Ma than in ST, LT and BF up to 210 days lower than in Di throughout gestation. BF, LT and ST muscles exhibited intermediate MHC1 proportions throughout foetal life and were not significantly different from each other. To summarise, CT contained the lowest MHC1 percentage while Di contained the highest one at all stages (*figure 6*). It is only just before birth that MHC1 percentage dramatically increased in Ma.

3.2.2. MHC2

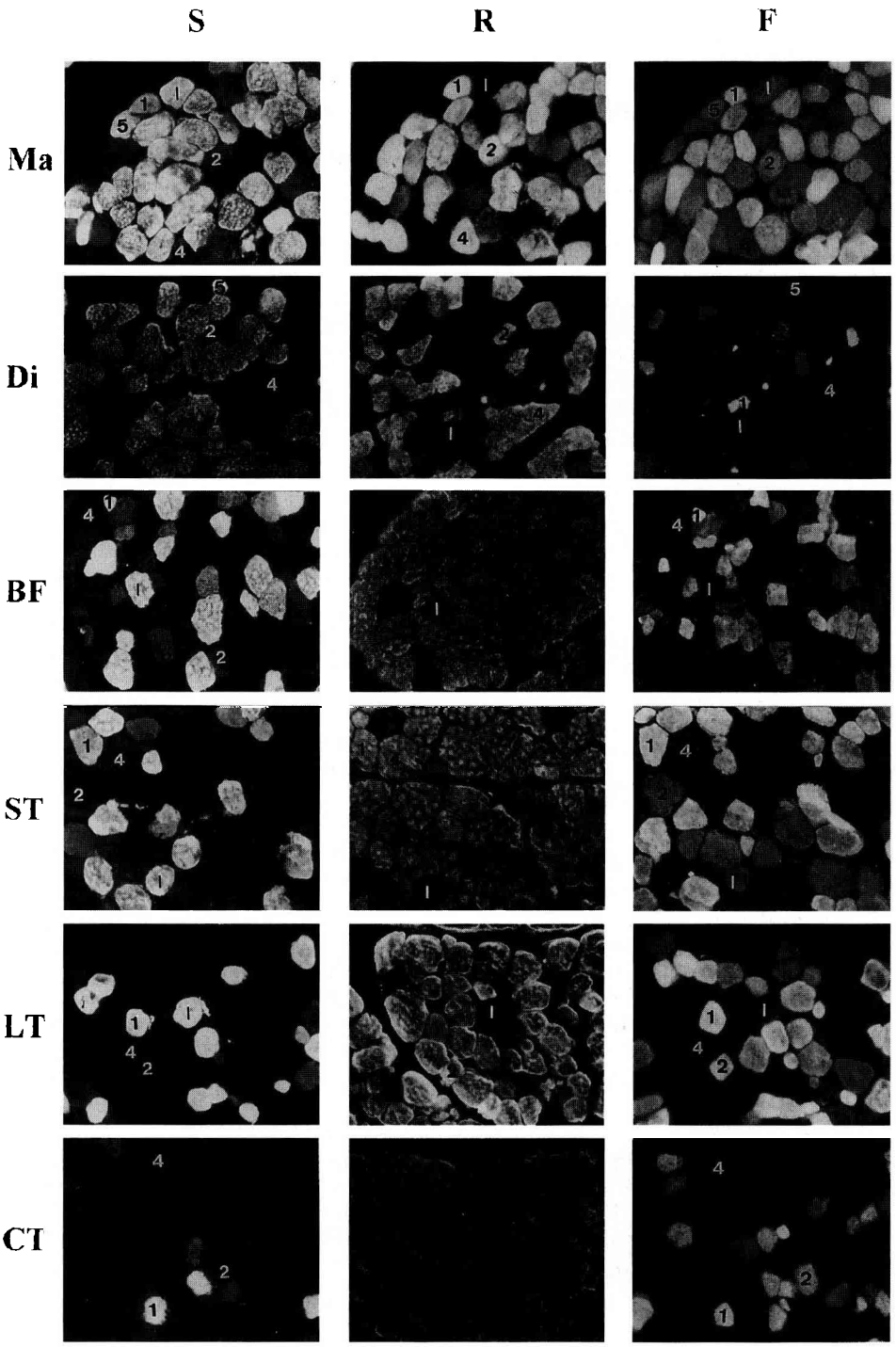
Fast MHC isoforms, 2a and 2b (or 2x), were recognised by antibody R. They exhibited the highest molecular weights and were detected at 230 days by electrophoresis in BF, CT, LT and ST. In Di and Ma, only MHC2a was identified at 230 days by electrophoresis (data not shown). Like MHC1, the percentage of MCH2 quantified by the ELISA test was low, stable and not different between muscles (around 2 %) up to 180 days (*figure 6*). It significantly increased after 180 days in LT ($P < 0.05$) and ST ($P < 0.01$), and after 210 days in other muscles ($P < 0.001$). At 210 days, ST was composed of a significantly higher MHC2 proportion than other muscles ($P < 0.001$) while the percentage of MCH2 was significantly lower in CT than in other muscles ($P < 0.05$ for BF, $P < 0.01$ for Di, $P < 0.001$ for LT and ST). At 230 days, the highest MHC2 percentage was observed in BF ($P < 0.05$ for LT, $P < 0.01$ for Di and CT, $P < 0.001$ for Ma) and the lowest one in Ma. Just before birth, BF and LT contained significantly more MHC2 isoforms than Di and ST ($P < 0.05$).

