Control of the Murine Phosphofructokinase-A Gene during Muscle Differentiation[†,‡]

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ABSTRACT: The muscle-specific isoform of phosphofructokinase (PFK-A) is induced during muscle development. To understand expression of PFK at the molecular level, transcription of the mouse PFK-A gene was examined during C2 myoblast differentiation to myotubes. PFK-A gene transcription increased 5–7-fold during differentiation *in vitro*. To identify cis-acting elements which direct muscle-specific transcription of the PFK-A gene, its 5′-flanking region and first exon were cloned and characterized. S1 nuclease protection and primer extension assays showed four sites of transcription initiation at 106, 105, 88, and 87 bp upstream of the translation initiation codon. Stable transfection of fusion genes linking −1900 to +99 of PFK-A 5′-flanking sequence to chloramphenicol acetyltransferase coding sequences into myogenic C2 cells did not confer muscle-specific expression. However, larger fragments of PFK-A 5′-flanking region (−5800 to +99) showed muscle-specific expression by transient transfection assay. The sequences directing muscle-specific transcription were further defined by linking various PFK-A upstream fragments to the luciferase gene under the control of the PFK-A proximal promoter, −335 to +99 bp. We found DNA sequence responsible for muscle-specific expression of the PFK-A gene between −4800 and −3900 bp.

Phosphofructokinase (PFK, EC 2.7.1.11)[1] catalyzes the committed and rate-limiting step in glycolysis, the conversion of fructose 6-phosphate to fructose 1,6-bisphosphate. PFK is allosterically regulated by numerous metabolites (Kemp & Foe, 1983; Reinhart & Lardy, 1980) which serve to integrate glycolysis with other cellular activities. Citrate and ATP inhibit while AMP and ADP activate PFK so that flux through glycolysis is decreased when energy within the cell is abundant and increased when energy is in demand. The most potent positive allosteric effector of PFK is fructose 2,6-bisphosphate, the intracellular concentration of which is hormonally regulated (Pilkis *et al.*, 1981). The relative abundance of fructose 2,6-bisphosphate serves to integrate glycolysis with the energy demands of the whole organism (Pilkis & Claus, 1991). The smallest active form of PFK *in vitro* is a tetramer with subunit molecular mass of ~85 kDa. There are three different subunit isoforms of PFK: PFK-A, PFK-B, and PFK-C (Tsai & Kemp, 1972; Dunaway, 1983), which combine to form homo- and heterotetramers of differing allosteric and catalytic properties (Vora, 1982). In muscle, PFK-A is the major isoform expressed (Gehnrich *et al.*, 1988) so that muscle PFK is made up of PFK-A_4 which gives glycolysis in this tissue unique regulatory properties. Muscle PFK is less sensitive to ATP and citrate inhibition. Muscle PFK has a higher specific activity (Kemp & Foe, 1983) and is better suited for binding to F-actin, which lowers its K_m for fructose 6-phosphate (Reid & Masters, 1986). Fetal muscle has been shown to express all three isoforms of PFK, which is presumably sufficient for the proliferation and housekeeping function of fetal muscle. Shortly after birth, however, skeletal muscle switches to the exclusive production of PFK-A and at a much higher level (Dunaway *et al.*, 1986).

How does muscle specifically turn on the proper PFK isozyme at the proper time in development? Embryonic myoblasts migrate from the myotomes surrounding the neural tube to the developing limbs. There they proliferate and later fuse to form the multinucleated myotubes of mature skeletal muscle. Proliferating myoblasts express the housekeeping enzymes and structural proteins typical of fibroblasts. Upon terminal differentiation, however, myotubes express a battery of muscle-specific genes that allow it to carry out its specialized function. Myogenesis is partly under the control of the MyoD family of genes, which activate the myogenic program when expressed in nonmyogenic cells (Weintraub *et al.*, 1989). Four members of the MyoD family (MyoD, myogenin, myf5, and MRF4) have been cloned and shown to activate many of the contractile protein genes [for a review, see Edmondson and Olson (1993)].

C2 myoblasts (Yafee & Saxel, 1977) proliferate rapidly in high mitogen medium, but when the cells grow to near-confluence and the mitogens are removed, they fuse to form multinucleated myotubes. Using this system, we have previously shown that myoblasts express all three isozymes of PFK at about the same level. Upon differentiation, PFK activity increases dramatically due to the specific increase in synthesis of the PFK-A isozyme, making PFK-A the major isoform synthesized in myotubes (Gekakis *et al.*, 1989). Furthermore, increased PFK-A synthesis is a direct result of an increase in the steady-state PFK-A mRNA level. The PFK-A gene has been cloned from both human (Nakajima *et al.*, 1990) and rabbit (Lee *et al.*, 1987), but in neither gene has the start site of transcription been identified nor has transcriptional regulation of the gene been studied. In the present study, we measured the rate of transcription of the PFK-A gene and showed that it specifically increases during C2 myoblast differentiation. We then cloned the mouse PFK-A (mPFK-A) gene and its 5′-flanking region and mapped its sites of transcription initiation. Furthermore, by transfection of chimeric genes in which the expression of a reporter

