INTRODUCTION

Muscle growth in agricultural animals, such as the pig, is of major scientific and commercial interest. However, the methods employed in the manipulation of growth are often empirical, with very little knowledge on the underlying mechanisms involved in particular processes operating at the molecular level. Growth is a highly complex, dynamic, multifactorial phenomenon, which involves the process of myogenesis, the determination of the number and size of muscle fibres, and the participation of the nervous and endocrine systems. In the latter, factors like growth hormones, insulin-like growth factors I and II (Magri et al., 1991; Szebenyi and Rotwein, 1991; Martelli et al., 1992) and thyroid hormone are important growth mediators. In the regulatory regions of the rat and human α-cardiac myosin heavy chain (HC) genes, for instance, are found thyroid hormone-responsive elements, which in vitro mediate thyroid hormone induction (Subramaniam et al., 1991). However, the mechanisms of actions for other hormones on the expression of muscle-specific genes remain poorly understood.

Since the final determinant of muscle growth is the laying down of myofibrillar proteins, one approach to elucidating the molecular processes of growth is to determine the regulation of muscle-specific genes. Like other myofibrillar protein components, the myosin HCs are encoded by a highly conserved multigene family (Swynghedauw, 1986) whose isoforms, each with its own particular ATPase activity that determines its velocity of muscle contraction (Edman et al., 1988), are expressed in a defined developmental pattern and are modulated by innervation and hormones (e.g. see Whalen et al., 1981; Condon et al., 1990; Sutherland et al., 1991; Dhoot, 1992). The myosin HC-β gene is of particular interest because, in all the mammalian species described to date, it is expressed at a very early stage during embryonic myogenesis, even before the appearance of the myogenic regulatory factor MyoD1 (Cusella-De Angelis et al., 1992). In the mouse, for example, myosin HC-β mRNA first appears in the myotomes at 9.5 days post-coitum (p.c.) whereas MyoD1 mRNA appears at 10.5 days p.c. (Lyons et al., 1990). During subsequent development, β isoform expression is widely distributed throughout the skeletal musculature, but confined to slow-twitch muscle fibers (La Framboise et al., 1991; Narusawa et al., 1987; Wigston and English, 1992). In addition, it is the predominant isoform in cardiac ventricular muscle throughout embryonic and fetal development and in the human it remains predominant throughout adult life (Bouvagnet et al., 1987). However, in the adult rat ventricle, the myosin HC-α replaces its β counterpart as the dominant isoform (Lompre et al., 1981, 1984).

To date there is no report on the isolation of any porcine myosin HC gene. In order to address some fundamental...
aspects of structural muscle gene expression, we report here on the isolation and characterization of the porcine slow muscle myosin HC-β gene and the functional and structural analysis of its promoter. In vivo expression studies revealed a highly regular rosette pattern of fiber arrangement, with a slow fiber occupying the central core.

MATERIALS AND METHODS

Isolation and mapping of the pig myosin HC-β clone

A 0.9 kb porcine myosin HC-β genomic DNA was used as a probe to screen a pig (Large white/Landrace cross) genomic library, cloned in λEMBL3 vector. This probe was derived from pig genomic DNA by PCR. It was obtained with the use of an upstream primer (5′-dCCCAACATCTTCACATCTCCG-3′) and a downstream primer (5′-dCTTCTCCCTGCTCCGATTCTCCG-3′) that corresponds to the conserved ATP-binding region of the human myosin HC-β exon 5 and exon 7, respectively (Jaenicke et al., 1990). This PCR clone was confirmed as a myosin HC by the dideoxynucleotide method of sequencing (Sequenase kit, USB). A clone, λPMHCβ, containing about 14 kb of insert, was isolated from the genomic library. λPMHCβ was extensively restriction mapped and subcloned into pBS (pBluescript SK+, Stratagene). Sequencing at selective sites was performed to determine the locations of putative exons and the orientation of λPMHCβ.