3.2.3. MHCF

An MHC isoform with an intermediate molecular weight between MHC1 and MHC2a was labelled by immunoblotting with antibody F. It was termed foetal MHC according to Picard et al. [20]. It was observed in all muscles throughout foetal life. The proportion of foetal MHC measured by the ELISA was high up to 180 days in all muscles, except in CT where it gradually decreased from 110 days onwards (*figure 6*). After 180 days, foetal MHC percentage significantly decreased in BF, Di, Ma and ST muscles ($P < 0.01$), and only after 210 days in LT ($P < 0.01$). CT contained the lowest proportion of foetal MHC from 110 to 260 days. On the contrary, LT contained a significantly higher foetal MHC percentage than other muscles at 210 days of foetal life ($P < 0.05$ for ST, $P < 0.01$ for Di, $P < 0.001$ for BF, CT and Ma). Other muscles, BF, Di, Ma and ST exhibited intermediate percentages of foetal MHC and did not significantly differ between each other.

A fifth MHC isoform, already described by Picard et al. [20], was observed by SDS-PAGE in all muscles from 60 days onwards. It had the same molecular weight as the adult MHC2a form, but was not recognised by

Figure 3. Immunohistochemical analysis of transverse serial sections of *Masseter* (Ma), *Diaphragma* (Di), *Biceps femoris* (BF), *Semitendinosus* (ST), *Longissimus thoracis* (LT) and *Cutaneus trunci* (CT) muscles of 260-day-old foetuses with monoclonal antibodies directed against slow MHC (S), fast MHC (R) and foetal MHC (F). Cells only recognised by S were called I corresponding to adult type I fibres. The secondary generation of cells was composed of cells recognised by S, R, F (1) corresponding to type IIC fibres; cells recognised by R, F (2); F (3) and cells only labelled with R (4) corresponding to adult type IIA and IIB fibres. Cells recognised by S, F (5) were only observed in Di and Ma.


