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Transcriptome Analysis of Two Bovine Muscles during Ontogenesis

Karine Sudre[1], Christine Leroux[1], Geneviève Piétu[4], Isabelle Cassar-Malek[1], Elisabeth Petit[2], Anne Listrat[1], Charles Auffray[3], Brigitte Picard[1], Patrice Martin[2] and Jean-François Hocquette[1*]

[1]INRA, Unité de Recherches sur les Herbivores, Theix, 63122 Saint-Genès-Champanelle, France; [2]INRA, Laboratoire de Génétique Biochimique et de Cytogénétique, 78352 Jouy-en-Josas, France; [3]Genexpress, CNRS, FRE 2571, 94801 Villejuif, France; and [4]Genexpress, CNRS, FRE 2571, 94801 Villejuif, France and present address CEA, Service de génomique fonctionnelle, 2 rue Gaston Crémieux, 91057 Evry Cedex, France

Macro-arrays, on which 1339 human skeletal muscle cDNA clone inserts had been spotted as PCR products, were used to make large-scale measurement of gene expression in bovine muscles during ontogenesis. Ten complex cDNA targets derived from two mixed muscle samples, *Rectus abdominis* (rather red oxidative muscle, RA) and *Semitendinosus* (rather white glycolytic muscle, ST), were taken from foetuses at 4 different stages (110, 180, 210, and 260 days post-conception) and from 15-month-old young bulls to generate differential expression patterns. Each sample analysed was prepared from a pool of RNA extracted from muscle tissues sampled from at least 6 different animals. Approximately 200 expression signals were validated and taken into account to provide a first "bovine" muscle gene repertoire. Despite the relatively small number of probes and the heterologous approach, this made it possible to identify up to 7 genes differentially expressed between RA and ST, depending on age. From 110 days post-conception to 15 months of age, differences in the expression levels of 110 genes were detected in the four comparisons between two consecutive ages. By comparing 260 days post-conception foetal muscles and adult muscles, up to 87 genes were overexpressed, whereas only 7 genes were shown to be down-regulated. Among these genes, 33% have unknown biological functions. Taken together, the results reported here underline the importance of the last three months of gestation in muscle myogenesis, and highlight new genes involved in this process.

Key words: bovine, macro-array, muscle, ontogenesis.

Abbreviations: PCR, polymerase chain reaction; RA, *Rectus abdominis* muscle; ST, *Semitendinosus* muscle.

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Although the main functions of skeletal muscle are postural support and movement, it also has a major role as a metabolic organ with consequent implications for meat quality in farm animals (*1*). Skeletal muscle is composed of heterogeneous fibre types that vary in contraction velocity, endurance capacity, and metabolic enzyme profile. According to their major fibre type content, muscles can be classified as red or white. Red muscles have the most aerobic oxidative metabolism, because of their higher percentage of capillaries, myoglobin, and mitochondria (*2*). Thus, red muscles, which are rich in type I fibres, are better adapted to long-duration activities, such as postural maintenance. In contrast, white muscles have a contractile apparatus tailored for higher velocity contractions and require anaerobic glycolytic metabolism to support a high transient energy demand (*2*).

The muscle metabolic profile is a determinant for meat quality in meat-producing animals (*3*). Glycolytic metabolism is associated with a greater glycogen content, which is implicated in meat ageing after slaughter, and hence tenderness. Glycolytic fibres generally contain less intramuscular fat. Such a feature is prejudicial for flavour. In contrast, oxidative metabolism is associated with higher contents of intramuscular lipids and of myoglobin, which is responsible for meat colour (*3*). Consequently, a global gene expression profiling of red *versus* white muscles should provide a better understanding of the gene regulation that underlies the metabolic and contractile differences between muscle fibre types (*4*) and controls muscle characteristics, which determine meat quality.

There is a great deal of literature indicating that the development of muscle properties depends to a large extent on foetal events. As muscle energy metabolism regulates whole-body fuel homeostasis (*5*), changes in metabolic traits before birth also influence muscle metabolic characteristics. These changes can thus play a role in meat quality traits. Among factors influencing such traits, the level of maternal nutrition at critical periods of foetal life has long term consequences for postnatal life. For instance, experimentally triggered under-nutrition during pregnancy has been reported to induce a decrease in total muscle fibre number in various mammalian species (*6*). Conversely, a high nutrition level either increases or expands myoblast proliferation (*7*). Furthermore, muscle mass and therefore body composition depend also on nutritional supply to foetuses. Thus, meat producers could satisfy the consumer's requirement for a consistently satisfactory product not only by optimising breeding factors during growth and before slaughter (*1*, *3*), but also by optimising muscle development during foetal life (*8*).

*To whom corresponding should be addressed. Tel: +33-4-73-62-42-53, Fax: +33-4-73-62-46-39, E-mail: hocquet@clermont.inra.fr

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Table 1. **Characteristics of the 1339 human muscle cDNA probes printed onto nylon filters.**

Characteristics	Numbers	Proportion of the total
With no information of sequence	264	19.70%
With information of sequence	1075	80.30%
Including:		
Expressed Sequence Tags (with no information of function)	257	19.20%
Titin	48	3.60%
Other proteins involved in contractile elements	70	5.20%
Proteins involved in transcription and hormonal transduction	122	9.10%
Proteins involved in cell regulation	73	5.50%
Proteins involved in differentiation	33	2.50%
Proteins involved in metabolism	74	5.50%
Proteins involved in proteolysis	24	1.80%

Muscle ontogenesis has been extensively described using biochemical and morphological approaches, but an overall view of mechanisms involved in its regulation is still lacking. Until now, only few genes involved in myogenesis have been studied simultaneously. Today, with the development of new molecular techniques (macro- and micro-array technologies) allowing large-scale gene expression measurement (*9*) in various physiological or pathological conditions, it is appropriate to consider the functional genomic tools for the assessment of gene expression during myogenesis.

The objective of this work was to acquire a better overall understanding of the evolution of gene expression during myogenesis of two skeletal muscles, differing in their contractile and metabolic properties, and having a commercial value after their conversion into meat. Unfortunately, the recent sequences arising from four pooled-tissue bovine cDNA libraries (*10*) were not available at the beginning of our study, and resources of bovine-specific expressed sequence data focused on muscle physiology were still lacking. Consequently, we started to perform gene expression profiling studies in bovine muscles by using cDNA macro-arrays comprising 1339 printed PCR products representing muscle-specific human cDNA clones (*11*). It was anticipated that transcriptome analysis of bovine muscle could be conducted using human filters, and that the resulting data would be useful to characterise different muscle types at various ages to identify markers, which in turn could be used to improve the quality of meat. Samples of two bovine muscles, *Rectus abdominis* (red oxidative muscle, RA) and *Semitendinosus* (white glycolytic muscle, ST) (*12*) were therefore analysed.