5′ end cloning by reverse transcription (solid phase) - polymerase chain reaction (RT-PCR)

Based on the sequence data, derived from the lambda PMHCβ, two primers corresponding to putative exon 1 and exon 6 were synthesized. Their sequences are 5′-dCTTCTTTCCTGGT-GCTCTCA-3′ (sense, upstream primer) and 5′-dTGATCAGGAT-CAGGCATTGT-3′ (antisense, downstream primer), respectively. Solid-phase reverse transcription was performed with the downstream primer, biotinylated at the 5′ end, bound to streptavidin-coated magnetic beads (Dynabeads M-280, Dynal). Briefly, 100 µg of total RNA, derived from the biceps femoris of a 10-day-old piglet (Chomczynski and Sacchi, 1987), was annealed to 20 pmoles of biotinylated downstream primer. The resulting RNA-primer complex was captured with Dynal M-280, isolated and washed with the use of a magnet. Reverse transcription was performed, as described by Sambrook et al. (1989), in a rotating oven. The primer-extended single-stranded cDNA was released from the magnetic beads by heating the suspension at 97°C for 10 min. PCR was conducted for 30 cycles, annealing at 60°C for 2 min, polymerization at 72°C for 1 min and denaturation at 95°C for 1 min. A PCR product of the predicted size of 0.5 kb was isolated and cloned into pBS. A third primer, based on the putative exon 5, was also made (5′-dAGCATGTACTG-GCTCTCA-3′, antisense). Further PCR was performed with the original upstream primer (exon 1) and this exon 5 antisense primer to confirm the authenticity of the first-round PCR product. The 0.5 kb PCR-derived cDNA was sequenced and compared with the corresponding sequences of the genomic clone, λPMHCβ. Exon-intron junctions on λPMHCβ were determined by sequencing.

In situ hybridization and ATPase staining

Preparation of the various tissues, the generation of 35S-labelled myosin HC-β RNA probe, by in vitro transcription from the T7 and T3 RNA polymerase promoters of pBS and in situ hybridization under high stringency, including the use of RNase A during the washing steps, were performed as described by Chang et al. (1990a,b). The 5′ end of the PCR-derived myosin HC-β cDNA was used as probe. It was 340 nucleotides in length, covering exons 1 to 3 and part of exon 4. This probe contained the entire 5′ untranslated region. Sense RNA probe was routinely used as a negative control.

In addition to in situ hybridization, serial sections were taken for ATPase staining to demonstrate the histochemical distribution of muscle fibers. A range of alkaline and acidic pre-incubations were performed as described by Guth and Samaha (1970). The ATPase activity of slow twitch fibers is acid-stable and alkaline-labile. Generally, fast-twitch fiber ATPase is acid-labile and alkaline-stable.

Southern and northern analyses and RNase protection assays

The PCR-derived 0.9 kb genomic DNA and 0.5 kb cDNA were used as probes in Southern analyses on porcine genomic DNA (Sambrook et al., 1989). This was to verify the specificities of the two probes. Total RNA was extracted from the liver, cardiac ventricle and biceps femoris of a 2-day-old pig (Large white/Landrace cross). Northern analysis was performed (Chang et al., 1990b) with the 0.3 kb myosin HC-β cDNA as probe, which is a subclone of 0.5 kb cDNA. RNase protection assay on total RNA was carried out to determine the transcriptional start site of the porcine myosin HC-β gene. The protocol used was essentially similar to the one that uses RNase A and RNase T1 as described by Sambrook et al. (1989) except that RNase I, that cleaves at every ribonucleotide, was used instead (Promega).

Plasmids construction

The 2.0 kb HindIII-KpnI fragment from λPMHCβ, housing the promoter region and the transcriptional start site, was blunt-ended with Mung Bean nuclease and cloned into the Smal site of pBS. The resulting p2.0-BS clone, with its insert oriented in the same direction as the HindIII to Sall sites, was identified by restriction mapping. The insert was released with HindIII and Sall digestion and cloned into pCAT-basic (Promega), digested with the same enzymes. Hence, the original 2.0 kb HindIII-KpnI insert was correctly inserted into a CAT (chloramphenicol acetyltransferase) reporter vector (p2.0-CAT). A series of 5′ deletions, based on p2.0-CAT, were made by carrying out selective restriction digests and re-ligations. The constructs created were p1.5-CAT, p1.0-CAT and p0.6-CAT. Another construct, p0.41-CAT, contained a 0.41 kb Psrl insert, derived 5′ from the start site, cloned into the PstI site of pCAT-Basic (see Fig. 6, below).