S
R
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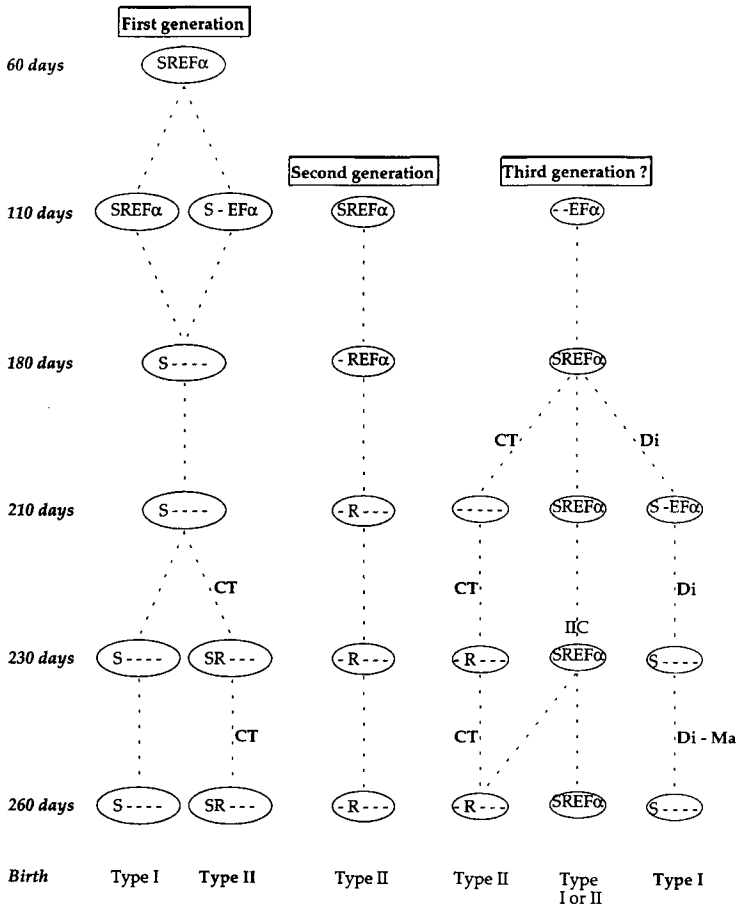

Figure 4. Schematic evolution of the different generations of cells during muscle differentiation in bovine foetuses. The primary generation of cells would give rise to type I fibres in all muscles and type IIC only in *Cutaneus trunci* (CT). The secondary generation of cells was completely differentiated in type II in all muscles just before birth. The third generation of cells would give rise to type IIC fibres from 180 days which could differentiate into different categories of cells according to muscle type. They could give rise to cells not recognised by all antibodies used, were only present in *Cutaneus trunci* (CT) at 210 days and they would be transformed into type II fibres. Adult type I fibres could occur in the third generation of cells in slow adult muscles such as *Diaphragma* (Di) and *Masseter* (Ma). After birth, type IIC fibres could give rise to type I or type II fibres in mixed muscles.

any of the antibodies used (data not shown). We were unable to determine the stage when it disappeared or if it was still present before birth.

The electrophoretic results and the quantification of different MHC isoforms by ELISA showed that, in all muscles, foetal MHC disappeared concomitantly with the accumulation of adult isoforms, in particular of MHC2 after 180 days.

4. DISCUSSION

Our results confirm and complete those reported by Robelin et al. [25] in ST muscle

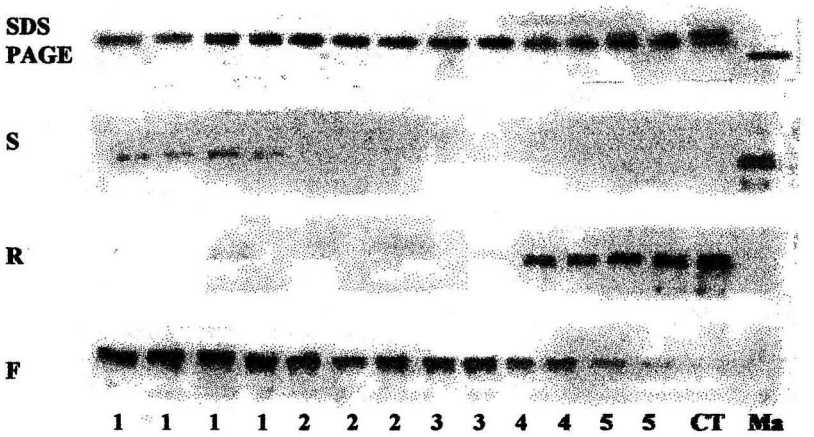

Figure 5. Electrophoresis (SDS-PAGE) and immunoblotting with S, R and F antibodies in *Cutaneus trunci* muscle. 1: 110 days; 2: 150 days; 3: 180 days; 4: 210 days; 5: 260 days.

and Picard et al. [18] in four bovine muscles showing that at least two generations of muscle cells appeared sequentially during foetal life.

