Functional activity of the two promoters of the myosin alkali light chain gene in primary muscle cell cultures: comparison with other muscle gene promoters and other culture systems

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ABSTRACT

Proximal upstream flanking sequences of the mouse myosin alkali light chain gene encoding MLC1_F and MLC3_F, the mouse α -cardiac actin gene and the chicken gene for the α -subunit of the acetylcholine receptor were linked to the bacterial chloramphenicol acetyl transferase (CAT) gene and transfected into primary cultures derived from mouse skeletal muscle or into myogenic cell lines. We demonstrate that the mouse MLC1_F/MLC3_F gene has two functional promoters. In primary muscle cultures, a 1200 bp sequence flanking exon 1 (MLC1_F) and a 438 bp sequence flanking exon 2 (MLC3_F) direct CAT activity in myotubes, but not in myoblasts or in non myogenic 3T6 and CV1 cells. Developmentally regulated expression is also seen with the α -cardiac actin (320 bp) and acetylcholine receptor α -subunit (850 bp) upstream sequences in the primary culture system. Transfection experiments with myogenic cell lines show different results with a given promoter construct, reflecting possible differences in the levels of regulatory factors between lines. Different muscle gene promoters behave differently in a given cell line, suggesting different regulatory factor requirements between these promoters.

INTRODUCTION

The two myosin alkali light chains expressed in adult fast skeletal muscle, MLC1_F and MLC3_F, are encoded by the same gene (1, 2, 3). The COOH terminal parts of the proteins and the 3' non coding sequences of their corresponding mRNAs are encoded by 5 common exons, preceded by an exon specific for MLC1_F, two exons specific for MLC3_F and a further 5' upstream exon specific for MLC1_F. The NH₂ terminal coding sequences of MLC1_F and MLC3_F, together with the 5' non coding mRNA sequences are thus located within exons one and two respectively. Primer extension and nuclease protection experiments demonstrate the presence of two transcripts with distinct cap sites in the steady state mRNA population (1, 2, 3, 4). Furthermore, two distinct precursor transcripts are present in the nuclear polyA⁺ RNA population (2). These results, therefore, suggest that the gene has two distinct

promoters, which give rise to the MLC1_F and MLC3_F transcripts. This is further indicated by in vitro "run off" experiments using total cell extracts from Hela cells (5). However, in order to show conclusively that the gene has two functional promoters, it is necessary to demonstrate transcriptional activity from these two regions in differentiated muscle cells, where the endogenous gene is expressed. The putative promoter region of MLC3_F in the mouse, rat or chicken genes for example, does not contain a perfect CAAT type consensus sequence, nor the standard TATA box, but rather CAACT and AAATAA (4). While similar sequences have been described in other RNA pol II promoters, it is particularly important to demonstrate muscle specific functional activity in the case of this postulated internal gene promoter. We have therefore examined the capacity of the putative MLC1_F and MLC3_F promoter regions to initiate transcription of the bacterial chloramphenicol acetyl transferase (CAT) gene when transfected into differentiated muscle cells in culture.

Such experiments are also important in order to establish a functional assay system for transcriptional regulatory sequences in the promoter regions of these muscle genes. In order to ascertain whether some tissue specific regulatory elements are present, transcriptional activity directed by the transfected fragment is compared with that of the endogenous muscle gene. In most muscle cell lines, the latter is characterized by (a) strong expression in differentiated muscle cells (myotubes), (b) no expression in undifferentiated muscle cells (myoblasts) or in non-muscle cells. Most transfection experiments with muscle genes or their 5' upstream sequences, to date, have been performed with rat or mouse myogenic cell lines. Variable results have been obtained ranging from a high level of activity in differentiated myotubes only, for example with the 5' promoter region of the chicken gene coding for the α -subunit of the acetylcholine receptor (AChR) (6), to expression but no apparent regulation between myoblasts and myotubes with avian and mammalian α -cardiac actin genes (7, 8). In general these results would indicate that although sequences adjacent to or within a muscle gene may be sufficient to mimic the expression of the endogenous gene in transfection experiments, in most cases some significant level of expression in myoblasts is also observed. This may reflect the fact that other sequences not included in the genomic fragments tested are important for developmental specific regulation. It may also indicate that the muscle