MATERIALS AND METHODS

Animals and Muscle Samples—Forty-eight Charolais foetuses were obtained by artificial insemination of Charolais cows with Charolais bull sperm. Sixteen double-muscled foetuses were obtained by transplantation of frozen embryos of strain INRA95 (synthetic strain composed of a mixture of breeds with muscle hypertrophy). Retained embryos contained around 75% of Charolais breed. Muscle samples were obtained from foetuses at various ages: 110, 180, 210 and 260 days post-conception (d.p.c.). Two carcass muscles differing in their adult contractile and metabolic characteristics were excised from each foetus: *Rectus abdominis* and *Semitendinosus*. Twelve 15-month-old young bulls of the same breed (*i.e.*, Charolais) were sampled. Muscle samples were immediately frozen in liquid nitrogen and stored at –80°C until analysed.

Target Preparation and Labelling—Total RNA was extracted using RNAble™ (Eurobio, Les Ulis, France) reagent followed by chloroform and acid-phenol extractions to remove traces of DNA. Total RNA from at least six animals was pooled to obtain one sample per muscle and per age, with identical proportions of each group of animals in all samples. This procedure resulted in 10 representative samples of total RNA (*i.e.*, from RA and ST at five ages). Poly(A)+ RNA (mRNA) was isolated from the 10 samples using Dynabeads™ mRNA Purification Kit (DYNAL, Oslo, Norway) following the manufacturer's protocol.

Poly(A)+ RNA (500 ng) was reverse-transcribed using Superscript II reverse transcriptase (RNAse H) (Bethesda Research Laboratories, Beverly, MA, USA) as described in the manufacturer's protocol, using random hexamers for priming (*13*).

Labelling was performed during reverse transcription by incorporating 1.85×10^6 Bq of [α-^{33}P]dATP (1.11×10^{14} Bq/mmol) (Amersham International, Bucks, UK). Unincorporated radioactive nucleotides were removed from the labelled target by filtration on a Quick Spin Column (Boehringer Mannheim, Mannheim, Germany) according to the instruction manual. On average, 80% of the radioactive label was incorporated into cDNA, and the specific activity of the targets ranged from 40 to 70×10^6 cpm/μg.

Hybridisation—High density filters were prepared as described (*11*) from a human muscle cDNA library. As indicated in Table 1, these filters contain: (i) cDNA with sequences similar to known sequences allowing putative identification of the corresponding genes and (ii) cDNA with sequences unrelated to any known sequences, referred to as unknown genes. However, constant updating of the public nucleic data bases may improve the information available for each cDNA.

Four filters were prehybridised in glass tubes in a hybridisation oven (Appligène, Illkirch, France) in a volume of 20 ml of ExpressHyb™ hybridisation solution (Clontech, Palo Alto, USA) overnight at 68°C. Hybridisation of filters was performed for 2 h at 68°C in the same solution with ^{33}P-radiolabelled targets. The membranes were washed twice for 30 min in 2 × SSC/0.1% SDS at room temperature and again twice for 30 min in 0.1 × SSC/0.1% SDS at 42°C. Exposure to phosphor screens lasted 15 to 18 h.

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Table 2. **Classification of hybridisation intensities between unvalid values, values lower than the background and values kept for the analysis.** Among the latter, values exceeding twice the background are indicated. Results for each age from 110 days post-conception (dpc) to 15 months (mo) are the means of analysis of *Rectus abdominis* and *Semitendinosus* muscle samples.

	Unvalid and lower than the background*	% of the 1339 cDNAs	Valid intensities kept for analysis	% of the 1339 cDNAs	Above 2-fold the background	% of the valid intensities
110 d.p.c.	1034	77.3	305	22.7	243	79.6
180 d.p.c.	857	64.0	482	36.0	286	59.3
210 d.p.c.	996	74.4	343	25.6	285	83.1
260 d.p.c.	758	56.6	581	43.4	289	49.7
15 mo	1050	78.4	289	21.6	247	85.3
Means	911	**68.1**	427	**31.9**	275	**64.4**

*Background is at 0.8 value of nIm for samples taken at 110 days post-conception, 0.7 value of nIm for samples from 15-month-old bulls and 0.6 value of nIm for samples taken at 180, 210 and 260 days post-conception.

Dehybridisation of membranes was performed by two successive immersions in a solution of 0.4 M NaOH, 0.1% SDS at 65°C for 15 min. Membranes were rinsed twice in 0.2 M Tris-HCl (pH 8.0) for 10 min at room temperature. They were hybridised a maximum of five to six times.

Hybridisation Signal Detection and Quantification—Acquisition and analysis of hybridisation intensities were performed as previously described (*14*). Different types of values were obtained for quantification of the dot intensity (*11*). We chose to use the mean of pixel intensities for each dot after subtraction of non-specific signals in a local correction mode, noted as Im. To take into account experimental variations in specific activity of cDNA target preparations or exposure time that might alter the signal intensity, data from different hybridisations were normalised by dividing the Im for each dot by the average of the intensities of all the dots to obtain a normalised Im value (nIm).

Hybridisation Signal Analysis—For each set of four results from the same sample, all values outside the range corresponding to the mean ± 25% were omitted. Results for one sample were considered as valid when at least three repetitions were within the range of mean ± 25% (*14*). In agreement with previous experiments (*11*), only 8% of the results were discarded following this validation procedure. The mean hybridisation signal intensity tended to slightly increase after four hybridisation/dehybridisation cycles with the same filter. This bias was cancelled by the normalisation step. In addition, the population of the lowest intensities (nIm between 0 and two-fold the median value) was compared to the theoretical Normal Distribution (*11*) using the UNIVARIATE procedure of SAS (SAS 1987).

Results were analysed as nIm values minus the background. Background was equal to the hybridisation signal intensity obtained with buffer dotted on the Nylon filter without any cDNA probe. It varied from 0.6 to 0.8 according to the experiment, whereas nIm varied up to 6 or even 8 for the highest values. Background was subtracted from each nIm, resulting in cIm (corrected nIm). About 21 to 44% of the cIm were above the background and, hence, considered to represent positive signals (Table 2). The remaining cIm were considered not to differ significantly from the background. Among the valid cIm, 49 to 85% were above twice the background level. Only a minority showed high values (less than 8% with values higher than sevenfold the background level, Table 2).

The mean membrane-to-membrane reproducibility was then calculated for two different sets of hybridisation, namely, hybridisation of RA sample and of ST sample from 180-day-old foetuses. The mean standard deviation for hybridisation signal intensities above the background (cIm) was equal to 0.25 in both samples. The smallest significant difference between two samples was calculated from the t test formula, taking into account the above standard deviation: it is equal to 0.50 on average at $p < 0.05$ (*i.e.* two standard deviations) and 0.76 at $p < 0.01$. We decided to consider all cIm below 0.76 not to differ significantly from the background ($p < 0.01$) due to high variability of low cIm values. Similarly, between 0 and 1.5–2 times the background, a minimum absolute difference of 0.5 was required to declare with $p < 0.05$ the difference in expression between two samples for one gene. Above 2.5–3 times the background, we considered all the genes with a 1.5-fold ratio in cIm between two samples as significant, which ensured a minimum of 0.5 absolute difference.

