Myosin expression in semitendinosus muscle during fetal development of cattle: immunocytochemical and electrophoretic analyses

J Robelin 1, B Picard 1, A Listrat 1, C Jurie 1, C Barboiron 1, F Pons 2, Y Geay 1

1 INRA-Theix, laboratoire Croissance et Métabolismes des Herbivores, 63122 Saint-Genès-Champanelle; 
2 INSERM-U300, Unité de Recherche de Physiopathologie Cellulaire et Moléculaire, Av Charles Flahaut, 34100 Montpellier, France

(Received 12 March 1992; accepted 29 October 1992)

Summary — The pattern of expression of different types of myosin and the development of different muscle cell populations were studied in the semitendinosus muscle of cattle from 39 d of gestation to 30 d of post-natal life. Monoclonal antibodies specific to different myosin heavy chains were used. Two cell generations were identified during myogenesis. They appeared successively and were characterized by different patterns of expression of myosins. The first population, which was present from the first stage studied (39 d of gestation), gave rise to type I fibers, which, in the mature animal, express only slow myosin. A second generation became differentiated at about 120 d of fetal life and then developed into type II fibers (Ila, Ilb or Iic). The beginning of differentiation was characterized in all the cell populations by the expression of specific types of embryonic or fetal myosins. A comparison of these results with findings from previous works shows a marked similarity between species in the pattern of myogenesis but great differences in the length of the different stages of development. In this respect, myogenesis in cattle closely resembles that in man.

Résumé — Expression de différentes formes de myosine dans le muscle semitendinosus au cours du développement fœtal chez le bovin : étude immunocytochimique et électrophorétique. Le profil d’expression des différents types de myosines, et le développement de différentes générations de cellules musculaires ont été étudiés au cours de la vie fœtale chez le bovin, à l’aide d’anticorps monoclonaux dirigés contre les chaînes lourdes de myosines. On a identifié 2 populations cellulaires apparaissant successivement au cours du développement, et caractérisées par des profils d’expression des myosines différents. La première population, déjà présente lors du premier stade fœtal étudié (39 j de gestation), est à l’origine des fibres de type I qui expriment chez l’adulte une myosine de type lent. Une seconde population est reconnaissable dès l’âge fœtal de 120 j, et conduit ultérieurement aux fibres de type II (Ila, Ilb et Iic). Enfin, le début de la différenciation des fibres musculaires est caractérisé dans les 2 générations de cellules par l’expression de myosines spécifiques de type fœtal ou embryonnaire que l’on ne retrouve pas chez l’adulte. La comparaison de ces résultats avec ceux obtenus chez d’autres mammifères fait apparaître une grande similitude.
entre espèces dans le profil général de la myogenèse, mais aussi des différences importantes dans la durée des différentes étapes du développement musculaire. À cet égard, la myogenèse chez le bovin ressemble beaucoup à ce que l'on observe chez l'homme.

bovin / fœtus / fibre musculaire / différenciation / myosine

INTRODUCTION

In mammals and birds, most muscles are made up of a heterogeneous population of muscle fibers. In the adult animal, these fibers are classified into 3 types (I, Ila and IIb) according to their rate of contraction and energy metabolism (for a review, see Gauthier, 1986). The classification of fiber types in the mature animal was first established by histochemical and histoenzymatic methods (Brooke and Kaiser, 1970). The characteristics of muscle fibers are related to the expression of different types of contractile proteins, particularly myosins (Lowey, 1986).

The development of the different fiber types occurs in the process of differentiation and growth of the muscle cells during embryonic and fetal life (for a review, see Müntz, 1990). In adult animals, the phenotype of a muscle fiber results from a combination of genetic and environmental effects, in which innervation is thought to play a role (Pette and Vrbova, 1985). However, it is not sufficient to account for fiber type diversity, as shown by Hughes and Blau (1992) on the basis of earlier work (Butler et al, 1982; Harris et al, 1989; Condon et al, 1990).