A series of 3′ deletions were made based on p1.0-CAT which was linearized at the 3′ end with SalI and subjected to exonuclease III/Mung Bean nuclease treatment (Stratagene; Chang et al., 1991). The deleted inserts were released by HindIII restriction, blunt ended and cloned into Smal-cut pBS. Inserts of the appropriate sizes and correct orientation were released with HindIII and Sall digestion and cloned back into pCAT-basic. In this way, p0.59-CAT, p0.47-CAT, p0.4-CAT and p0.38-CAT were made. p0.38-CAT, in turn, was subjected to further 5′ exonuclease III/Mung Bean nuclease deletions, following the previously outlined steps. This resulted in p0.34-CAT, p0.25-CAT and p0.12-CAT. The boundaries of all constructs were determined by sequencing.

Cell cultures, transfections and CAT assays

Murine skeletal C2C12 myoblasts (Blau et al., 1983) and human embryonic lung cells L132 (fibroblasts, ATCC no. CCL 5) were cultured in Dulbecco’s modified Eagle’s medium (DMEM, Flow) containing 10% fetal calf serum, 2 mM L-glutamine, 100 i.u./ml penicillin, 100 µg/ml streptomycin and 2.5 µg/ml amphotericin B
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(proliferation medium), at 37°C under a humidified atmosphere of 95% air and 5% CO₂. Myogenic differentiation medium for C2C12 myoblasts contained 4% horse serum in place of fetal calf serum. Primary cultures of porcine muscle satellite cells were derived from the biceps femoris muscle of a 2-day-old piglet. The protocol used was as described by Doumit and Merkel (1992).

Cell cultures were grown to about 50% confluency and transfected by the calcium phosphate co-precipitation method as described by Sambrook et al. (1989). A 20 µg sample of test CAT plasmid and 5 µg of reference pCH110 (Pharmacia), a control for β-galactosidase (β-gal) activity, were used per transfection. Cells were harvested 72 h later. Transfected C2C12 myotube cultures were switched to differentiation medium the following morning. C2C12 myoblasts, upon transfection, were grown in proliferation medium for a further 48 h. Harvested cells were resuspended in 230 µl of 0.25 M Tris-HCl (pH 7.8) and lysates were prepared for CAT assays as described by Sambrook et al. (1989). A 40 µl sample derived from each transfection was used for β-gal assay to normalize the amount of lysate used in each set of CAT reactions. CAT conversion was quantified by a Chromoscan densitometer (Joyce-Loebl).

RESULTS

Isolation and characterization of the porcine myosin HC-β clone

In order to facilitate the screening for the porcine myosin HC-β gene, a homologous myosin probe was first isolated. This was achieved by performing PCR on porcine genomic DNA. The two primers chosen were derived from exon 5 and exon 7 of the human myosin HC-β (Jaenicke et al., 1990). Exons 5 to 7 code for the highly conserved ATP-binding site of myosin HC. A discrete 0.9 kb PCR product was obtained. The sequence of its exons confirmed that it was myosin HC in origin. Southern analysis on genomic DNA, digested with various enzymes, demonstrated that this probe was myosin species specific (data not shown). Screening of a λEMBL3 pig genomic library yielded a clone (λPMHCβ) with an insert of about 14 kb. This clone was extensively restriction mapped and subcloned into pBS. Selective Southern hybridization and sequencing led to the orientation and localization of exons 5 to 7 (Fig. 1). In order to define the transcriptional start site(s), RNase protection assay was performed. The selection of probe for this assay was facilitated by the high homology that exists between orthologous myosin HC isoforms of different mammalian species (Parker-Thornburg et al., 1992). A 0.12 kb fragment (Fig. 7, below) that included the putative exon 1 was cloned into pBS and in vitro transcribed to generate antisense ³²P-labelled RNA. A number of similar size frag-
ments were protected from RNase I digestion (Fig. 2A). This finding was not surprising, given the ability of RNase I to cleave any base mismatch. The largest fragment was about 50 bases in length. The smaller fragments might reflect alternative start sites or partial degradation of myosin HC-β mRNAs. Interestingly, when a sequence comparison was made at the CAP region of various myosin HC-β genes (Shimizu et al., 1992a,b; Thompson et al., 1991; Yamauchi-Takahara et al., 1989), the start site was located within a conserved pentameric sequence, CAGCT, in three out of the four mammalian species examined (Fig. 2B). In contrast, two such pentamers were found in the pig.