cell culture systems used to test the transfected gene are transcriptionally "leaky" at some level. The transfection protocol does not seem to be an important factor. Similar results were obtained for example with promoter CAT constructs (CAT assays) or with the complete gene (S1 mRNA analysis), in both transient and permanent transformants with the human α -cardiac actin gene (8). Primary muscle cell cultures offer an alternative system where the regulation of exogenous muscle genes may reflect more closely the *in vivo* situation. This is suggested by transfection experiments with chicken α -skeletal actin gene sequences in primary cultures from chick skeletal muscle (9, 10) and with the rat cardiac myosin heavy chain gene in primary cardiac cell cultures where a regulatory effect of thyroid hormone was reproduced with the exogenous transfected gene promoter (11). We report here the results of experiments in which the 5' upstream regions of the mouse myosin alkali light chain gene (4), the mouse α -cardiac actin gene (12) and the chicken gene for the α -subunit of the AChR (6) were transfected into myoblasts and myotubes of primary cultures derived from mouse skeletal muscle. The degree of regulation seen with these promoter regions in primary cultures is compared with the more variable results obtained in transfection experiments with different established muscle cell lines.

MATERIALS AND METHODS.

Cell culture conditions.

Primary cultures of mouse myoblasts. Primary cultures of mouse muscle cells were prepared using two different methods, both of which gave the same experimental results. Using the first protocol, muscle cells were prepared from 1-2 days old C3H mice. Leg muscles were enzymatically digested in medium MCDB 202 containing 0.1% trypsin (Boehringer) and type V collagenase (Sigma) at 0.5 mg/ml during 10 min. at 37°C. The enzymatic action was stopped by addition of fetal calf serum (FCS). This procedure was repeated 4 times and the cells were pelleted by centrifugation at 1500 g for 5 min. The cells were plated at 10^3 cells by cm^2 on tissue culture dishes (Falcon) in MCDB 202 containing 20% FCS and 10^{-6} dexamethasone and grown at 37°C in a humidified atmosphere of 95% air: 5% CO_2 . The medium was changed every 3 days. After 6 days, the cells were frozen in 90% FCS and 10%

DMSO (Merk). All the experiments were performed 2-10 passages after thawing. Undifferentiated muscle cells were obtained by growing cells during 4 days in MCDB 202 containing FCS and dexamethasone. Myotubes were obtained from undifferentiated cells by changing the medium to Dulbecco's modified Eagle medium (DMEM) containing 10 µg/ml of bovine insulin. After 48 hours, more than 80 % of nuclei were incorporated into muscle fibers.

Using the second protocol, hind limbs of fetal or newborn C3H mice were aseptically dissected to separate skeletal muscle from skin and bone tissue. Enzymatic dissociation of muscle tissue was carried out according to (31) using 5 ml of sterile enzyme solution composed of 0.05 % type II collagenase (Sigma), 0.15 % trypsin and 0.1 % of bovine serum albumin in DMEM per 150 mg of tissue. After incubation in a shaking water bath at 37°C for 15 min., supernatant was removed and neutralized with an equal volume of DMEM supplemented with 10 % of fetal bovine serum (FBS). Fresh enzyme solution was added to the remaining tissue and incubated as before. The whole procedure was repeated 2-3 times. Neutralized cells were pooled and centrifuged, and then plated on gelatin coated 100 mm-diameter plates in DMEM supplemented with 20 % FBS (10⁶ viable cells per plate), medium was changed the following day for fresh medium and then changed to DMEM containing 5 % horse serum, to promote fusion.

Cell lines. Primary cultures of Soleus mouse muscle were prepared from 4 weeks old C3H mice using the first protocol previously described. After the second splitting, the cells were cloned by limiting dilution in 24 multiwell dishes. 10 clones were randomly selected. Their growth kinetics (doubling time of 16-18 hours) and their ability to differentiate in the presence of FCS or in the presence of insulin were very similar. The experiments described here were performed with clone 8 and we named this new cell line sol8. The characteristics of growth and differentiation of these cells have not changed after 12 months in culture.

Myogenic C2.7 cells (32) were grown in DMEM medium supplemented with either 20 % FCS, or 2 % FCS as the fusion medium. Non myogenic 3T6 mouse fibroblasts and CV1 monkey kidney cells were cultivated in DMEM containing 7 % FCS. Mouse T984 clone 10 cells were cultivated as described in (23) and quail Q NY ts68 cells as described in (22).

Construction of recombinant plasmids and preparation of plasmid DNA.