Enormous advances in the study of embryonic origin of the muscle fiber types have been made by the development of monoclonal antibodies raised against different types of myosins (Gauthier et al, 1978) and by the use of molecular probes corresponding to these proteins (Lyons et al, 1990; Miller, 1990). The most detailed results have been obtained in birds (Bandman et al, 1982; Crow and Stockdale, 1986; Miller and Stockdale, 1986; for reviews, see Stockdale and Miller, 1987, and Stockdale, 1990), in small mammals, such as mice (Rubinstein and Kelly, 1981; Whalen et al, 1981; Lyons et al, 1983; Vivarelli et al, 1988) and cats (Hoh and Hughes, 1989). Studies have also been made on the human fetus (Pons et al, 1986; Draeger et al, 1987). There are still large gaps in our knowledge of how the muscle fibers develop into different types, in species whose advanced stage of maturity at birth (cattle, sheep, humans) suggests that their myogenesis is characterized by specific features (Robelin et al, 1991).

For this reason, we studied the expression of different types of myosins during muscle differentiation in the fetal calf. Two techniques were used, immunocytochemistry, to identify the different types of muscle cells, and electrophoresis, followed by immunoblotting to analyse the expression of the different types of myosin in the muscle tissue.

This study shows that 2 distinct cell populations develop in succession during myogenesis in the fetal calf, and describes the pattern of expression of the different types of myosins in these populations. It also shows that there are many similarities between cattle and humans in muscle differentiation, especially in the temporal sequence of events.
MATERIALS AND METHODS

Semitendinosus muscle was sampled from 10 bovine fetuses, obtained from cows who had been artificially inseminated and killed after gestation periods of 39, 53, 64, 84, 98, 122, 143, 180, 224 and 270 d, and from 1 calf aged 30 d after birth. Samples of the median part of the muscle were prepared and frozen in liquid nitrogen as described previously (Robelin et al, 1991).

Immunocytochemistry

The myosins present at the different stages of fetal life were evidenced by immunofluorescence on serial frozen sections, 10 microns thick. The procedure used was that described by Pons et al (1986). We used 4 monoclonal antibodies (MAb) raised against different types of human myosin heavy chains: a β type (slow) myosin from the heart ventricle of an adult (antibody A), a fast myosin immunopurified from the quadriceps muscle of an adult (antibody B), a myosin from a 22-wk-old fetus (antibody C) and a myosin from adult atrium (antibody D). The specificity of these antibodies has been described in earlier works (Pons et al, 1986; Marini et al, 1990). However, in preliminary experiments, we analysed their reaction with myosin from fetal and adult cattle. Immunofluorescence and classical histochemistry (ATPase activity) analyses were carried out on frozen cut sections of cutaneus trunci muscle (entirely fast), masseter muscle (entirely slow) and semitendinosus muscle (predominantly fast) from an 8-yr-old cow. They showed (data not presented) that antibodies A and B reacted with type I and type II fibers respectively. Immunoblotting confirmed that antibody A reacted with type I myosin and antibody B with the myosins of type Ila and IIb. Antibody C did not react with adult muscle fibers (data not shown), but during fetal life reacted with all muscle cells up to = 140 d of gestation, and with some until 220 d (results presented in this article). Immunocytochemical analysis and immunoblotting showed that antibody D raised in human cardiac muscle reacted strongly with adult bovine cardiac muscle (data not shown). It also reacted with fetal muscle cells at the beginning of differentiation (fig 1). However, it did not react with any of the myosin heavy chain in immunoblotting analysis. Tests made with 2 other antibodies raised against human cardiac muscle yielded similar results.

Electrophoresis

Samples of semitendinosus muscle were frozen in liquid nitrogen, stored at −80 °C and then minced in a solution containing 30% (v/v) glycerol, 5% (w/v) sodium dodecyl sulfate (SDS), 1 M Tris–HCl (pH 6.8), 7.5% (v/v) β-mercaptoethanol and 0.05% (w/v) pyronin Y. After 10 min at room temperature, the samples were heated for 10 min at 90 °C. An amount of solution corresponding to 4 µg of proteins was placed in each well. The myosin heavy chains (MHC) were separated on a polyacrylamide gradient gel 5–8% (Laemmli, 1970; Bar and Pette, 1988) on plates measuring 160 x 160 x 15 mm. As in the study of Sugiura and Murakami (1990), a gradient of glycerol was also used, 30% (v/v) in the solution at 5%, and 40% (v/v) in the solution at 8%, to improve the separation of the different MHCs. Migration was carried out at 50 constant volts for half an hour, then at 110 constant volts for = 18 h at 4 °C. The temperature was an important factor in the successful resolution of the different MHCs. After migration, the gels were stained with Coomassie blue or used for immunoblotting.