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[1] Abbreviations: PFK, phosphofructokinase; DMEM, Dulbecco's modified Eagle's medium; EDTA, ethylenediaminetetraacetic acid; Tris, tris(hydroxymethyl)aminomethane; PCR, polymerase chain reaction; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid; PIPES, piperazine-*N,N′*-bis(2-ethanesulfonic acid); MB, myoblasts; MT, myotubes; PBS, phosphate-buffered saline; CAT, chloramphenicol acetyltransferase.

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gene is controlled by regions upstream of the mPFK-A gene, we showed that a cis-acting element responsible for muscle-specific transcription of PFK-A lies between −4800 and −3900 bp relative to the start site of transcription.

EXPERIMENTAL PROCEDURES

Tissue Culture. Myogenic C2 cells (Yafee & Saxel, 1977) were plated at a density of $10^4/cm^2$ and grown in DMEM supplemented with fetal bovine serum to 20% (growth medium). Proliferative undifferentiated muscle cells (myoblasts) were passaged every other day and were maintained at 70% confluence or less. Differentiation was initiated by switching myoblast cultures, at 85% confluence, to DMEM supplemented with fetal bovine serum to 0.5% (fusion medium). After being switched to fusion medium, cells were fed with fresh medium every day. Typically 50% of the cells were terminally differentiated muscle cells (myotubes) after 2 days in fusion medium.

Nuclei Isolation and Run-On Transcription Assay. Nuclei were isolated from myoblasts 2 days after plating or from myotubes 3 days after switching to fusion medium, as described previously (Moustaïd & Sul, 1991). Equal numbers of nuclei [(4–8) × 10^6] from myoblasts or myotubes were used in a run-on transcription assay as described. Equal counts from myoblast and myotube nuclei [(0.5–1.0) × 10^7 cpm] were hybridized for 3 days at 65 °C to denatured plasmid DNA immobilized on nitrocellulose (10 μg/slot). The plasmids used were full-length mPFK-A cDNA in pBluescript II SK+, pBluescript II SK+ vector without insert, muscle creatine kinase (generously provided by Dr. S. Hauschka, University of Washington), or $\alpha_{skeletal}$-actin. The nitrocellulose filters were washed at 65 °C in 0.1 × SSC (15 mM sodium chloride/1.5 mM sodium citrate)/0.1% SDS before autoradiography for 24–48 h with an intensifying screen. Hybridization of probe to immobilized plasmids was quantitated directly by phosphorimaging of the radioactive nitrocellulose. The fold increase in transcription rate was determined by comparing the rate of transcription (in cpm of newly synthesized transcript/45 min) of PFK-A in myotubes to that in myoblasts.

Screening and Isolation of Genomic Clones. A mouse embryo genomic library in Charon 4A (generously provided by Dr. L. Hood, California Institute of Technology; Davis *et al.*, 1980) was screened with random-primer-labeled, near-full-length mPFK-A cDNA and with an end-labeled 45mer corresponding to mPFK-A amino acids 15–30 according to standard protocols (Sambrook *et al.*, 1989). A total of 600 000 plaques at a density of 20 000 pfu/150-mm plate were screened. Clones which were positive with both probes were picked and plaque-purified. Phage DNA, purified by the plate lysate method, was mapped by single and double restriction digestion followed by Southern blotting using different cDNA fragments as probes. Once identified, 5′-flanking portions of the mPFK-A gene were subcloned into pBluescript II SK+ (Stratagene, LaJolla, CA) to generate nested deletions, using Stratgene's exonuclease III/mung bean nuclease deletion kit, which were then sequenced by the dideoxy chain termination method (Sanger *et al.*, 1977) using Sequenase (USB, Cleveland, OH). The phage DNA was also used to subclone various mPFK-A promoter fragments upstream of chloramphenicol acetyltransferase (CAT) coding sequences in the vector pBLCAT3 (Luckow & Schutz, 1987) or upstream of luciferase coding sequences in the vector pGL2-basic (Promega, Madison, WI).