4.1. Differentiation of the primary generation

The primary generation of cells was observed from 60 days onwards. Its distinction from the secondary generation was possible up to 210 days. During this period, primary cells were larger than secondary ones and the S antibody marked them with a high intensity. From 210 days onwards, the size of the fibres was homogeneous and some secondary cells were also recognised by the S antibody. Indeed, the data of Robelin et al. [24] in ST, show that the total number of fibres is fixed at 230 days. Added slow fibres are necessarily issued from the secondary generation. Thus, at 210 days we could not distinguish slow fibres coming from the first and second cells.

Our results showed that the primary cells gave rise to type I fibres from two-thirds of foetal life onwards in BF, Di, LT, Ma and ST, whereas no type I fibres were observed in CT muscle. Therefore, all primary myotubes mature to fast muscle fibres which are the only fibre types in CT of adult cattle [19]. Thus, the primary generation of cells could not only give rise to type I fibres but could also form other fibre types depending on the muscle. In cat masseter, Hoh and Hughes [9] showed that some primary cells mature into superfast fibres during the first weeks after birth. In pig ST muscle, Lefaucheur et al. [13] showed that primary myotubes mature towards type I fibres in the mixed deep red portion and type II fibres in the superficial fast white portion. These results are in agreement with previous results reported in the anterior tibialis and crural muscles of the rat [1, 3].

4.2. Differentiation of the secondary generation

The secondary generation of cells was observed later than the primary one. Its differentiation in cattle muscles was complex because of multiple secondary cell categories observed at mid-gestation. It is therefore difficult to determine the destiny of each one. We speculate that small myotubes