Immunoblotting

The gels for immunoblotting were obtained by the previously described technique, with a smaller amount of protein (0.4 µg) per well. To determine the nature of the different isoforms of the myosin heavy chains, the proteins were transferred from the polyacrylamide gel on to a membrane of polyvinyl (Millipore, Immobilon P), in a semi-dry system, at 24 constant volts for 1 h (Towbin et al, 1979). The blot was treated with one of the anti-myosin antibodies mentioned above (A, B, C or D), and then by a second antibody raised against the first, and labelled with alkaline phosphatase (mouse IgG; Jackson Immunotech) according to the method of Matus et al (1980). In all the buffers used in this procedure, bovine serum albumin was replaced by 3% (w/v) skimmed milk.

Image-analysis

An image-analysis system was used to make a quantitative analysis of the muscle slices. After
Fig 1. Serial cryosections of semintendinosus muscle of 84- and 143-d-old bovine fetuses. At 84 d of age, all the cells reacted simultaneously with anti slow (A84) and anti-alpha cardiac (D84) myosin antibodies. At 143 d of age, a small population of large primary myotubes reacted with anti slow (A143) antibody, but not with anti-alpha cardiac (D143). This antibody reacted only with the secondary myotubes beginning their differentiation. White bar = 50 μ.
digitizing the images of the muscle cut sections under the microscope, we carried out a threshold to isolate the cells that emitted fluorescence, and measured their total surface (Si). With another threshold, we measured the total surface of all the muscle fibers visible in the field of the microscope (ST). This surface corresponds to the total surface visible in the field minus the interfascicular tissue. For each group of cells that reacted with an antibody, we calculated the proportion of surface they occupied: 100* Si/ST. This measurement gives only an indicative value, since it was made in just 1 animal per stage of development, and has no decisive incidence on the conclusions of this study, which are drawn from the presence or absence of expression of various types of myosins in the muscle cells.

RESULTS

**Expression of the different types of myosins in the muscle fibers**

**Age 39–64 days**

The muscles were not yet well organized at this age, and it was not possible to clearly distinguish muscle masses in the back or limbs. However, numerous fibers already reacted with antibodies A, C and D, which shows that muscle differentiation and the synthesis of myosin had actually begun.

**Age 84 days**

The muscle masses were now better organized and most fibers had a myotube-like appearance (fig 2). All the fibers reacted uniformly with the 3 antibodies A, C and D as before, but also with antibody B. The population of myotubes therefore seemed perfectly homogeneous in terms of myosin synthesis.

**Age 122 days (fig 3)**

Two muscle fiber populations were observed at this age. The first, which represented = one-third of the total population, reacted strongly with antibody A and weakly with antibody B. These slow fibers, resembling large myotubes, corresponded to the primary generation. The second population of fibers that appeared at this age were smaller, and all reacted very weakly with antibody A, but strongly with antibodies C and D. Some of them also reacted with antibody B.

**Age 143 days (fig 3)**

The large primary fibers reacted with antibody A but not at all with B. Their proportion was now close to 10% only, probably because of the increase in the number of secondary myotubes. These smaller secondary fibers (30% of the total population) did not react with antibody A. Their differentiation program is therefore unlike that of the primary generation, and they constitute a real second population and not merely a continuation of the proliferation of the primary one. All the secondary fibers reacted with antibodies C and D, but only some of them reacted with antibody B. Thus, at this age we distinguished 3 fiber populations, which differed in size and in the type of myosin they synthesized at this time, and which, to make designation simpler, can be called primary-large-slow (10%), secondary-small-fast (30%) and secondary-small-embryonic (60%).

**Age 180 days (fig 4)**

The group of large primary fibers that reacted very strongly with antibody A, no longer reacted with antibody C or antibody D. In this respect, these fibers were exactly the same as type I fibers in the adult. The
population of smaller fibers reacted uniformly with antibody B, while a small proportion (5% of the total) also began to react with antibody A. The small fibers that reacted with antibodies A and B also reacted with C and D. The fibers of the second generation therefore seemed to begin differentiation by expressing only myosins that react with C and D, and then to continue their development by expressing either fast myosins or, simultaneously, fast and slow myosins.

Age 224 days (fig 4)

The proportion of type I fibers, which express only slow myosins, remained practically stable at around 10%. The group of fibers that had previously expressed slow and fast myosins simultaneously was now clearly distinctive, as the reaction with antibody B was stronger. They also represented about 10% of the total but were larger and close in size to those of the type I fibers previously described. These fibers, which expressed simultaneously fast and slow myosins, correspond to type IIc fibers. The third group of fibers, which was by far the most numerous (about 80% of the total population), were fibers which now expressed very clearly a fast myosin but no slow myosin. The reaction with antibodies C and D became increasingly variable and yielded no additional information.