Bovine Probe Preparations—For some genes which were declared differentially expressed, bovine cDNA probes were prepared with specific primers. The human cDNA sequences spotted on the macro-arrays were blasted against sequences present in GenBank to check the identity of the genes, and specific primers of these genes were designed from bovine sequences when the latter were available. First-strand cDNA was synthetised from 2.5μg of total RNA extracted from bovine muscles, using a Superscript™ II RNAse H$^-$ reverse transcriptase (Gibco BRL, Life Technologies, Bethesda, MD, USA) and oligo-dT primers. PCR reactions primed by specific synthetic primers were performed using Taq polymerase (Promega, France) on cDNA generated by reverse transcription. The resulting PCR fragments were analysed on agarose gels and purified with a Quiaquick purification kit (Qiagen S.A., Courtaboeuf, France) in order to remove unused primers. Low quality probes were not used. For some genes declared differentially expressed, bovine cDNA probes from a bovine cDNA library currently *in preparation* (Sudre *et al*., in preparation) were used. To prepare this library, messenger RNA from various bovine muscles was first reverse transcribed. The resulting cDNAs was cloned in a pUC18 vector, amplified and sequenced. Clones showing similar identities to the human cDNA of interest present on human high density filters were used as cDNA probes in Northern blot analyses.

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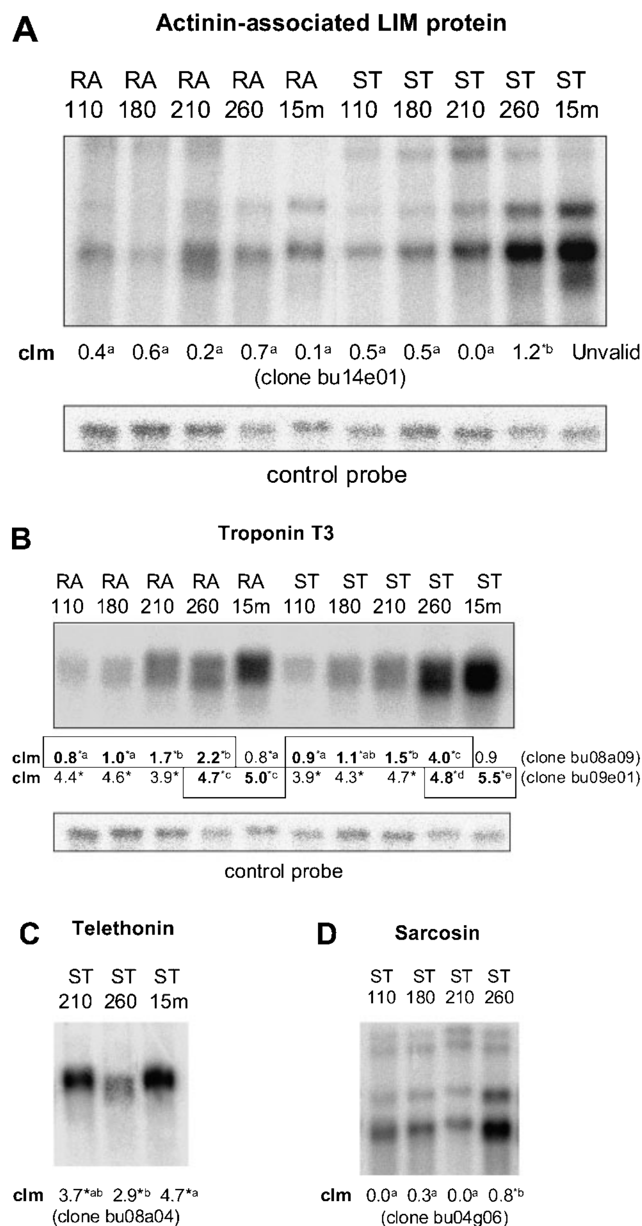

Fig. 1. **Northern blot analysis.** Northern blot analysis of poly(A)+ RNA (500 ng) from bovine muscle samples hybridised with cDNA fragments corresponding to the differentially expressed genes detected by using macro-arrays. A/ actinin-associated LIM protein in the two muscles, B/ the two different clones of troponin T3 (foetal isoform: clone bu08a09 ; adult isoform: clone bu09e01), C/ telethonin and D/ sarcosin. Two different Northern-blot analyses were carried out for each clone and the results were compared to the results observed with a control probe from the human muscle library. Values of hybridisation signal intensities above the background level (cIm) from macro-array experiments were indicated under Northern-blot figures. * indicates values which significantly differ from the background at $p < 0.01$. Two values with different superscripts were declared significantly different at $p < 0.05$.

Northern Blot Analysis—The profiles of expression for four genes, identified as differentially expressed, were checked by Northern blot analysis after extraction of poly(A)+ RNA as described above. The quality (integrity and amount) of the poly(A)+ RNA samples was carefully checked by analysing electrophoregrams obtained using the Agilent 2100 Bioanalyser. The poly(A)+ RNA samples (500 ng) were denatured in 1.2 M formaldehyde and 50% formamide (10 min, 65°C), then size-separated by electrophoresis in agarose (1%) and 2.15 M formaldehyde dissolved in electrophoresis buffer (sodium phosphate 10 mM, EDTA 1 mM, sodium acetate 5 mM, pH 7.0). Migration was performed at constant voltage for 15 min at 110 V, then for 3 h at 80 V at room temperature. RNA was transferred to GeneScreen membranes (NEN Life Science Products, Boston, MA, USA) by capillary blotting overnight under high ionic strength (10 × SSC = 1.5 M sodium chloride, 0.15 M sodium citrate). Transfer was checked by assuming its completion was achieved after total disappearance of ethidium bromide on the gel.

The bovine cDNA probes were labelled using a random priming DNA labelling kit (Roche, Mannheim, Germany), according to the manufacturer's protocol, with a [^{32}P]dCTP (3000 Ci/mmol; ICN, Orsay, France). Hybridisations were performed overnight at 42°C using Ultrasensitive Hybridisation Buffer (ULTRAhyb™ Ambion, Clinisciences, France), according to the manufacturer's protocol. Membranes were washed twice for 5 min in 2 × SSC, 0.1% SDS at 42°C. Membranes were exposed to PhosphorImager screens overnight, and signals were detected using a Storm Imager and Image-Quant software (Molecular Dynamics, Sunnyvale, CA).

Analysis of the poly(A)+ RNA samples with the high sensitivity Agilent 2100 BioAnalyser system was shown to be the best procedure to verify intactness and to quantify the mRNA. One cDNA probe from the human muscle library was shown to hybridise at very similar levels with all samples and was therefore used as an internal control probe (Fig. 1, A and B).