5′ Myosin HC-β cDNA cloning by RT(solid-phase)-PCR

RT(solid-phase)-PCR consistently yielded a 0.5 kb fragment. This was cloned into pBS and sequenced (Fig. 3A). Sequence comparison was made between the cDNA and putative exons found in the genomic clone, λPMHCβ. Interestingly, there were four base mismatches, in the coding region, with no alteration in the deduced amino acid sequence (Fig. 3A,B). Since the genomic and cDNA clones were derived from different animals, these mismatches are more likely to reflect allelic variations rather than PCR associated errors. The genomic organization of the exons were also determined by locating the exon-intron junctions (Fig. 1). The deduced pig myosin amino acid sequence was compared with the myosin HC-β isoform of human, rat and rabbit (Fig. 3B). The degree of homology amongst the four species is remarkably high, indicating that this pig myosin gene is indeed the β slow isoform. Most of the differences are substitutions by amino acids of similar biochemical properties, e.g. isoleucine for valine. However, there are found three mismatches (position 4, 11 and 65) where the amino acid substitutions are biochemically disparate, e.g. at position 4 there is alanine (uncharged), serine (positively charged) or arginine (hydrophobic). It is noteworthy that all three mismatches are located 5′ to the ATP-binding site.

Fig. 3. 5′ End cDNA sequence and comparative amino acid sequence of the porcine myosin HC-β (GenBank accession no. L10129). (A) Nucleotide sequence of the 5′ cDNA obtained by RT(solid-phase)-PCR. Allelic variations are evident at position 117, 465, 468 and 561, without change in the amino acid sequence. (B) Deduced 5′ end amino acid sequence was compared with mammalian species. There is high homology in all the species examined. Interestingly, the 3 main disparate amino acid mismatches (positions 4, 11 and 65) are all situated 5′ to the ATP-binding site.
corresponds to the myosin HC-β RNA from a 2-day-old pig: heart ventricle (H), tissue- and isoform-specific. Each lane, only one hybridization band was obtained that are in kb. Results from (A) and (B) show that the probe is muscle genomic Southern and northern analyses. (A) Genomic Southern (S) and (B) Northern analysis. Each lane was loaded with 30 µg total porcine genomic DNA digested with HindIII (H), EcoRI (E), PstI (P), BamHI (B), SalI (S) and KpnI (K), respectively. U, undigested genomic DNA. In each lane, only one hybridization band was obtained that corresponds to the myosin HC-β genomic restriction map (Fig. 1). (B) Northern analysis. Each lane was loaded with 30 µg total RNA from a 2-day-old pig: heart ventricle (H), biceps femoris (S) and liver (L). Only the heart and skeletal muscle RNA hybridized specifically to the probe in the 6 kb size region. Markers shown are in kb. Results from (A) and (B) show that the probe is muscle tissue- and isoform-specific.