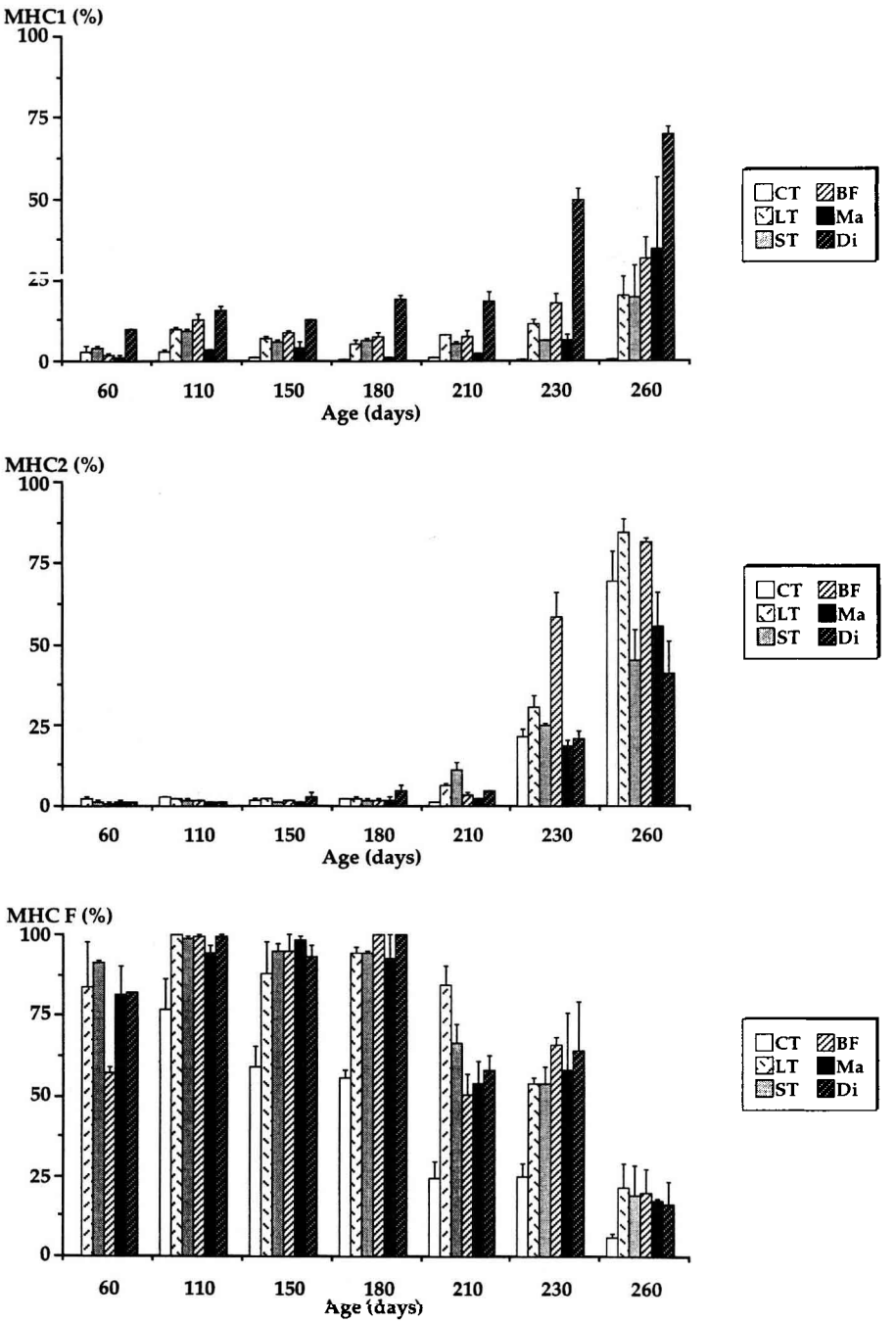

Figure 6. ELISA determination of MHC1, MHC2 and foetal MHC percentages in *Cutaneus trunci* (CT), *Longissimus thoracis* (LT), *Semitendinosus* (ST), *Biceps femoris* (BF), *Masseter* (Ma) and *Diaphragma* (Di) muscles of bovine foetuses at different stages of development.

recognised by the three antibodies used at 90 days in all muscles could stop expressing the slow isoform and then developmental isoforms during the last third of foetal life and give rise to type II fibres. The sequential expression of the different MHC isoforms was similar in slow and fast muscles, which is not in accordance with the results of Lefaucheur et al. [13] in pig ST muscle. In the future fast white portion of this muscle, secondary fibres sequentially expressed MHC genes for embryonic, foetal and adult fast myosin. In the future red portion of the ST, however, secondary fibres stained positively with the anti-fast MHC antibody as soon as they were formed. This suggests differences in the differentiation of secondary myotubes between pigs and cattle. It is also noteworthy that secondary fibres are distributed randomly in cattle muscles as in rat [16] and human [1] muscles, whereas in pig muscle, secondary fibres are highly organised around primary ones [13].

4.3. Differentiation of a third generation

Small cells which only expressed developmental MHC isoforms during the first two-thirds of gestation were still present just before birth in Di, ST and BF muscles. We speculate that this population of cells was a third generation because of the smaller cell size and the MHC isoform composition. In fact, during foetal development of human muscle, Draeger et al. [6] observed a new population of small-diameter fibres which could be distinguished from primary and secondary myotubes by their specific staining with an adult fast MHC antibody. This third generation of cells was already suggested in sheep [36] and in pigs [13, 15], but during the first weeks after birth. It could contribute to the formation of a great number of fibres in large mammals, while two generations of cells would be sufficient in small laboratory rodents [36]. Initially, all tertiary cells would only express developmental MHC and then some of them could express adult isoforms during the last third of foetal life. They could give rise to specific fibres in specialised muscles or type IIC fibres in mixed ones such as BF, LT and ST during the last third of gestation. In future slow muscles, such as Di and Ma, fibres recognised by S, F antibodies were only observed at 230 days. They could arise from this third generation of cells and form type I fibres in slow adult muscles. In contrast, small third generation cells could be transformed into fibres not recognised by the antibodies used in CT at 210 days of foetal life. The results of the ELISA test showed that at 210 days, CT contained the lowest foetal and MHC2 proportions and few MHC1, which would suggest the existence of another MHC isoform in this muscle. Nevertheless, just before birth, CT was composed of the highest percentage of fast fibres but not of the greatest proportion of MHC2. Therefore, cells not recognised by any of the antibodies used could give rise to fast fibres which would coexpress an unidentified myosin isoform with MHC2a and 2b (or 2x) from the last stages of gestation onwards. We have to confirm, however, the existence of this third generation and verify that it does not come from the second one.