RESULTS

Analysis of the Hybridisation Signals—One major difficulty of this work was the molecular hybridisation of nucleic acids in a heterologous system. Radiolabelled cDNAs transcribed from bovine skeletal muscle poly(A)+ RNA were hybridised against human skeletal muscle cDNA clones. Generally, working in a heterologous system requires low stringent conditions for hybridisation and washing because of the weaker bonds between-non homologous cDNA. In addition, one of the causes of false signals is non-specific hybridisation of labelled targets with probes containing a long poly(A) tail (*15*). To avoid this bias, relatively high stringent conditions for washing and hybridisation were necessary. Therefore, we conducted preliminary experiments with different washing temperatures after hybridisation. Four temperatures, 27, 32, 42, and 52°C, were tested on a set of four filters hybridised with two of our complex targets (one for RA and one for ST). For each of these temperatures, signal hybridisation was analysed and the distributions of hybridisation signals (Fig. 2) were tested for normality. Based on statistical analysis, we observed that the distribution of the lowest intensities (which are considered to represent clones that do not hybridise significantly with signal intensities above the background) was the closest between RA and ST samples and close to the normal distribution at a washing temperature of 42°C.

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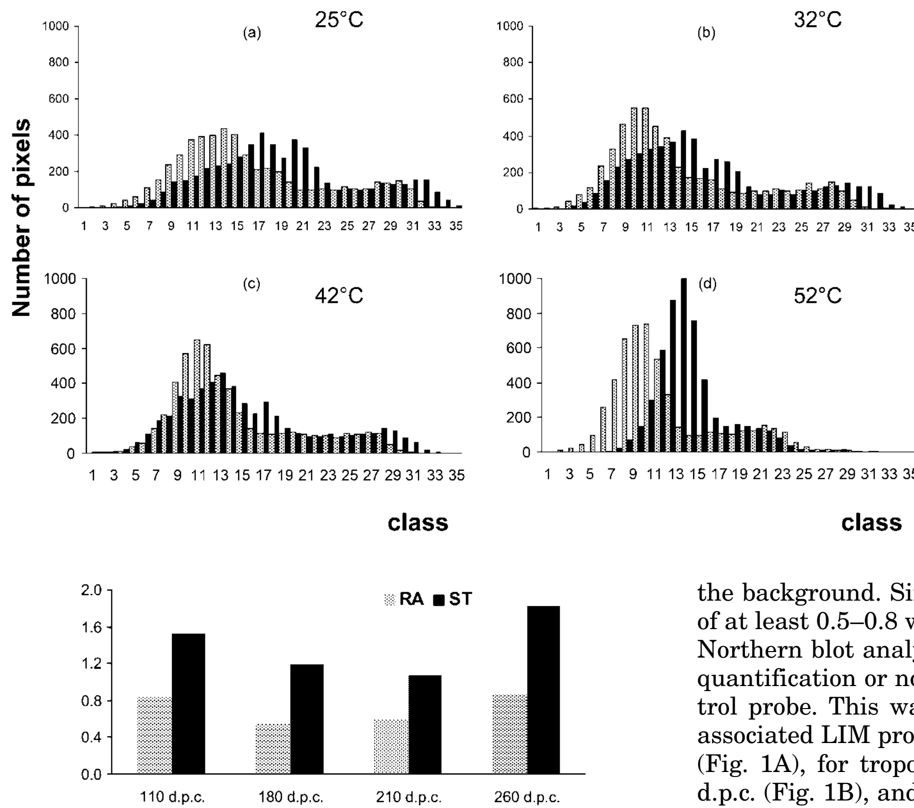

Fig. 2. **Distribution of hybridisation signals according to washing temperatures.** Numbers of hybridisation signals are represented in each of 35 classes of intensity for: 25 (a), 32 (b), 42 (c) and 52 (d) °C. gray: in ST, dark: in RA.

Fig. 3. **Hybridisation intensity values (cIm) obtained for LEU5 cDNA.**

Therefore, we decided to wash filters at 42°C in the subsequent experiments, thus allowing comparisons between membranes under similar background level conditions. This choice is also the best compromise between the low stringency required by the heterologous system and the high stringency required to prevent non-specific hybridisation.

Generally, transcripts of genes with cIm lower than 0.8 from macro-array experiments were barely detectable by Northern blot analysis, as shown for sarcosin in ST (Fig. 1D). However, Northern blotting may be either more or less sensitive than macro-arrays depending on the gene. But, on average, the results confirm our calculation that cIm lower than 0.76 were not significantly different from the background. Similarly, an absolute difference in cIm of at least 0.5–0.8 was necessary to be easily observed by Northern blot analysis with confidence, but without any quantification or normalisation of the signals to the control probe. This was the case, for instance, for actinin-associated LIM protein in ST between 180 and 260 d.p.c. (Fig. 1A), for troponin T3 in RA between 180 and 210 d.p.c. (Fig. 1B), and for telethonin (Fig. 1C) and sarcosin (Fig. 1D) in ST between 210 and 260 d.p.c. This fits well with the smallest significant difference of 0.5 in cIm between two samples calculated for macro-array experiments taking into account additional parameters such as three to four replications, and precise quantification of hybridisation signals by image analysis and normalisation.

Among the 1339 probes printed on macro-arrays, 694 probes gave a signal in at least one muscle at one stage. Finally, 197 probes were kept for RA *versus* ST comparison and 204 for kinetic analysis in the two muscles.

Comparison of Gene Expression Patterns between RA and ST Muscles—In this study, comparison of gene expression between RA and ST samples showed only few significant differences for the five stages considered (Table 3). For all stages studied, only one gene, which encodes tumour suppressor LEU5, was found systematically more expressed in ST than in RA (Fig. 3). The other

Table 3. **Genes found differentially expressed between *Rectus abdominis* (RA) and *Semitendinosus* (ST) muscles at the various stages of development.** Ratios of expression in RA to ST are indicated. (A) Genes more expressed in RA than in ST. (B) Genes more expressed in ST than in RA.

Correspondence	cDNA clone	Ratio	Stages (d.p.c.)
(A) Genes more expressed in RA than in ST			
Nebulin	bu02g10	1.72	260
unknown gene	bu03d10	1.63	210
Trip15 (thyroid receptor interacting protein 15)	bu01a01	1.61	260
(B) Genes more expressed in ST than in RA			
LEU5 (candidate tumour suppressor)	bu04g08	0.55, 0.46 & 0.47	110, 180 & 260
unknown gene	bu09a10	0.43	210
Troponin T3	bu08a09	0.56	260
unknown gene	bu01f05	0.40	260
unknown gene	bu04h11	0.61 & 0.53	180 & 260
Actinin-associated LIM protein	bu14e01	0.56	260