S1 Nuclease and Primer Extension Assays. To generate a probe for the S1 nuclease assay, two oligonucleotides were synthesized: one complimentary to the mPFK-A mRNA at amino acids 15–21 (3′ oligo) and the other 463 bp upstream of the translation initiation codon and identical to the coding strand (5′ oligo). The two oligos were used to amplify genomic DNA in the Charon 4A clone by PCR. The 526 bp PCR product was end-labeled with [γ-^{32}P]ATP and polynucleotide kinase (New England Biolabs, Beverly, MA). ^{32}P was removed from the 5′ end by digestion with *Nhe*I, which cuts 12 bp downstream of the 5′ end of the fragment, followed by passage through a G-50 spin column, leaving the fragment labeled only at the 3′ end. The labeled PCR product (10^5 cpm) and 30 μg of total myotube RNA were ethanol-precipitated and resuspended in 20 μL of hybridization buffer (80% formamide, 400 mM NaCl, and 40 mM PIPES, pH 6.4). After denaturation by boiling for 2 min, DNA and RNA were hybridized at 52 °C for 4 h; 300 μL of S1 nuclease buffer (280 mM NaCl, 50 mM sodium acetate, pH 4.5, and 4.5 mM $ZnSO_4$) and 1 μL of the indicated amount of S1 nuclease (Pharmacia) were added. Digestion was carried out at 37 °C for 30 min. The digested probe was precipitated by adding 80 μL of 3.8 M ammonium acetate, 20 mM EDTA, 50 mg/mL tRNA, and 800 μL of ethanol and storing at −80 °C for 2 h followed by microcentrifugation. The probe was dried and resuspended in polyacrylamide gel loading buffer (38% formamide, 0.02% bromophenol blue, 0.02% xylene cyanol FF, and 8 mM EDTA) and denatured at 80 °C for 2 min before running on a sequencing gel.

The primer extension assay was done according to Ntambi *et al.* (1988). The 3′ oligo (above) was end-labeled with [γ-^{32}P]-ATP and T4 polynucleotide kinase; 10^6 cpm of labeled oligo and 20 μg of poly(A+) RNA from myoblasts or myotubes were ethanol-precipitated and redissolved in 10 μL of hybridization buffer (10 mM Tris, pH 8.3, 250 mM KCl, and 1 mM EDTA). The DNA/RNA solution was incubated at 65 °C for 45 min and then at 45 °C for 45 min and then slowly cooled to room temperature. Reverse transcription was carried out in a final volume of 40 μL containing 50 mM Tris, pH 8.3, 70 mM KCl, 10 mM dithiothreitol, 10 mM $MgCl_2$, 0.5 mM each dNTP, 1 unit/μL AMV reverse transcriptase, and 1 unit/μL human placental ribonuclease inhibitor at 37 °C for 5 min and then at 42 °C for 60 min. The extended products were ethanol-precipitated, redissolved in 6 μL of polyacrylamide gel loading buffer, and fractionated on a sequencing gel.

Transfection. Plasmid DNA was transfected into MB 1 day after plating by the calcium phosphate coprecipitation method (Ausubel *et al.*, 1989). The DNA precipitate was left on the cells for 10–16 h after which it was washed off by two rinses with PBS and replaced with growth medium. Forty-eight hours after transfection myoblasts were harvested or switched to fusion medium, and myotubes were harvested 2–3 days after the switch to fusion medium (Sternberg *et al.*, 1988). To compensate for the variation in transfectability of a given plasmid from one preparation to another, all experiments were done at least 3 times with at least two different preparations of each plasmid. Alternatively, a control plasmid in which expression of lacZ is driven by the cytomegalovirus promoter (kindly provided by Dr. G. R. MacGregor; MacGregor *et al.*, 1991) was included in the transfection.

CAT and Luciferase Assays. After being rinsed twice in PBS, transfected cells were harvested by scraping them in 1 mL of TEN (40 mM Tris, 1 mM EDTA, and 15 mM NaCl) into 1.5-mL centrifuge tube; the cells were collected by centrifugation, then resuspended in 150 μL of 0.25 M Tris, pH 7.8, and lysed by four rounds of freezing (in ethanol/dry