4.4. Myosin isoform transition

MHC1 and MHC2 proportions increased during the last third of foetal life when foetal MHC disappeared. The MHC1 proportion was low and stable from 60 to 230 days in most muscles except in Di muscle where it started to increase before 180 days. The increase in MHC1 was the highest in future slow muscles such as Di and Ma, while in CT, a future fast muscle, the MHC1 proportion remained low throughout gestation. This increase in MHC1 corresponds to a de novo expression of this isoform in fibres of the secondary generation. In pig muscle Lefaucheur et al. [13] showed that this

increase occurs at the end of gestation and after birth. This illustrates a higher maturity of bovine muscle development compared to pig muscle development. The MHC2 percentage was low up to 180 days and increased thereafter when foetal MHC proportion decreased. Thus, we have shown that adult MHC isoforms accumulated when developmental MHC disappeared during the last third of foetal life. A similar result was obtained in human foetuses [1, 23] and in some bovine muscles [18, 25]. In this study, we confirmed that this transition occurs during the last third of gestation. At birth, the foetal isoform practically disappeared. In other species, this isoform disappears during the weeks after birth (in pig [13]; in rabbit [8]; in rat [5]). Thus, these results show that bovine muscles are more mature at birth compared to other species.

The appearance of the adult MHC isoform in the secondary generation of fibres coincides with the beginning of metabolic differentiation. Robelin et al. [24] showed that SDH activity increased from 210 days in ST muscle of bovine foetuses. It was the highest in type I fibres and some fast type fibres. Elsewhere, we studied metabolic differentiation in three cattle muscles displaying different metabolic properties in adult animals. We showed that enzymatic activity increases during the last third of foetal life whatever the muscle type [7]. It therefore seems that there could be a relationship between the acquisition of contractile properties and metabolic differentiation of muscle from the last third of gestation.

4.5. Concordance between the different methods used

The different methods used in this study give complementary results. Electrophoresis, immunoblotting and ELISA give information on MHC at the tissue level and immunohistochemistry at the cellular level. Our results show the limit of detection of electrophoresis for low values. For example, no MHC1 was detected in CT muscle during gestation by electrophoresis, but it was detected by immunoblotting at 110 days and quantified by ELISA at 3 %. Electrophoresis, however, permits the distinction between fast MHC which is not possible with immunochemical methods. Indeed, immunoblotting shows that the isoform with the same molecular weight as MHC2a is not MHC2a but another isoform, perhaps an embryonic isoform. Electrophoretic or ELISA techniques do not permit this distinction.

It is very difficult to make a rigorous correspondence between tissue and immunohistochemical analyses. For example, in CT muscle at 110 days, 50 % of the fibres were recognised by the S antibody. Only 3 % of MHC1 was, however, detected by ELISA. We could speculate that the fibres recognised by S contained a small proportion of MHC1.

4.6. Precocity of different muscles

Our results show that muscles which differ by their contractile properties in adult cattle exhibit more or less precocious foetal development: Di and CT were followed by BF, ST, LT and Ma. Di seems to be in advance particularly during the first two-thirds of gestation. On the contrary the precocity of CT was more evident during the end of gestation.