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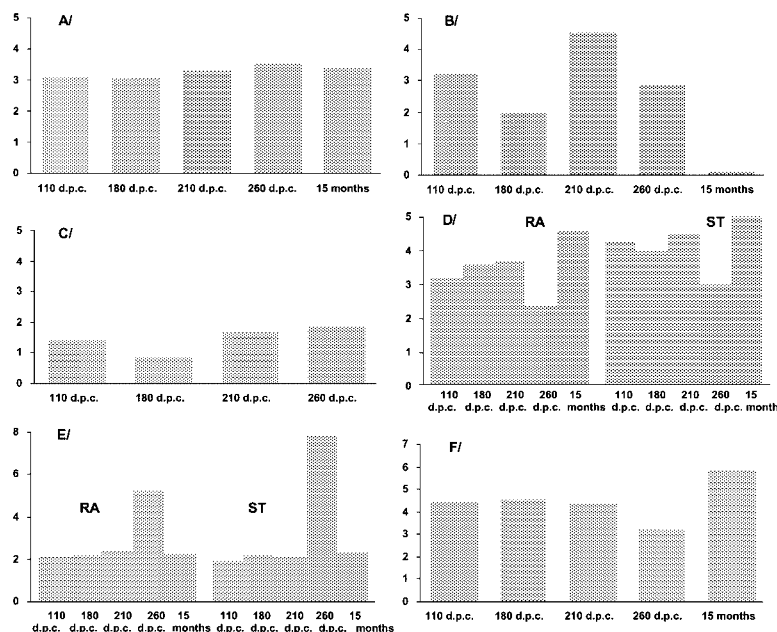

Fig. 4. **Hybridisation values (cIm) obtained for cDNA of genes representative of the five groups of variation revealed by our study.** Tropomyosin 1 (A) represents here genes with no variation of expression in RA during the period studied. Unknown gene (bu10a01) (B) was regulated during the whole period in RA. Oxoglutarate dehydrogenase (C) was strongly regulated around 180 d.p.c. in ST. Ras inhibitor (D) and subunit non ATPase 4 of proteasome 26S (E) were under- and over-expressed at 260 d.p.c., respectively in both muscles. Transcriptional activator hSNF2b (F) was regulated around birth in ST.

significant differences between RA and ST were observed mostly at 260 d.p.c. For example, nebulin and Trip15 (thyroid receptor interacting protein 15) were more expressed in RA than in ST. Conversely, actinin-associated LIM protein and foetal fast troponin T3 (clone bu08a09) were more expressed in ST than in RA at this stage (1.2 *vs* 0.7 cIm and 4.0 *vs* 2.2 cIm, respectively). These latter results were confirmed by Northern blot analysis (Table 3 and Fig. 1, A and B). Finally, the expression of genes involved in muscle metabolic (phosphofructokinase 2, aldolase A, dihydrolipoamide dehydrogenase and carnitine palmitoyl transferase complex 1) or contractile (tropomyosin 1, centractin alpha, α–actinin 2 and desmin) traits did not significantly differ between the two studied muscles.

Within Muscle Type Comparison of Gene Expression at Various Developmental Stages—Gene expression with valid cIm above the background was analysed step-by-step for two successive developmental stages (Table 4).

When kinetics of expression were analysed from 110 d.p.c. to the age of 15 months, genes could be classified into five major groups according to their expression pattern: (i) genes with no significant variations in their expression (Fig. 4A), (ii) genes up- or down-regulated during the whole studied period, at least in one muscle (Fig. 4B), (iii) genes highly regulated around 180 d.p.c. (Fig. 4C), (iv) genes highly regulated around 260 d.p.c. (Fig. 4, D and E) and (v) genes showing a differential expression between foetal life and 15 months of age (Fig. 4F). In the first group, 4 genes were listed encoding a tumour necrosis factor alpha-inducible cellular protein containing leucine zipper domains, subunit 4 of eukaryotic translation initiation factor 3, tropomyosin-1 and an uncharacterized protein (Table 4). The only gene belonging to the second group (bu10a01) was regulated in RA at each stage and also encodes an uncharacterised protein (Table 4). Five genes were particularly regulated around 180 d.p.c., among which those encoding α2,8-sialyltransferase in both muscles and oxoglutarate dehydrogenase in ST (Table 4). The two last groups are larger. Sixty-one genes were found highly regulated around 260 d.p.c., especially in ST (43 genes); and 89 genes were found differentially expressed between foetal life and 15 months of age, of which 84 were found overexpressed after birth, especially in ST (52 genes). Despite quantitative differences between these five groups of genes, each group had a physiological significance with regard to myogenesis.

Thirteen genes were found differentially expressed between 110 and 180 d.p.c., of which 5 were underexpressed at 180 d.p.c. and eight overexpressed at this stage. Among them, 4 genes were found differentially expressed in RA, 7 in ST and two in both RA and ST (data not shown). These latter two genes encode the α2,8-sialyltransferase and a protein of unknown function (bu10a01) (Table 4). Genes encoding semaphorin 6A and oxoglutarate dehydrogenase were found 1.5- and 1.7-fold less expressed in RA and in ST at 180 d.p.c. than at 110 d.p.c., respectively. Conversely, an unknown gene (bu06b01) and Trip15 were found 1.6- and 2.5-fold more expressed at 180 d.p.c. than at 110 d.p.c. in RA and in ST, respectively. Taken together, all these results indicate small differences in overall expression profiles between 110 and 180 days of gestation.

Eleven genes were found differentially expressed between 180 and 210 d.p.c., of which 5 genes were less expressed and 6 more expressed at 210 d.p.c.. Among them, 6 were found differentially expressed in RA and 5 in both muscles. The genes encoding a protein of unknown function (bu10a01) and α2,8-sialyltransferase were again differentially expressed in both muscles (Table 4). Oxoglutarate dehydrogenase was more expressed at 210 d.p.c. by 4.3-fold in RA and 2-fold in ST (Table 4). Moreover, 3 other genes were found affected in RA only: those encoding fœtal troponin T3 and semaphorin 6A were also found more expressed at 210 d.p.c., whereas a gene localised on chromosome 2 encoding a protein of unknown function (bu13g11) was found 1.75-fold less expressed at this stage (Table 4). Northern blot analysis

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Table 4. **Examples of genes found differentially expressed between two consecutive developmental stages for the two studied muscles: *Rectus abdominis* (RA) and *Semitendinosus* (ST).** Typical ratios of RA to ST for hybridisation intensity values corrected for the background are indicated. When the expression differs in both muscles, the mean of the ratio is represented.