ice) and thawing (at 37 °C). Extracts to be assayed for chloramphenicol acetyltransferase (CAT) activity were heated at 65 °C for 10 min to inactivate endogenous acetyltransferases; 20–100 μL of extract was assayed in a final volume of 150 μL containing 0.25 M Tris, pH 7.8, 0.5 mg/mL acetyl-CoA, and 0.1 mCi of [^{14}C]chloramphenicol (10 mCi/mol) at 37 °C for 1–20 h. Acetylated products were separated from substrate by extraction with 300 μL of xylene (Shaw, 1975). After one back-extraction with 100 μL of 0.25 M Tris, pH 7.8, 200 μL of the organic phase was counted in a scintillation counter. CAT activity was expressed as the percent of [^{14}C]-chloramphenicol converted to acetylated products, minus background conversion of mock-transfected cells. Preparation of extract for luciferase and β-galactosidase assays was the same as for CAT assay except that the extract was not heated. Luciferase activity was assayed according to Brasier *et al.* (1989); 5–50 μL of extract was added to 300 μL of assay buffer (25 mM glycylglycine, pH 7.5, 4 mM EGTA, 15 mM $MgCl_2$, 15 mM potassium phosphate, 1 mM dithiothreitol, and 2 mM adenosine triphosphate), and luminescence was measured for 20 s in a luminometer (Lumat 9501, Berthold) after the addition of 150 μL of luciferin solution [0.2 mM luciferin (Sigma Chemical Co., St. Louis, MO), 25 mM glcylglycine, pH 7.4, 4 mM EGTA, 15 mM $MgCl_2$, and 2 mM dithiothreitol]. Luciferase activity was expressed as light emitted (relative light units, RLU) per minute per microgram of protein extract. β-Galactosidase activity was assayed as described (MacGregor *et al.*, 1991) and was expressed as the increase in A_{410nm} in a 30-min reaction per microgram of protein extract. Luciferase assay, β-galactosidase assay, and protein determination were all done in duplicate.

RESULTS AND DISCUSSION

Transcription Rate of mPFK-A during Myogenesis. We have previously shown that PFK activity increases significantly when C2 myoblasts are allowed to differentiate into myotubes and that this increase is due to an increase in synthesis of the muscle isoform of PFK (PFK-A) (Gekakis *et al.*, 1989). Furthermore, we demonstrated a 90-fold increase in steady-state mRNA levels of PFK-A during C2 differentiation. To see if this accumulation of mRNA is due to an increase in transcription of the gene, the rate of PFK-A transcription in isolated nuclei was measured. Nuclei were isolated from proliferating myoblasts or from myotubes after 3 days in differentiation medium. Nuclear run-on transcription was done in the presence of [α-^{32}P]UTP, and the labeled transcripts were hybridized to excess, denatured, plasmid DNA immobilized on nitrocellulose. The plasmids used were near-full-length PFK-A cDNA, muscle creatine kinase (MCK) cDNA, and mouse $\alpha_{cardiac}$-actin, which also cross-hybridizes to cytoplasmic β- and γ-actins. Figure 1 clearly shows that transcription of both PFK-A and MCK increases upon C2 myoblast differentiation to myotubes. In the experiment shown, PFK-A transcription increased 7-fold as quantitated by phosphorimaging when background hybridization to vector alone was subtracted. In each of two other experiments, each employing a separate preparation of nuclei, PFK-A transcription increased 5-fold. We therefore conclude that the accumulation of PFK-A mRNA during C2 myoblast differentiation is due, at least in part, to an increase in transcription of the gene. However, since the steady-state PFK-A mRNA level increases 90-fold during C2 myoblast differentiation, PFK-A mRNA stability may also be regulated.

Although not previously measured in C2 cells, MCK transcription reportedly increases 9-fold during differentiation

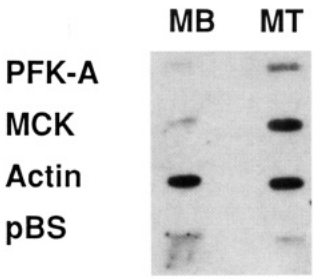

FIGURE 1: Transcription rate of PFK-A, MCK, and actin genes during C2 myoblast differentiation. Isolation of nuclei, preparation of ^{32}P-labeled RNA probe, and hybridization of probe to DNA are described under Experimental Procedures. The *in vitro* transcribed probes were MB (myoblast) and MT (myotube). The plasmids used were as follows: pBS, pBluescript II SK+; PFK-A, near-full-length mouse muscle phosphofructokinase cDNA in pBluescript II SK+; MCK, mouse muscle creatine kinase cDNA; actin, mouse $\alpha_{skeletal}$-actin cDNA.

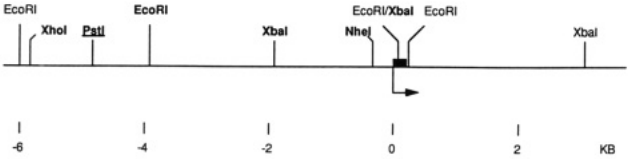

FIGURE 2: Partial restriction map of the 5′ portion of the mouse PFK-A gene. Genomic cloning and restriction mapping are described under Experimental Procedures. The filled box represents the first exon. The transcription start site (see below) is represented by an arrow. The restriction sites used in constructing CAT or luciferase fusion genes are shown in boldface type.

of a mouse myogenic cell line, MM14 (Jaynes *et al.*, 1986). In agreement with the data obtained in MM14 cells, MCK transcription increased in C2 cells as shown in Figure 1. However, the magnitude of the increase was somewhat smaller, only 5-fold. In myotubes, the rate of transcription of the MCK gene appears greater than that of PFK-A (Figure 1). However, transcription of MCK is also greater in myoblasts, leading to a greater induction of the PFK-A gene. When measured in MM14 cells, actin transcription increases dramatically during myogenesis. In C2 cells, however, we and others (Buonanno & Merlie, 1986) measured a slight decrease in total actin transcription during myogenesis. This decrease represents the combined effects on several structurally similar actin isoforms; transcription of α-actin increases (Seiler-Tuyns *et al.*, 1984) while that of cytoplasmic β- and γ-actins decreases.