Age 270 days (fig 5)

The main observation was an increase in the size of the secondary fast fibers, which were now as large as types I and IIc. The reaction to antibodies C and D had become very weak.

Age 30 days after birth (fig 5)

The fibers were all larger at this age. A significant proportion of them were still synthesizing simultaneously fast and slow myosins, but none reacted to antibodies C and D.

Expression of different types of myosins

The results of electrophoresis and immunoblotting shown in figure 6 are presented in diagrammatic form in figure 7. The mus-

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Fig 2. Serial cryosections of semitendinosus muscle of an 84-d-old bovine fetus. The primary population of muscle cells was composed of large myotube-like cells, which all reacted with anti-slow (A84), anti-fast (B84), anti-fetal (C84) and anti-alpha cardiac (D84) myosin antibodies. White bar = 50 µ.

Fig 3. Serial cryosections of semitendinosus muscle of 122- and 143- d-old bovine fetuses. At 122 d of age, a population of large myotube-like cells indicated by S in the figure reacted strongly with anti-slow (A122) myosin antibody, but weakly with anti-fast (B122) antibody. A population of smaller cells identified by F on the figure reacted with anti-fast (B122) antibody. A similar pattern was observed at 143-d of age (A143 and B143) with a stronger contrast between the 2 populations of cells. In particular, larger cells (S) did not react at all with anti-fast. White bar = 50 µ.

Fig 4. Serial cryosections of semitendinosus muscle of 180- and 224- d-old bovine fetuses. At 180-d of age, the 2 populations of primary and secondary cells were clearly recognizable. The large primary fibers indicated by S reacted with anti-slow (A180) and not with anti-fast (B180) myosin antibody. The secondary smaller cells all reacted with anti-fast (B180), and a small proportion of them, indicated by S/F, also reacted weakly with anti-slow (A180). At 224-d of age, this population of cells indicated by S/F reacting simultaneously with anti-slow (A224) and anti-fast (B224) antibodies was well identified. White bar = 50 µ.
Fig 5. Cryosections of semitendinosus muscle of a 270 d-old fetus and of a 30-d-old newborn calf. The size of cells increased rapidly between these two stages of growth. At both stages, a small proportion of cells reacted with anti-slow (A270 and AP30) myosin antibody. Some indicated by S/F- also reacted with antifast (B270 and BP30) antibody, and correspond to the type IIC muscle fibers. White bar = 50 μ.
Fig 6. Characterization of different isoforms of myosin heavy chains in developing muscle of fetal and new-born calves. SDS–PAGE (s) and immunoblotting analyses with anti-slow (a), anti-fast (b) and anti-foetal (c) myosin antibodies. In the 4 figures, lanes 1–8 correspond respectively to muscle samples of 64, 84, 122, 143, 180, 224, 170 d of age, and 30 d after birth. Lanes C and M corresponding to adult cutaneous trunci and masseter are presented as controls. The interpretation of this analysis is shown in figure 7.
cle tissue expressed a slow myosin from the earliest ages onwards (64 d). The signal grew in intensity at about 180 days of fetal life and decreased after birth. The fetal myosin (recognized by antibody C), which migrated just above the slow myosin, was expressed throughout fetal life but was no longer detectable on the gels after birth. Fast myosins (types Ila and IIb) recognized by antibody B were only expressed from 143 days of gestation. Its signal increased at around 210 days and remained high thereafter.

Electrophoretic analysis showed a protein which migrates at the same molecular weight as type Ila myosin from 56 d to the end of the period analysed. However, this protein did not react with antibody B in immunoblotting analysis (fig 6).