A 2.2 Kb BamHI fragment and a 1.85 Kb HindIII fragment, containing respectively exon 1 and exons 2, 3 and 4 of the mouse MLC1_F/MLC3_F gene, were purified from a recombinant phage λ 492 (phage C: see reference 1) and subcloned in the vector pEMBL8+ (33) in order to obtain plasmids B3 and H4. A 474 base pair HaeIII-HpaI fragment and a 1200 base pair A_{va}I fragment were purified from plasmid B3, and the cohesive ends were converted to blunt ends by filling in with *E.coli* DNA polymerase large fragment (Klenow enzyme, Boehringer). These fragments were respectively subcloned in the HindIII site of vectors pSVOCAT (13) and pSB1 (14) either by blunt end ligation or ligation after addition of 5' phosphorylated linkers (Biolabs), giving rise to plasmids pMLC1 CAT 0.47 (+) and (-), and pMLC1 CAT 1.2 (+) and (-). A 453 base pair HaeIII-Fnu4H1 fragment was purified from plasmid H4 and, after conversion of cohesive ends into blunt ends and addition of HindIII linkers, this fragment was cloned into the HindIII site of the pSVOCAT vector to generate plasmids pMLC3 CAT (+) and (-). A 439 base pair SstI-BamHI fragment was purified from the recombinant phage λ IG 10 containing the 5' portion of the mouse α -cardiac actin gene (Garner et al., 1986), and, after addition of HindIII linkers, was cloned into the HindIII site of pSVOCAT in order to obtain plasmid p α _CA CAT (+). Construction of plasmid p α ACh CAT (+) has been described in (6). Large scale preparations of plasmid DNA were prepared either from cleared bacterial lysates or from SDS-sodium hydroxide preparations by banding on ethidium bromide-caesium chloride gradients. All preparations to be used for CAT assays were purified on two successive gradients.

Cell transfection and CAT assays.

Non myogenic 3T6 and CV1 cells were split 1:10 into 100 mm-diameter dishes the day before transfection. For myogenic cells, they were transfected either at low density, when they were semi-confluent and still proliferating, and the cells were harvested 24 hours after transfection, or when they were confluent and beginning to fuse and they were harvested 24 or 48 hours after transfection. Fresh medium was added to the cells 2 to 4 hours before transfection. Transfection mixtures were prepared as follows per 100 mm-diameter dish : to 0.5 ml of 2 X N-2-

hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES)-buffered saline (42mM HEPES pH 7.1, 270mM NaCl, 10mM KCl, 1.4 mM Na₂HPO₄) (HBS) was added dropwise to 0.5 ml of a solution of 250mM CaCl₂ containing 10 µg of plasmid DNA, with constant mixing of the 2 X HBS solution. This quantity of DNA was checked to be within the linear range of CAT activity for the plasmid used. The calcium phosphate DNA precipitate was left for 20 min at room temperature and added dropwise to 10 ml of medium in the tissue culture dish. The cells were left for 5 to 15 hours at 37°C and then medium was changed for fresh medium and cells were left for an additional 24 to 48 hours. Cells were rinsed with phosphate buffered saline (PBS) and scraped of the dish in 1 ml of Tris-HCl pH 7.4 / 1 mM EDTA / 0.15 M NaCl. Cells were pelleted and resuspended in 150 µl of Tris-HCl pH 7.8 250mM, 15% glycerol, and cell extract prepared by sonication in a cell disruptor. After heating at 60°C for 10 min, 5 to 70 µl of extract was incubated with 0.1 to 0.5 µCi of D-threo-(dichloroacetyl-1-¹⁴C) Chloramphenicol (Amersham, U.K.) in the presence of 0.5mM of acetylCoA, at 37°C for 15 to 150 min. Chloramphenicol was extracted and analysed on thin-layer silica gels (Polygram sil-G, Macherey- Nagel) as described by (13). Quantitation of CAT assays was performed by scintillation counting of the appropriate areas of the chromatograms, and all CAT measurements were carried out under conditions in which no diacetylated chloramphenicol was detectable on the chromatograms. CAT activity was expressed as the percentage of chloramphenicol acetylated by 10 µl of cell extract in a 5 min reaction.

RESULTS

Sequences Immediately 5' of the Cap sites of MLC1F and MLC3F in the gene coding for the two myosin alkali light chains promote tissue specific expression of the bacterial CAT gene in primary muscle cell cultures.

Genomic DNA fragments encompassing the proposed Cap sites and 5' upstream regions of the first exons coding for MLC1_F and for MLC3_F in the mouse fast skeletal myosin alkali light chain gene, were isolated from the appropriate plasmid subclones derived from the original lambda phage recombinant (1). In the case of MLC1_F, two fragments were used. One of 1200 base pairs located between two Ava II restriction sites contains 7 base pairs of 5' non-coding mRNA sequence ,

Figure 1. A) Schematic representation of the 5' portion of the mouse MLC1_F/MLC3_F gene and of the MLC1_F and MLC3_F promoter regions used to construct the CAT recombinant vectors. Exon 1, specific for MLC1_F, is separated from exon 2, specific for MLC3_F, by an intronic region of 10 Kb. Fragments used for constructing pMLC1 CAT and pMLC3 CAT recombinant vectors are represented. Numbering, for the different restriction sites, is given taking the transcription start sites of either MLC1_F or MLC3_F as +1. Positions of CAAT and TATA boxes and of ATG initiation codons are indicated. Dashed boxes and open boxes represent respectively the 5' non coding and coding sequences in exons 1 and 2; numbers above them refer to their length in terms of nucleotides.