In Di, most primary myotubes stopped expressing MHC2 at 90 days of foetal life, whereas primary myotubes expressing MHC1, MHC2 and developmental MHC were the most numerous in other muscles. Moreover, the primary generation of cells expressed only MHC1 from 150 days onwards in Di, while in other muscles this cell type was only observed from 170 days onwards in most muscles and from 210 days onwards in Ma. Type IIC fibres were, however, present from 170 days onwards in Di and they were more numerous in this muscle. Secondary cells recognised by S, F were

only present in Di from 210 days onwards and would give rise to type I fibres just before birth. Therefore, transition from foetal MHC to adult isoforms occurred earlier in Di of cattle as in rats [5]. In fact, respiratory muscles (intercostal and *diaphragma*) are the first muscles to function very actively and regularly at birth and display the most precocious isomyosin transition [5]. Johnson et al. [10], however, who described a correlation between the expression of fast MHC isoforms and a progressive increase of contractile properties during early postnatal development in the Di of rats, suppose that the functional impact of the developmental changes in Di in sustaining ventilation would be uncertain.

CT also displayed an early contractile differentiation. From 230 days on CT contained very few type I fibres. It was composed of an average of 10 % of type IIC, 90 % fast type fibres and less than 1 % of type I fibres. Thus, the adult phenotype, completely fast in cattle [19], was already reached just before birth. CT was the only muscle containing fibres not recognised by any of the antibodies used at 210 days of foetal life. This cell type was already observed in several muscles of double-muscled foetuses [20]. These authors supposed that these fibres contain either another developmental isoform present transitorily or a fast myosin, different from MHC2a and MHC2b (or 2x) forms but specific to muscles rich in fast type glycolytic fibres such as CT and muscles of double-muscled cattle.

Ma was characterised by an important delay both in tissue organisation and in differentiation compared with other muscles. MHC expression was delayed in both cell generations. The primary generation of cells still expressed developmental MHC isoforms at 170 days while in other muscles it was only recognised by the S antibody. The secondary generation of cells was also delayed. Just before birth, Ma was composed of a major proportion of type IIC fibres and many type II fibres. Moreover, the fibres recognised by the S and F antibodies only present in slow muscles, were observed earlier in Di than in Ma (data not shown). They were still observed just before birth in Ma, while in Di those cells no longer expressed developmental MHC isoforms. In other species such as rabbits, rats and humans, however, developmental MHC are still observed in adult Ma [4, 5]. Thus, Ma differentiation seems to be characterised by very different kinetics of foetal isoform transition. This could be related to a different embryonic origin. Ma derives from the first branchial arch. It is innervated by cranial nerves while limb muscles, which arise from somites, are innervated by spinal nerves [26]. Final differentiation of Ma, however, must also be due to functional demand in relation to feeding behaviour. According to Suzuki [32], Ma of adult cattle, which displays a slow and regular rate of chewing, is composed of one slow fibre type. It is only after weaning that the adult phenotype, completely slow, appears in Ma of cattle [21] in adaptation to a functional demand.

BF, LT and ST, mixed muscles, displayed an intermediate kinetics of differentiation. Nevertheless, the primary generation of cells seemed to differentiate later in BF than in ST. Moreover, type IIC fibres appeared only from 210 days in BF, whereas they were already observed at 170 days in ST and LT. This delay of contractile differentiation of BF muscle could be related to an anteroposterior gradient of development in adult cattle as described by Totland and Kryvi [34].

5. CONCLUSION

This study confirms the presence of at least two generations of muscle cells during myogenesis in cattle. The primary generation of fibres would give rise to adult type I fibres from 180 days onwards in Ma, Di, BF, ST and LT muscles and also type IIC in CT. The differentiation of the secondary generation of cells is very complex,

in particular between 150 and 210 days when many cell categories exist. Therefore, it would be appropriate to study this period in more detail in order to specify the lineage of each category. Later, secondary fibres can be transformed into type II fibres in future fast muscles and type I fibres in future slow muscles. In mixed muscles, the secondary generation of cells can give rise to either fast or slow fibres during the last third of gestation. Elsewhere, the kinetics of differentiation are different according to muscle type. Each muscle is subjected to its own programme of differentiation. Thus, the earliness of foetal development is related to muscle function at birth.

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