Groups	Address	Names	180/110 d.p.c.				210/180 d.p.c.				260/210d.p.c.				15m/260d.p.c.			
			ratio	RA & ST	only in RA	only in ST	ratio	RA & ST	only in RA	only in ST	ratio	RA & ST	only in RA	only in ST	ratio	RA & ST	only in RA	only in ST
Always regulated	bu10a01	unknown gene	0.61	x			1.90	x			0.63		x		0.03		x	
Regulated around 180 d.p.c.	bu05c01	sialyltransferase 8	0.62	x			1.83	x										
	bu12g12	unknown gene	0.55		x		4.07		x									
	bu09b04	Superoxide dismutase 2	1.54			x	0.49			x								
	bu09b06	EST	1.65			x	0.54			x								
	bu10d02	semaphorin 6A	0.66		x		1.71		x									
	bu13d03	oxoglutarate dehydrogenase (lipoamide)	0.59			x	1.99			x								
Regulated between 110 and 180 d.p.c. only	bu04c03	unknown gene	4.84			x												
	bu01a01	Trip15	1.60			x												
	bu03f12	Kelch-like ECH-associated protein 1	1.68			x												
	bu09a10	unknown gene	0.43			x												
	bu06b01	unknown gene	2.47		x													
	bu08a08	nucleoporin	5.14		x													
	bu02g12	laminin receptor I	1.75		x													
Regulated between 180 and 210 d.p.c. only	bu08a09	troponin T3					1.69		x									
	bu13d03	oxoglutarate dehydrogenase (lipoamide)					3.18	x										
	bu03g01	unknown gene					0.57		x									
	bu13g11	gene on chromosome 2 with unknown function					0.57		x									
Regulated around 260 d.p.c.	bu02b01	NADH-ubiquinone oxidoreductase NDUFS2 subunit									0.60	x			1.74	x		
	bu04c12	eukaryotic translation initiation factor 4γ2 (p97)									0.64	x			1.80	x		
	bu05c12	ras inhibitor									0.66	x			1.81	x		
	bu05f04	proteasome 26S subunit non-ATPase 4									2.89	x			0.37	x		
	bu10b06	dolichol-phosphate-mannose synthase									0.60	x			1.84	x		
	bu13b02	nebulin									0.60	x			1.87	x		
	bu08g01	NADH dehydrogenase 1b subcomplex									0.58		x		1.74		x	
	bu09b01	helicase II (RAD54L)									0.65		x		1.80		x	
	bu10b11	CPT I (carnitine palmitoyl transferase I)									0.61		x		1.55		x	
	bu14e01	actinin-associated LIM protein									4.13		x		0.10		x	
	bu02a11	PBX/knotted 1 homeobox 1, homeobox-containing protein									0.54			x	2.11			x
	bu02c05	precursor of P100 serine protease									0.52			x	2.10			x
	bu02g10	nebulin									0.47			x	2.52			x
	bu04d07	INE1 (inactivation escape I)									0.63			x	1.62			x
	bu04g01	microtubule-associated protein, RP/EB family, member 3 (MAPRE3)									0.66			x	1.83			x
	bu04g03	heat shock 90kD protein 1, alpha									0.59			x	1.90			x
	bu04g09	activin A receptor type I									0.48			x	2.48			x
	bu04h09	carbonic anhydrase III									1.57			x	2.05			x
	bu05e02	ubiquitin protein ligase E3A									0.65			x	1.79			x
	bu05e07	NADH dehydrogenase 1a subcomplex 4 (9kD, MLRQ) (NDUFA4)									0.59			x	1.60			x
	bu07c06	COQ 3									0.54			x	2.10			x
	bu07f04	clone YR-29									0.58			x	1.74			x
	bu07f07	proteasome subunit b type 7									0.66			x	1.69			x
	bu08a09	troponin T3, skeletal, fast									0.35			x	0.22			x
	bu08b07	ubiquinol cytochrome-c reductase core I protein									0.54			x	1.94			x
	bu08d05	TRC8 (multiple membrane spanning receptor)									0.63			x	1.80			x
	bu08h02	small zinc finger-like protein									0.66			x	1.73			x
	bu08h04	cationic amino acid transporter, y+ system									0.51			x	1.92			x
	bu09b08	myomesin I									0.64			x	1.90			x
	bu09c01	protein kinase inhibitor a									0.67			x	1.97			x
	bu09e05	peroxisomal antioxydant enzyme									0.59			x	1.69			x
	bu10b03	DNA-dependent protein kinase catalytic subunit (DNA-PKcs)									0.64			x	1.91			x
	bu11a03	dihydrolipoamide dehydrogenase									0.61			x	1.65			x
	bu11d04	M phase phosphoprotein 10 (U3 small nucleolar ribonucleoprotein)									0.62			x	1.56			x
	bu13b10	DNA bp A									0.62			x	0.60			x
	bu14e12	chromosome 16									0.59			x	1.61			x

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Table 4. **(Continued)**

Groups	Address	Names	260/210 d.p.c.				15m/260 d.p.c.			
			ratio	RA & ST	only in RA	only in ST	ratio	RA & ST	only in RA	only in ST
Regulated between 210 and 260 d.p.c. only	bu08d02	EST	10.65	x						
	bu06b07	nebulin-related anchoring protein (N-RAP)	8.10	x						
	bu01e01	tropomyosin-1 (TM-β)	10.20	x						
	bu02g01	heat shock 90kD protein 1α	5.85	x						
	bu01a01	Trip15	0.56	x						
	bu04g08	LEU5	1.69			x				
	bu02e01	TNFα-inducible cellular protein	5.00			x				
	bu11f01	tristetraproline (TTP) (zinc finger protein)	0.54			x				
	bu05b11	mhc IIa	0.56			x				
	bu02c09	myoglobin	0.59			x				
	bu01c05	mortalin II	0.62			x				
	bu10c05	proteasome subunit beta type 4	0.67			x				
	bu13g11	unknown gene	1.57		x					
Regulated between the age of 15 months and the foetal life	bu03a09	thymus-specific serine peptidase					1.58	x		
	bu03c01	titin					1.65	x		
	bu03d01	RAS-related protein R-RAS2					1.59	x		
	bu04a09	proteasome 26S subunit non-ATPase 2 (p97)					1.66	x		
	bu04b11	BTB (POZ) domain containing 1 (BTBD1)					1.58	x		
	bu08b10	centractin α					1.80	x		
	bu08b12	eukaryotic translation initiation factor 3 subunit 5					1.77	x		
	bu08d12	ribosomal protein L15					1.62	x		
	bu08h10	transcriptional activator hSNF2b					1.72	x		
	bu09f07	DNA-directed RNA polymerase II					1.57	x		
	bu10a06	dolichol-phosphate-mannose synthase					1.74	x		
	bu04a05	unknown gene					0.33		x	
	bu04h09	carbonic anhydrase III					1.57		x	
	bu07f08	GDP dissociation inhibitor 2					1.69		x	
	bu08a09	troponin T3, skeletal, fast					0.35		x	
	bu01c04	sterile 20					1.75			x
	bu01h03	NEK3 (serine/threonine protein kinase)					1.75			x
	bu02a05	MAP kinase kinase 6 (MKK6)					1.78			x
	bu02c04	LIM-protein FHL1					1.74			x
	bu02g07	inducible 6-phosphofructo-2-kinase/fructose 2,6-bisphosphatase (IPFK-2)					1.59			x
	bu02h07	proteasome subunit β type 4, β chain precursor					1.68			x
	bu02h10	B-cell receptor associated protein (BAP)					1.59			x
	bu03a08	motor protein					1.51			x
	bu03f11	LIM-protein FHL3					1.62			x
	bu03g07	nascent-polypeptide-associated complex alpha polypeptide (αNAC)					1.90			x
	bu04e01	ribosomal protein L4					1.75			x
	bu05b03	proteasome 26S subunit non-ATPase 1 (p112)					1.67			x
	bu05c02	phosphatidylethanolamine bp					1.82			x
	bu05g11	CPT 1					1.68			x
	bu05h03	NADH dehydrogenase Fe-S protein 3 (NADH-coenzyme Q reductase)					1.66			x
	bu06a03	calpain, skeletal muscle-specific, large polypeptide L3					1.89			x
	bu06b01	unknown gene					0.17			x
	bu07b03	stress-associated endoplasmic reticulum protein 1 (SERP1)					1.59			x
	bu07c05	kinase A (PRKA) anchor protein 1					1.68			x
	bu08a04	telethonin					1.60			x
	bu08c06	prenylcysteine carboxyl methyltransferase (PCCMT)					1.56			x
	bu11a05	amphiphysin II					1.61			x
	bu11a06	TGFβ1-induced anti-apoptotic factor 1					1.76			x
	bu11a07	supervillin					1.67			x
	bu12a09	amino-terminal enhancer of split					1.58			x
	bu12c11	NADH-ubiquinone oxidoreductase B22 subunit (Bos Taurus)					1.50			x
	bu12e05	THRA1, triiodothyronine receptor					1.57			x
	bu13h02	smoothelin					1.74			x