Isolation and Characterization of the mPFK-A Gene. As a first step toward identifying the element(s) controlling muscle-specific transcription of the mPFK-A gene, the PFK-A gene was cloned by screening a mouse embryo genomic library. A 5′-end-labeled synthetic oligonucleotide, corresponding to bases 145–190 (amino acids 15–45) of the mPFK-A mRNA, and random-primer-labeled, near-full-length mPFK-A cDNA were used as probes to screen the library. A single clone which hybridized to both probes was picked and plaque-purified. A partial restriction map of this clone's 16-kb insert is shown in Figure 2; it contains the first several exons of PFK-A as well as 6 kb of 5′-flanking sequence. Sequencing a 197 bp *Eco*RI fragment revealed that it contains all the coding sequences of the first exon plus the 5′-end of the first intron; the coding sequence is identical to the corresponding sequence of the murine muscle cDNA which we have also cloned and sequenced (data not shown). The deduced amino acid sequence agrees with that of human PFK-A in 27 out of 28 amino acids (Nakajima *et al.*, 1987). 5′-Untranslated and

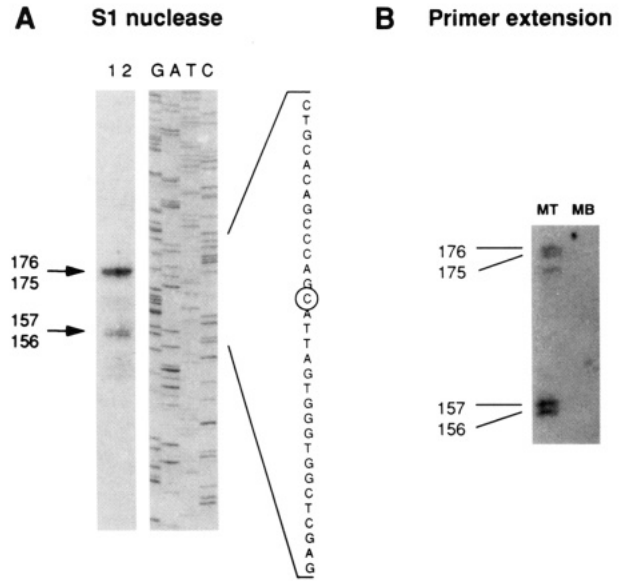

FIGURE 3: Mapping the transcription start site of the mouse PFK-A gene. (A) S1 nuclease assay. A portion of the mPFK-A gene was amplified with the oligonucleotides indicated in Figure 4 and labeled just at the 3′ end; the probe was hybridized to total myotube RNA, digested with S1 nuclease, and fractionated on a sequencing gel (see Experimental Procedures). The concentrations of S1 nuclease used were 18 (lane 1) and 90 (lane 2) units/mL. The resulting bands are presented next to a sequencing ladder primed with the 3′ oligonucleotide. The sizes of the digested DNA fragments are indicated. (B) Primer extension assay. The 3′ oligonucleotide indicated in Figure 4 was end-labeled, hybridized to myoblast or myotube poly(A+) RNA, extended with reverse transcriptase, and fractionated on a sequencing gel (see Experimental Procedures). The sizes of the resulting bands are indicated.

5′-flanking sequences are contained in an adjacent 4-kb *Eco*RI fragment.

Mapping of the mPFK-A Transcription Start Site. In order to identify the site of initiation of transcription, a 523 bp fragment of double-stranded DNA, which presumably spans the transcription start site, was amplified by PCR, end-labeled, and restriction-digested so that just the 3′-end was labeled. The 3′-end-labeled fragment was hybridized to myotube mRNA and digested with S1 nuclease. The products of digestion were fractionated on a sequencing gel. As a size marker on the gel, a sequencing ladder was run using the 3′ oligo from PCR as the primer. Figure 3A shows 4 major protected fragments of 176, 175, 157, and 156 nucleotides in length, representing 4 distinct transcription start sites 106, 105, 88, and 87 bp upstream of the translation initiation codon. A primer extension assay using 3′ oligo (Figure 3B) gave the same results when myotube mRNA was used. However, no bands were visible when myoblast mRNA was used, indicating a very low level of PFK-A mRNA present in myoblasts. This agrees with our previous finding that the steady-state PFK-A mRNA level in myoblasts is only 1% that in myotubes (Gekakis *et al.*, 1989). mRNA secondary structure can give rise to bands from primer extension and S1 nuclease analysis that are actually artifacts, leading to errors in the interpretation of such results. However, the fact that both S1 nuclease and primer extension analysis gave the same pattern of bands supports our interpretation: that there are four distinct transcription start sites in this area.