To determine the specificity of the PCR derived myosin HC-β cDNA, 0.3 kb of its 5′ end was subcloned into pBS and used as a probe for Northern and Southern hybridizations (Fig. 4A,B). This probe, which housed the entire 5′ untranslated region, was found to hybridize, in a muscle-specific manner, to a message of about 6.0 kb, which is the approximate size of a myosin HC mRNA. Genomic Southern analysis showed that the probe was bound only to one specific band in each of the genomic digests. This indicated that the 0.3 kb cDNA, hybridizing in an isoform-specific manner, was suitable for use as a probe for in situ hybridization.

In vivo expression of porcine myosin HC-β
Various tissues, from a 2-day-old Large white/Landrace piglet, were taken for in situ hybridization and ATPase studies. The 0.3 kb 5′ end cDNA was used for 35S-labelling by in vitro transcription. The sense probe did not give in any significant hybridization, as with the antisense probe on brain, liver and gut sections (data not shown). The in situ hybridization results on skeletal and cardiac sections were particularly distinctive (Fig. 5). One salient feature is that the myofiber hybridization pattern, as detected by silver grains, in all the skeletal muscle sections examined, mirrored very closely the well recognized rosette arrangement of primary and secondary fibers found in porcine skeletal muscles. In cross-sections, most positive fibers could be seen to adopt a regular spatial arrangement, each surrounded by a ring of negatively stained fibers. This finding, together with the comparative sequence data, provides compelling evidence that the myosin gene in question is indeed the myosin HC-β isoform. In muscles of known slow phenotype, such as the soleus, there were evidently more slow fibers, in contrast to the fast muscle phenotype such as the gastrocnemius and mylohyoid, where proportionally fewer slow fibers were detected (Fig. 5, A2, B2, A4, B4). Myosin HC-β expression, where it occurred, was evident throughout the fiber (Fig. 7, A5, B5). In some muscle bundles, like the biceps femoris, there was a gradation of slow myosin HC-β expression. Slow fibers, found only in the deeper region, were altogether absent in the superficial part of the bundle (Fig. 5, A5, B5, B7). This feature was not found in the diaphragm (Fig. 5, A3, B3). To provide histochemical evidence for myosin HC-β protein expression, ATPase staining on muscle serial sections was performed. Slow myosin is alkaline-labile and acid-stable (Guth and Samaha, 1970). Acid-stable ATPase-positive fibers were found to co-localize with myosin HC-β message expressing fibers (Fig. 5, A6, B6). In the heart, myosin HC-β was expressed diffusely throughout the ventricle and atrium. Expression in the ventricle was greater than in the atrium (Fig. 5, A8, B8, A9, B9). This preferential expression in the ventricle is similar to that seen in the human heart after birth.

Functional and structural analysis of the 5′ regulatory region
The 5′ regulatory region of the porcine myosin HC-β gene was sequenced (Fig. 6). Like its amino acid sequence, the regulatory domain also showed extensive homology with its counterpart in other mammalian species. There is a homology of 82% with the human myosin HC-β, suggesting that mechanisms governing the expression in both genes are similar. A series of 5′ and 3′ deletions were performed on this 5′ region and spliced, in the correct orientation, into pCAT-basic, a promoterless CAT reporter vector. They were used in transient transfection assays, conducted on murine C2C12 cells (Fig. 7). Comparison of CAT activity in the different constructs suggests the presence of positive and negative modulating elements within the 2.4 kb 5′ region, on either side of the start site. 5′ Deletions, from p2.0-CAT to p1.0-CAT, led to a steady increase of CAT activity, from 24.75 to 83.63 relative units (ru). There was an abrupt decline to 22.73 ru of CAT activity (p0.59-CAT) as p1.0-CAT was deleted from the 3′ end. Further 3′ deletions (p0.47-CAT, p0.4-CAT and p0.38-CAT) resulted once again in the elevation of CAT activity, peaking at 89.21 ru. This pattern of CAT expression suggests the presence of a positive and a negative element, located in close proximity to each other, within intron 1. A similar pattern
Fig. 5
Fig. 5. In vivo expression of the porcine myosin HC-β gene. In situ hybridization was performed on different tissues, taken from a 2-day-old piglet, using the 0.3 kb porcine myosin HC-β cDNA as probe. This probe comprises exons 1 to 3 and part of 4. The A series are brightfield micrographs with their corresponding darkfield counterparts in the B series. 1, biceps femoris; 2, mylohyoid; 3, diaphragm; 4, soleus and gastrocnemius; 5, biceps femoris in oblique section; 6, cranial tibia; 7, biceps femoris showing gradation of myosin HC-β expression from deep (d) to superficial (s) regions; 8, cardiac ventricle; 9, cardiac atrium. A6 is a bright-field micrograph of the cranial tibial stained for acid-stable ATPase. B6 is corresponding dark-field serial section subjected to in situ hybridization. a and b are orientation markers. Colocalization of acid-stable (pH 4.6) ATPase- and myosin HC-β mRNA-expressing fibers can be seen. Sections on ventricle and atrium (8 and 9) were treated identically and photographed under the same exposure conditions. Note the higher level of expression in the ventricle. Bar in A1, 20 µm. All other micrographs except, A6 and B6, are on the same scale. Bar for A6 and B6, 10 µm.
of promoter behavior was also reported recently in the murine myosin HC IIB gene (Takeda et al., 1992).