**DISCUSSION**

The aim of this work was to analyse the temporal sequence of myogenesis during the development of fetal calves, and in particular to determine the various myosin heavy chain isoforms expressed in the different populations of muscle fibers, no published information being available in

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<th>F56/64</th>
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<td>Primary</td>
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| Type II b                                              |        |      |      |      |      |      |      |      |
| Type II a                                              | *****  | ***** | ***** | ***** | ***** |      |      |      |
| Foetal                                                 |        |      |      |      |      |      |      |      |
| Type I                                                 |        |      |      |      |      |      |      |      |

**Fig 7.** Characterization of different populations of muscle cells in developing fetal and newborn calves. Diagrammatic representation of the expression of myosin heavy chain isoforms identified by monoclonal antibodies on tissue slices (upper part) and with SDS-PAGE and immunoblotting analyses (lower part). In the upper part, the letters (A, B, C, D) indicate that populations of muscle cells identified on cryosections reacted with the corresponding myosin antibodies: A = anti-slow, B = anti-fast, C = anti-foetal, D = anti-alpha cardiac. In the lower part of the figure, the level of expression of each type of myosin is represented diagrammatically by the width of the drawing. Owing to the small amount of muscle, the first step of the development analysed by electrophoresis and represented in this figure was 64 d, whereas histological analysis began earlier. Fast type myosin was detected by immunoblotting after 180-d of age only. For this reason, the protein that migrated at the same molecular weight as type Ila myosin between 64 and 180 days was interpreted as an embryonic fast-type isoform, and is represented by a dotted line.
this species. The main points of discussion are: the number and nature of the myosin isoforms detected, the characterization of the muscle fiber populations observed, the chronology of their development during fetal growth, and the specificity of myogenesis in cattle in comparison with that of other species.

Expression of the different types of myosin

Our study shows the presence of 4 types of MHC during myogenesis identified by electrophoresis and immunoblotting, in the following increasing order of migration rate: 2 fast types which react with antibody B, a fetal type recognized by antibody C and a slow type recognized by antibody A. We also evidenced, by immunocytochemical analysis alone, a myosin that reacts with antibody D. It is likely that the myosin characterized by this antibody is an embryonic isoform, since it is only expressed in cells as they begin their differentiation. This embryonic myosin may share a common epitope with alpha cardiac myosin, as evidenced by the reaction in immunocytochemistry. This protein did not react in immunoblotting, possibly because of its low abundance. It was therefore not possible to situate it on the electrophoretic gel.

Electrophoretic analysis also showed another type of myosin that is expressed at the beginning of fetal life, and which migrates with the same molecular weight as type Ila myosin but without reacting with antibody B or any other antibodies. This fast-like myosin may also be an embryonic isoform of the fast myosin.

Adult slow myosin is expressed from the first stages of muscle development in cattle, as in the human fetus (Draeger et al, 1987), in cats (Hoh et al, 1988), in rats (LaFramboise et al, 1991), in mice (Lyons et al, 1990) and in chickens (Stockdale and Miller, 1987). All the above studies confirmed that this expression is not necessarily dependent on innervation, which is not fully developed at this stage of growth. However, it is now widely recognized that the last stage of myogenesis is dependent on environmental influence (see reviews of Stockdale, 1990; Hughes and Blau, 1992).

Adult fast myosin is expressed at about 180 d of gestation in cattle, thus, somewhat later than slow myosin. A similar sequence was observed in the human fetus by Pons et al (1986) and Draeger et al (1987), who reported that the fast myosin was not expressed until the appearance of the secondary generation, at = 100 d of gestation. In rats, the adult fast myosin is only expressed after birth (LaFramboise et al, 1991), at an even later stage than in humans.

Characterization of the fiber generations in developing muscle

Two cell populations, with very different cell sizes, called primary and secondary fibers, were first observed in mammal fetuses, in particular in the fetus of sheep, almost 20 years ago (Ashmore et al, 1972). In a previous study on cattle (Robelin et al, 1991), we obtained results with histochemistry that showed the differentiation of 2 populations of fibers ≈ 140 d of fetal life. In the present study, by characterizing the types of myosin synthesized by the cells, we have added to these results and shown that these fibers expressed different kinds of myosin during their differentiation programs.

During its development, the primary generation began by expressing slow myosins, then 2 fetal or embryonic myosins, and afterwards another embryonic fast-like myosin. This finding closely agrees with the results of Vivarelli et al (1988) in mice.
and those of Draeger et al (1987) in humans. In cattle, as in humans (Draeger et al, 1987), this primary population evolves during growth and expresses only a slow myosin at maturity.

The second generation, which appeared around the age of 120 d, differs from the primary in initially expressing only fetal and embryonic myosins. Later, it expresses a fast myosin and in some cells a fast and a slow myosin simultaneously. This second population is similar to that found in the 100-d-old human fetus (Pons et al, 1986; Draeger et al, 1987) or to that observed in 15–17 d-old rat fetuses (Rubinstein and Kelly, 1981). Its change into slow or fast types is probably dependent on innervation, as in other species.