Across the 106 bp untranslated region there is 75% nucleotide identity between human and mouse muscle PFK and <30% identity between mouse muscle and mouse liver PFK (Rongnoparut *et al.*, 1991). As with the coding sequence, there is greater conservation of the 5′-untranslated sequence between the same isozymes from different species than between different isozymes in the same species. In both rabbit and human, there is evidence of an additional transcription start site and upstream exon for PFK-A (Nakajama *et al.*, 1990; Valdez *et al.*, 1989; Li *et al.*, 1990) which splices to an acceptor 8 bp upstream of the translation initiation codon. Our S1 nuclease assay also shows evidence of an upstream exon; a 78-base band is present which would correspond to a splice acceptor 8 bp upstream of the translation initiation codon (data not shown). However, this band is <10% the intensity of the major bands.

Figure 4 shows the sequence of the first exon (191 bp) as well as 1267 bp of 5′-flanking sequence and 95 bp of the first intron. Also shown ar *Xba*I and *Nhe*I sites, used in constructing PFK-A/CAT fusion genes. There is no discernible TATA box; however, there are three putative Sp1 binding sites. There are no consensus G/C boxes, GGCGGG; however, Gustafson and Kedes (1989) defined another consensus Sp1 binding site of (G)GGGAGGG(G) by electrophoretic mobility shift assay and footprinting of the human $\alpha_{cardiac}$-actin promoter with purified Sp1. This sequence is present 3 times in the mPFK-A promoter (Figure 4). Several other sequences with similarity to previously identified muscle-specific elements are indicated in Figure 4. The E-box is the core binding site of the basic helix–loop–helix (bHLH) family of transcription factors, which includes muscle-specific (MyoD, myogenin, myf5, and MRF4) and general (E12 and E47) transcription factors (Blackwell & Weintraub, 1990). Its consensus sequence, CANNTG, is present in the mPFK-A promoter at −174. An E-box in the muscle creatine kinase enhancer which binds MyoD, CACCTG (Lassar *et al.*, 1989), is present in the 5′-untranslated region of mPFK-A. Also present are three copies of the M-CAT sequence CATTCCT, which is present in the distal promoter region of cardiac troponin T gene and is required for its muscle-specific transcription (Mar & Ordahl, 1990).

Presence of Sequences Responsible for Muscle-Specific Expression of PFK-A. Plasmids containing PFK-A 5′-flanking sequences linked to coding sequences of the bacterial chloramphenicol acetyltransferase (CAT) gene were cotransfected into C2 myoblasts with a plasmid containing coding sequences for the neomycin resistance gene driven by the SV40 promoter and enhancer (pSV2Neo). Initially plasmids were constructed using PFK-A sequences from −1900 (1900CAT) or −335 (335CAT) bp to +99 bp (relative to the start site of transcription) to drive expression of the CAT gene. Cell lines were established by pooling neomycin resistant colonies (>100 colonies/plasmid) for each CAT construction transfected. For each cell line, cells were plated in six 100-mm plates and grown for 2 days in growth medium. Three plates were then harvested as myoblasts while the other three were switched to fusion medium and harvested 2 days later as myotubes. Greater than 50% of the cells were fused after 2 days in fusion medium. Each plate was assayed separately for CAT activity. The average activity of 335CAT in myoblasts and myotubes was 15% and 13%, respectively, of [^{14}C]chloramphenicol converted to acetylated products (see Experimental Procedures). 1900CAT gave activities of 24% and 25% conversion, respectively, in myoblasts and myotubes. In neither case was there a significant difference in CAT activity between myoblasts and myotubes. Transient transfection studies showed no muscle-specific enhancer activity up to −3900 bp (data not shown). These data indicate that the E-box sequence is not responsible or at least not sufficient for muscle-specific expression of the PFK-A gene.