Several selected constructs were transfected into human L132 fibroblasts and murine C2C12 myoblasts to evaluate their tissue- and differentiation stage-specificities. In L132 cells, CAT expression of all the constructs tested were consistently less than in C2C12 myotubes (p1.5-CAT, p1.0-CAT, p0.38-CAT to p0.12-CAT, Fig. 7). It is noteworthy that their relative expression within the same cell line mirrored that of C2C12 myotubes. With murine C2C12 myoblasts, the finding was similar to that of L132 cells (p1.5-CAT, p1.0-CAT and p0.38-CAT). It should be pointed out that a small degree of differentiation in the myoblast cultures could have occurred after the transfection step. Where the 5′ promoter was completely absent (p0.6-CAT), CAT activity was negligible in all cell types tested. It can therefore be concluded that the porcine promoter behaves in a muscle- and differentiation-specific manner in vitro.

There have been a number of recent reports on the identification of cis-acting regulatory elements close to the start site of the myosin HC-β genes of rabbit, human and rat (Shimizu et al., 1992a,b; Flink et al., 1992; Vosberg et al., 1992; Thompson et al., 1991; Gulick et al., 1991). In particular, two conserved elements seem to stand out as essential for conferring muscle-specific expression. They are element A (also known as βe2 or βF1-binding element) and element B (also known as βe3). These two elements are present in the porcine myosin-HC β promoter (element A from −266 to −288, element B from −179 to −209, Fig. 6).
Results based on constructs derived from 5' deletions of p0.38-CAT, which housed both elements, transfected into C2C12 cells, supported the importance ascribed to these two elements. Removal of element A (p0.34-CAT) resulted in about 50% reduction of CAT expression in C2C12 cells, from 78 to 41 ru (Fig. 7). When both elements A and B were deleted (p0.25-CAT), there was a further reduction to 27 ru of CAT activity. However, when the same series of constructs were transfected into primary porcine myotubes the results were somewhat different (Fig. 7). With p0.38-CAT, CAT expression level was 50 ru, which was comparable to the result obtained for C2C12 myotube transfection. The difference in CAT expression between C2C12 myotubes and primary porcine myotubes was evident when p0.34-CAT was used. The absence of element A in p0.34-CAT did not result in a reduction of CAT expression in...
primary porcine myotubes, unlike that seen in C2C12 myotubes. The other deletion constructs in the series, p0.25-CAT and p0.12-CAT, gave similar levels of expression to those obtained in C2C12 myotubes. This apparent anomaly in findings between C2C12 myotubes and primary porcine myotubes does, nonetheless, highlight the fact that both elements, A and B, are important for promoter function, although their importance may vary according to the cell type used. It is interesting to note that the potential loop-structure, located at the 3' end of βF1-binding element (or element A), found to be important for full promoter activity (Flink et al., 1992), is also present in the pig but not the rat or rabbit.