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confirmed the differential expression of the gene encoding fast troponin T3 (clone bu08a09) between 210 and 180 d.p.c. in RA muscle (Fig. 1B). Taken together, these results suggest that there are small differences in expression profiles between 210 and 180 d.p.c., with greater differences in RA than in ST at these stages.

Between 210 and 260 d.p.c., 92 genes were found differentially expressed (only the most important differences are indicated in Table 4). Twenty-eight of them were differentially expressed in both muscles, 5 in RA and 59 in ST. Seventy-four and 18 of the 92 differentially expressed genes were found less and more expressed at 260 than at 210 d.p.c., respectively. Eight of the 28 differentially expressed genes in both muscles were found more expressed at 260 than at 210 d.p.c., exemplified by proteasome 26S subunit non-ATPase 4 (Table 4). The transcript encoding nebulin-related anchoring protein (N-RAP) was barely detectable at any stage except at 260 d.p.c., and conversely NADH dehydrogenase was markedly less expressed at 260 d.p.c. than at any other stage (Table 4). Furthermore, in ST, 9 genes were found more expressed and 50 less expressed at 260 than at 210 d.p.c. Among the latter, the lower expression of the transcript encoding telethonin was confirmed by Northern blot analysis (Fig. 1C). Finally, 5 genes were found differentially expressed in RA but not in ST; in particular, a gene encoding an uncharacterised protein (bu13g11) was the only one found more expressed in RA at 260 d.p.c. Thus, both muscle types show important differences between 260 and 210 d.p.c. In addition, the expression of many genes appeared to be affected in ST but not in RA. For example, Northern blot analysis confirmed higher expression in ST at 260 than at 210 d.p.c. of the gene encoding actinin-associated LIM protein (Fig. 1A), foetal troponin T3 (clone bu08a09) (Fig. 1B) and sarcosin (Fig. 1D).

One hundred and forty-eight genes were found differentially expressed between the 260-day-old foetus and the 15-month-old bulls. Among them, 47 were found affected in both muscles, 13 in RA and 88 in ST. Thirteen were found less expressed (5 in both muscles, 7 in RA and 1 in ST) and 135 more expressed (42 in both muscles, 6 in RA and 87 in ST) at 15 months of age than at 260 d.p.c.. The higher overexpression of the gene encoding telethonin in ST after birth (4.7 *vs*. 2.2 cIm, ratio = 1.6) was subsequently confirmed by Northern blot analysis (Fig. 1C). However, the differences were small on average: 120 of these genes showed expression ratios ranging between 1.5 and 2 while only 28 genes were associated with a ratio greater than 2. Within the group of 13 genes found underexpressed after birth, the most important differences were recorded for those encoding troponin T3, oxoglutarate dehydrogenase, telethonin and some uncharacterised proteins, in both muscles (Table 4). In the case of troponin T3 gene, two different cDNA clones representing the foetal and the adult isoforms gave opposite results: a decrease in expression (clone bu08a09) for the foetal isoform and an increase in expression (clone bu09e01) for the adult isoform between 260 days of foetal life and 15 months of age. Only the decrease was significantly different according to our criteria for validating intensities (ratio lower than 0.67 or higher than 1.5). However, Northern blot analysis confirmed the increase of 0.5 cIm between the foetal life and the adult stage with the probe used in that experiment (Fig. 1B). Furthermore, the transcript encoding tumour suppressor LEU5 was barely detectable at 15 months of age, and only in ST. The most important differences in the group of 135 genes found overexpressed after birth were registered for those encoding Trip15 and activin A receptor type I in both muscles, and for nebulin and the precursor of P100 serine protease in ST (Table 4). As could be expected, all these results underline the existence of large differences in expression profiles between 260 d.p.c. and the age of 15 months after birth. Most of the differences, however, were observed for ST muscle (Table 4).

DISCUSSION

For the first time, cDNA clone inserts arrayed on high density nylon filters have been used to monitor the expression profile of a large number of genes in bovine muscles. This was done by using available macro-arrays of human muscle-specific cDNA clones (*11*).

The objective of this work was to follow changes in gene expression occurring during myogenesis in relation to muscle development (*6*, *8*, *16*), and meat quality (*1*, *17*, *18*). Ours results provide information in terms of: (i) identification of the genes differentially expressed between two different types of skeletal muscles, (ii) evaluation of the gene regulation pattern at each foetal age, thus allowing identification of crucial stages for the differentiation process and (iii) discovery of regulated genes very likely involved in a key physiological function, and warranting further studies.

Technical Adjustments for Analysis of the Hybridisation Signals in a Heterologous System—This study demonstrates that transcriptome analysis of bovine muscle can be performed by using human cDNA arrays. However, we first faced problems due to the heterologous feature of the system. The most evident difficulty was that excessively stringent conditions may have prevented hybridisation of genes with low sequence similarities between human and cattle. A second difficulty was that some cDNA probes had a long poly(A) tail, which may have induced non-specific hybridisation at the low stringent conditions for washing imposed by the heterologous system (*15*). We had to adjust conditions of hybridisation and to take them into account during the analysis of signals. As a result, the proportion of cDNAs on the filters which were hybridised to the radiolabelled targets is in agreement with previous studies in humans (*11*). However, further analyses of expressed genes by classical molecular biology techniques are necessary to definitely confirm the results, as was indeed done for some in the present study (Fig. 1).

Comparison of Gene Expression between RA and ST Muscles—In this study, only 2.5% of the 197 genes for which intensities of hybridisation were validated were found to be differentially expressed between RA and ST samples (Table 3).

The set of genes used was initially prepared to explore muscular dystrophy in humans. Consequently, genes encoding contractile proteins and metabolic traits only represent 5% each of the total number of genes (Table 1). In addition, we did not use the most extreme muscles such as *Masseter* (slow, oxidative) and *Cutaneus trunci*

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(fast, glycolytic) in adult cattle because they are not very relevant to meat production in farm animal species. Moreover, it is also well known that there are few metabolic differences between muscle types in large mammals (*19*, *20*), whereas differences between muscle types are more important in small mammals such as the rodents. This could partly explain some of the discrepancies between our results and those recently reported by Campbell *et al*. (*4*). Actually, using oligonucleotide arrays, these authors have detected about 12% of differentially expressed genes between *Soleus* (slow, oxidative) and *Quadriceps* (white, glycolytic) mice muscles. However, such a difference (2.5 *vs.* 12%) between these two studies may be also explained by the differences in identity, number and design of the genes arrayed.