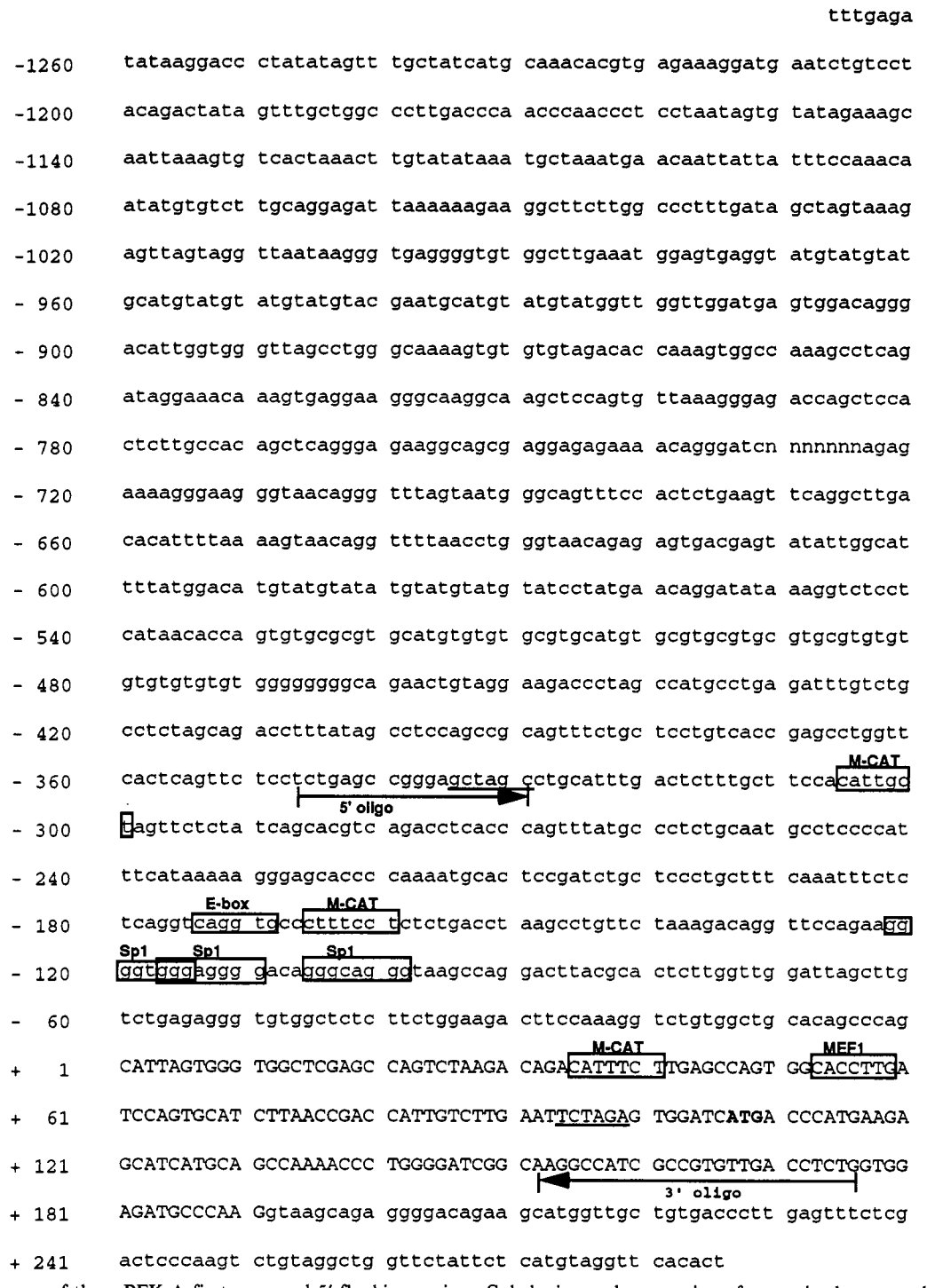

FIGURE 4: Sequence of the mPFK-A first exon and 5'-flanking region. Subcloning and sequencing of genomic clones are described under Experimental Procedures. The transcribed sequence is shown in capital letters. Sp1 binding sites, an E-box sequence, and M-CAT and MEF1 elements are boxed (see Results and Discussion). The translation initiation codon is indicated in boldface letters, and the synthetic oligonucleotides used for PCR and primer extension are indicated by arrows. Restriction sites used for promoter/reporter fusion gene construction are underlined.

Since no muscle-specific enhancer activity was found between −3900 and +99 bp of the PFK-A gene, a plasmid containing from −5800 to +99 of mPFK-A driving CAT expression (5800CAT) was constructed. C2 myoblasts were transfected with 5800CAT and 335CAT 1 day after plating; 2 days after transfection, myoblasts were harvested or switched to fusion medium. Myotubes were harvested after 2–3 days in fusion medium. Cell extracts were prepared by freeze/thaw and assayed for CAT activity in the presence of [^{14}C]-chloramphenicol. Figure 5 shows that 335CAT gave CAT activities of 0.32% and 0.21% [^{14}C]chloramphenicol converted to acetylated products in MB and MT, respectively. 5800CAT gave activities of 1.85% and 5.77% conversion, respectively, in myoblasts and myotubes, for a 3.11-fold induction in myotubes (significantly different than 1.00 at $p < 0.005$). This represents an average of seven experiments done with four different preparations of the same plasmid. Likewise, MCK3300CAT, which has been reported to increase 50-fold during muscle differentiation (Sternberg *et al.*, 1988), increased 7.82-fold in our experiments. Transient transfection assays with PFK-A/CAT constructs show that an element responsible for muscle-specific transcription is present between −5800 and −335 of mPFK-A while stable and transient transfections show no muscle-specific element between −3900