**DISCUSSION**

This study, on the isolation and characterization of the porcine myosin HC-β gene, is the first to be conducted on a farm animal. Not surprisingly, the porcine myosin HC-β gene shows remarkable homology with the same isoform of the rat, rabbit and human, at the nucleotide and amino acid levels. Exon 1 of the porcine myosin HC-β gene is conspicuously larger than its counterpart in the other mammalian species. In many eucaryotic genes, the CA doublet is frequently found to be the transcriptional start site with the A at position +1 (Nussinov, 1990; Bucher and Trifonov, 1986). This consensus is evidently present in the myosin HC-β pentameric sequence (Fig. 2B). Interestingly, in the pig, this pentamer occurs as a direct repeat. It is unclear, however, whether this feature has any regulatory significance. Comparison of the 5′ amino acid sequences of the different mammalian myosin HC-β genes shows three biochemically disparate amino acid substitutions at positions 4, 11 and 65 (Fig. 3B). Since all three mismatches are located just 5′ to the ATP-binding site (exons 5 to 7), it is possible that these positions may have a modulating effect on ATP-mediated energy generation specific for each species. Nucleotide sequence examination of the 5′ porcine myosin HC-β regulatory region revealed eight E-box motifs, four of which are completely conserved in both pig and human. The E-box motif is a consensus recognition sequence for myogenic regulatory factors (e.g. see Weintraub et al., 1991). Since critical cis-acting elements are more likely to be conserved between species, it would be logical, for future study, to focus on the possible functional significance of these four conserved E-boxes in skeletal and cardiac muscles.

The in vivo porcine myosin HC-β expression studies showed a characteristic rosette fiber arrangement, with the slow-twitch fiber occupying the central core position. This ordered pattern of fiber distribution in the pig, in contrasts to other mammals, persists into adulthood (Davies, 1972; Handel and Stickland, 1987), and allows a direct means of fiber type diversity with respect to primary and secondary fibers (Miller, 1992; Stockdale, 1992). For any given stimulus, for instance, the differential response between primary and secondary fibers can be more readily investigated. The distribution of myosin HC-β message seems to extend throughout each fiber type (Fig. 7, A5,B5). This contrasts with the report by Aigner and Pette (1990), who found that rabbit myosin HC-β message expression was confined to the perinuclear region of the fibers. It is likely that this difference is age related in that older rabbits were used. In the 2-day-old pig, myosin HC-β expression was higher in the ventricle than in the atrium (Fig. 5, A8 to B9). Interestingly, this distribution pattern is also found in man but in small animals of the same age, such as rat, myosin HC-β is mainly expressed in the atrium (Thompson et al., 1991).

Considering the high degree of nucleotide sequence homology, including the 5′ regulatory region, in the myosin HC-β genes of pig, man, rat and rabbit, one likely explanation for this difference in expression between the large mammals (pig and man) and small mammals (rat and rabbit) may rest with some of the divergent sequences in the 5′ flanking region.

Functional analyses, based on transient CAT transfection assays with a series of 5′ and 3′ deleted constructs, suggest the presence of a number of positive and negative modulating elements on either side of the transcriptional start site (Fig. 7). This is consistent with the expectation that multiple regulatory elements are in operation to effect the highly dynamic and complex process of myosin HC gene regulation (Sadoshima et al., 1992). Results from this study support the functional significance of the recently reported elements A and B, although the behavior of the porcine promoter construct does vary with the muscle cell type used. It is not clear why the absence of element A in p0.34-CAT did not result in a reduction of CAT expression in porcine primary myotubes. This finding may be artifactual or, more plausibly, reflect differences of specific trans-acting factors in the porcine myotube environment. Of particular importance is the identification of two putative regulatory elements in intron 1 of the porcine myosin HC-β gene. The first is a putative positive element located, between p1.0-CAT and p0.59-CAT, with a 0.4 kb region (Fig. 7). The second is a putative negative element, between p0.59-CAT and p0.47-CAT (from −318 to −188). The limits of these two elements require further defining and their phenotypic properties characterized.

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