Comparison of Gene Expression between Developmental Stages—When expressions of the 197 genes were considered over the period studied, it was possible to highlight some expression profiles. An overall survey of the results indicates small differences in gene expression during the first developmental period studied (110 to 210 d.p.c.) and, conversely, higher differences during the second developmental periods (*i.e* from 210 d.p.c. onwards). Later, differences in gene expression were more pronounced at 15 months of age. A similar profile was observed in both muscles, which gives greater confidence in the trend. However, a greater number of differentially expressed genes was detected in ST than in RA (Table 4). This may be explained by precocious development in RA as previously described for other muscle types (*21*). This is coherent with strong variations observed in characteristics (contractile and metabolic traits, lipid and collagen contents, *etc*.) of ST between 15 and 24 months of age in bulls, while characteristics of *Longissimus thoracis*, a muscle which has a composition close to that of RA, are barely modified (*8*). Ontogenesis is therefore associated with different waves of gene expression concerning genes such as those encoding transcription and growth factors regulating proliferation in the early phases, or genes involved in contractile and metabolic traits after 180 d.p.c., which were not represented in the DNA arrays used.

Indeed, muscle formation occurs in temporally distinct phases. The first one corresponds to the proliferation of myoblasts under the control of cell growth factors. Then the myoblasts withdraw from the cell cycle, align themselves, and fuse into multinucleated myotubes. These cells differentiate as demonstrated by numerous biochemical modifications including changes in their contractile and metabolic characteristics. This process is complicated by the existence of several generations of myogenic cells, probably three, which appear at about 60, 90, and 110 d.p.c. in both cattle (*22*) and humans (*23*). As a result, proliferation occurs before 180 d.p.c., and contractile and metabolic differentiation of fibres occur mainly after this stage (*8*).

From the present results, regarding the five stages considered, some genes appear to be highly regulated around 180 d.p.c., around 260 d.p.c. or between foetal life and 15 months of age. This underlines the physiological importance of the 180-day stage, which is confirmed as a transition between two different ontogenic phases. Moreover, these results particularly underline the importance of the differentiation phase (from 180 days of foetal age onwards), especially at the age of 260 d.p.c. Finally, these results are in agreement with evolution of muscle metabolic and contractile traits observed during this developmental period.

The absence of numerous differentially expressed genes during the proliferation phase, and especially between 110 and 180 d.p.c., may be explained by several considerations. Firstly, genes encoding proteins involved in cell ontogenesis represent less than 3% of the total number of genes printed on the arrays and only 15% of the genes retained for the analysis. Secondly, growth factors regulating proliferation are often transiently expressed or at a low level, making it difficult to detect their mRNA, especially in heterologous conditions. Thirdly, since multiplication of secondary and tertiary fibres occurs between 110 and 180 d.p.c., most regulatory factors should have been expressed before 110 d.p.c. (*24*).

An interesting finding of this study is the distinction between two different waves of gene expression during the differentiation period: a down-regulation of many genes between 210 and 260 d.p.c., and an up-regulation of many genes between the end of foetal life (260 d.p.c.) and the adult stage. As described in the literature for foetal myosin heavy chain isoforms (*21*) involved in muscle contractile traits or glucose transporter isoform 1 (*25*) involved in muscle metabolic traits, the first wave may correspond to the disappearance of foetal markers. In contrast, the second wave may correspond to structural and metabolic development of muscle as previously described for adult isoforms of heavy chains of myosin (*21*) and metabolic enzymes (*26*).

Identification of Differentially Expressed New Genes with Putative Physiological Functions—Among the cDNAs printed on the macro-arrays, a large proportion encodes genes with unknown biological functions. Indeed, no sequence information is available for about 20% of the printed cDNAs and *ca.* 19% correspond to expressed sequence tags (ESTs) with no associated functional information (Table 1).

However, some genes encoding proteins of known functions show interesting expression profiles. For instance, deletion in the tumour suppressor gene LEU5 is associated with B-cell chronic lymphocytic leukemia (B-CLL) (*27*). Like many tumour suppressor genes, LEU5 is probably involved in regulation of cellular proliferation and/or differentiation. Its higher expression at 260 d.p.c. and in ST than in RA confirms the notion that the proliferation/differentiation process is not synchronised between these two muscles. Trip15, the thyroid receptor interacting protein 15, plays a role in activation and stabilisation of *c-Jun* (*28*), which regulates the transition between proliferation and differentiation (*29*). It was found markedly down-regulated at 260 d.p.c. in both muscles, when muscle differentiation occurs (*8*).

α2,8-Sialyltransferase is responsible for the sialylation of adipoQ (*30*), which is an adipocyte-specific glycoprotein found in bovine serum and considered to play important roles in energy homeostasis. It was found down-regulated at 180 d.p.c. in both muscles concomitantly with the beginning of the differentiation phase.

Activin A, a factor involved in many reproductive processes (*31*), is a member of the TGF-β family. Its action is

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paracrine or autocrine and its physiological action in muscle remains to be studied. Nebulin is a sarcomeric component involved in the regulation of actin thin filament length in interaction with tropomodulin (*32*). These two genes were found markedly down-regulated at 260 d.p.c., *i.e.*, during the differentiation phase.

Oxoglutarate dehydrogenase is part of the mitochondrial 2-oxoglutarate dehydrogenase complex (*34*). It is therefore involved in the oxidative catabolism of nutrients. Its higher expression level after 210 d.p.c. is in accordance with the increase in isocitrate dehydrogenase activity (characteristic of oxidative metabolism) from 210 d.p.c. in bovine muscle (*26*).

Conclusion—Several interesting and new findings arise from this study. Firstly, the importance of the last three months of foetal life in bovine muscle differentiation, previously demonstrated by biochemical approaches, was confirmed here by medium-scale measurement of gene expression. Secondly, such an approach also allowed the identification of genes regulated during myogenesis. These genes often differ from those that could have been chosen on the basis of biochemical, metabolic and physiological considerations. Thirdly, this heterologous gene expression profiling study has also contributed to the discovery of new genes potentially regulated during myogenesis, since the biological function of approximately 33% of the declared differentially expressed genes is unknown as yet.

In conclusion, we demonstrate here that large-scale measurement of gene expression performed under carefully controlled conditions, even using human macroarrays, is a powerful tool for gene expression profiling during muscle myogenesis in cattle. This could have broad implications in various fields as ontogenesis of muscle traits associated with meat qualities in farm animals, normal muscle growth and physiology, and muscle-related diseases in several species. Indeed, given the close proximity between cattle and humans in terms of foetal ontogenesis, length of gestation, maturity at birth and chronology of events (*8*), our results could be tentatively extrapolated to humans and useful to diagnose and forestall chronic metabolic disease in humans (*4*, *35*, *36*).

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