	CAT Activity (% conversion) MB	MT	fold induction
PFK-A-5800 CAT	1.85 ±0.29	5.77 ±1.01	3.11
PFK-A-335 CAT	0.32 ±0.13	0.21 ±0.08	0.66
MCK-3300 CAT	3.30 ±0.97	25.79 ±4.01	7.82

FIGURE 5: Expression of PFK-A– or MCK–CAT fusion genes during C2 myoblast differentiation to myotubes. Transfection, harvesting of cells, and CAT assay are described under Experimental Procedures. The plasmids transfected were the following: PFK-A-5800, –5800 to +99 of mouse muscle phosphofructokinase linked to CAT coding sequences; PFK-A-335, –335 to +99 of mouse muscle phosphofructokinase linked to CAT coding sequences; MCK-3300, –3300 to +91 of mouse muscle creatine kinase linked to CAT coding sequences. The numbers for PFK-A-5800 are the average of seven transfections using four different preparations of the same plasmid, for PFK-A-335 two experiments, and for MCK-3300 four experiments.

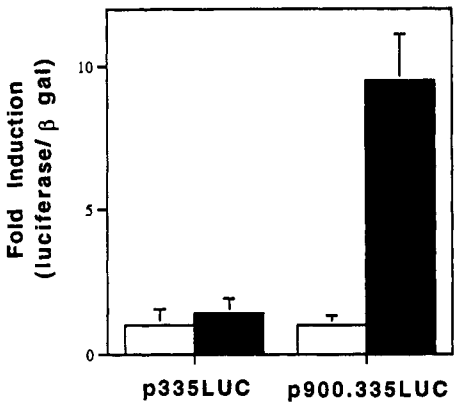

FIGURE 6: Expression of PFK-A/luciferase fusion genes in C2 myotubes. Transfection, harvesting of cells, and luciferase assay are described under Experimental Procedures. The plasmids transfected were as follows: 335LUC, mouse muscle phosphofructokinase sequences from –335 to +99 linked to luciferase coding sequences; 335LUC.900, mouse muscle phosphofructokinase sequences from –4800 to –3900 and from –335 to +99 linked to luciferase coding sequences. The graph represents an average of two experiments each done with two different preparations of each plasmid. In each experiment, duplicate plates of C2 myoblasts or myotubes were transfected. (□) MB; (■) MT.

and +99. We therefore conclude that an element responsible for tissue-specific expression of mPFK-A is present between –5800 and –3900.

To further delimit the regions of PFK-A's 5′-flanking sequence which direct muscle-specific transcription, plasmids were constructed in which luciferase expression was driven by the PFK-A proximal promoter (–335 to +99). We have found that this promoter drives high-level CAT expression in the presence of a heterologous enhancer (data not shown). Various fragments of PFK-A 5′-flanking sequence were cloned upstream of the promoter. Comparison of luciferase activity in myoblasts and myotubes transfected with the basal promoter construct (p335LUC) or with a construct which links a 900 bp fragment (–4800 to –3900) to the basal promoter (p900.335LUC) shows that the upstream 900 bp fragment contains muscle–specific enhancer activity (Figure 6). After correction for the activity of the cotransfected β-galactosidase expression plasmid, the luciferase activity of p335LUC remains approximately the same during *in vitro* muscle differentiation. p900.335LUC shows an approximately 10-fold increase in luciferase activity upon differentiation of myoblasts to myotubes, indicating the presence of a muscle-specific element in the 900 bp fragment from –3900 to –4800.

Muscle meets its specialized energy requirements by expressing muscle-specific isoforms of energy-metabolizing enzymes. The muscle-specific isozyme of phosphofructokinase, an important glycolytic enzyme, is encoded by a distinct gene which is expressed mainly in muscle cells. Like muscle creatine kinase, another important energy-metabolizing enzyme in muscle, the PFK-A gene is transcriptionally regulated during muscle differentiation. Transcription of the PFK-A gene increases (5.6 ± 1.2)-fold during *in vitro* myogenesis. Although we found muscle-specific enhancer-like sequences in the PFK-A proximal promoter, these sequences by themselves are not sufficient to direct muscle-specific expression of the mPFK-A gene. Transcriptional activation of PFK-A is controlled by a distal upstream cis-acting sequence between 3900 and 4800 bp upstream of the transcription start site.

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