**Chronology of myogenesis in cattle**

The synthesis of myosin in the bovine fetus begins very soon, before 39 d of age. Up to 122 d of gestation, we observed only 1 population of large myotubes, which successively expressed several types of myosins. These myotubes later transformed into type I fibers. This is practically the same development as that observed in humans (Pons et al, 1986; Draeger et al, 1987).

A secondary generation of smaller cells appeared at 122 d of gestation. Its pattern of expression of myosins was different from that of the primary population and it was made up of smaller cells, whose proportion increased rapidly until the end of fetal life. It is likely therefore that this secondary population is more directly involved in the increase in muscle fiber number observed during this period in cattle (Robelin et al, 1991). At a later stage of development, this second generation gave rise to 2 types of fibers, one of which expressed fast myosin and the other fast and slow myosins simultaneously.

At birth, we found the 4 muscle cell types commonly observed in other species: type I fibers (slow), types Ila and Iib (fast), and type IIC fibers (fast/slow). The distribution of the fiber types observed in this study was closely related to the kind of muscle we chose, semitendinosus, which is made up of ≈ 10, 30 and 60% type I, type Ila and type Iib fibers respectively in adult cattle (Holmes and Ashmore, 1972; Johnston et al, 1975; Robelin, unpublished data).

**Comparison of the temporal sequence of myogenesis in different species**

For the sake of simplicity, we compared only 4 animal species: chickens, rats, cattle and humans. There are considerable differences between these species in their adult size (a ratio of 1:1 000), and the length of their gestation period (from ≈ 20–280 d), and also variations in the extent to which weight at birth compares with that of the mature animal (< 1% in rats; = 2% in chicks; and between 4 and 6% in cattle and humans).

We used the results of various studies to compare myogenesis in these species: those of Crow and Stockdale (1984) on chickens, of Rubinstein and Kelly (1981), Lyons et al (1983) and LaFramboise et al (1991) on rats, and the findings of Pons et al (1986) and Draeger et al (1987) in humans. While the chronological periods varied greatly in length, there was, nevertheless, an overall similarity in the sequence of events. The first recognizable stage in myogenesis is signalled by the presence of tissue containing cells that synthesize myosin. It occurs at ≈ 4–6 d of gestation in chickens, 10–12 d in rats, and a little before 40 d in cattle. The following stage is marked in all the species by the appearance of a second cell population: at 8–12 d of gestation in chicks, 17 d in rats, and ≈ 100 d in humans and cattle. The third stage culminates in the maturation of the muscle fibers, by which time the fetal and embryonic myosins are no longer synthe-
sized or only in a very small number of cells. This takes place relatively late in post-natal life in chickens, at \( \approx 20 \) d in rats, and shortly after birth in cattle and humans. The muscle tissue of the last 2 species is thus more fully developed at birth than in chickens or rats. There is probably a link between this more advanced stage of development and the fact that the weight of newborn calves or humans is comparatively high in relation to that of the adult.

**CONCLUSION**

These results show that during myogenesis in cattle, and in mammals in general, 2 successive cell generations develop, which have different patterns of expression of myosin types. The first generation gives rise to type I fibers, while the second generation transforms into type II fibers (Ila, Ilb or IIc) in rapid muscles. Adult slow myosin is expressed early on in fetal life, whereas fast type is synthesized at a later time. The period of muscle differentiation in both cattle and humans is characterized by the expression of specific myosins, which are classically designated as embryonic or fetal. There are many similarities in myogenesis between cattle and humans, both in the sequence of events, which is practically the same, as in the number and characteristics of the cell populations involved, classically termed primary and secondary muscle fibers.

These findings should now be complemented by a study *in vitro* of the differentiation of myoblasts sampled at different stages in fetal life. A more detailed study should also be made of the precise role of these 2 generations in muscles with extreme characteristics, such as the masseter or cutaneous trunci, which in ruminants are composed of a single type of fiber, slow in the former and fast in the latter. Finally, cattle represent an interesting model for the study of the early stages of myogenesis because of the similarities in the temporal sequence with the fetal development of humans.

**ACKNOWLEDGMENTS**

The authors thank R Jailler and G Cuylle for animal management and slaughtering, and R Jailler for assistance in the dissection of muscles.

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