B) Structure of the CAT recombinant vectors used in the transfection experiments. DNA fragments of the promoter regions of MLC1_F, MLC3_F, α -cardiac actin and α AChR are represented. Numbering is given taking the different transcription start sites, indicated by a broken arrow, as +1; TATA and CAAT sequences are shown. Boxes represent 5' non coding sequences of the different genes. Plasmid pSB1 or pSV0CAT is drawn and the Hind III site, into which the different promoter fragments have been cloned, is indicated. The thin line, in the vector, represents either pBR322 or SV40 sequences.

the proposed Cap site, TATA and CAAT boxes (Figure 1). The second was a shorter fragment of 474 base pairs located between an Hae III and a Hpa I site, containing 105 base pairs of 5' non-coding sequence together with 369 base pairs upstream from the proposed Cap site including the same sequence elements mentioned above (Figure 1). For MLC3_F, a fragment of 453 base pairs delimited by an Hae III and an Fnu4HI site was isolated. This contained 15 base pairs of 5' non-coding mRNA sequence from the second exon of the gene which corresponds to the first specific exon encoding the NH₂ terminal end of MLC3_F, the major Cap site and the "pseudo" TATA box (AAATAAAA) of this supposed promoter region (4). The three genomic fragments were inserted into the Hind III site of the vectors pSVOCAT or pSB1 (13, 14) upstream of the bacterial chloramphenicol acetyl transferase (CAT) gene to give the constructions pMLC1 CAT 1.2 (+), pMLC1 CAT 0.47 (+) and pMLC3 CAT (+) (see Figure 1). None of these recombinants contain the initiation codon ATG present in the first or second exon of the myosin light chain gene; the initiation codon immediately downstream of the Hind III site is that of the bacterial CAT gene. As controls for transfection experiments, the different fragments were also integrated into the Hind III site of pSB1 in the opposite orientation (3'-5') from that in the gene to give pMLC1 CAT 1.2 (-), pMLC1 CAT 0.47 (-) and pMLC3 CAT (-). The following CAT reference vectors were also used as negative controls, namely pSB1, the promoterless (and enhancerless) vector used for the pMLC1 CAT 1.2 constructions, and the closely related pSVOCAT vector, used for pMLC1 CAT 0.47 and pMLC3 CAT. The vectors pRSV CAT and pSV2CAT (15, 13) in which the CAT gene is under the control of the LTR of the Rous sarcoma virus, or the early promoter and enhancer of the SV40 virus, respectively, were used as positive controls.

Primary cultures of myoblasts were prepared from newborn mouse C3H (or C57Bl, results not shown) skeletal muscle and either used as such, or myogenic cells were propagated and frozen in aliquots, to be re-plated as dividing myoblast cultures when required (see Materials and Methods). Muscle marker proteins and their mRNAs, such as the two fast skeletal myosin light chains MLC1_F and MLC3_F are not detectable in dividing muscle cell cultures, and only accumulate in myotubes (16, 17). Similar results were obtained with both culture procedures. Myosin light chain and control constructions were introduced into muscle cell

Figure 2. Chromatograms of CAT assays after transfection of muscle cells in primary cultures and of non-muscle cells.

CAT activity was assayed after transfection with different CAT constructs :

A) in extracts from undifferentiated myoblasts in primary cultures, harvested 24 hours after transfection. Lane 1, pSB1 ; lane 2, pRSV CAT; lane 3, pMLC1 CAT 1.2 (+); lane 4, pMLC3 CAT (+); lane 5, p $\alpha_{\text{C}}\text{A}$ CAT (+); lane 6, p αACh CAT (+). Half of the extract, eg. 70 μl /150 μl , was used in these assays, in a 30 min incubation for pRSV CAT and 160 min for the others.

B) in extracts from differentiated myotubes in primary cultures, harvested 48 hours after transfection. Lane 1, pSB1 ; lane 2, pRSV CAT; lane 3, pMLC1 CAT 1.2 (+); lane 4, pMLC1 CAT 1.2 (-); lane 5, pMLC1 CAT 0.47 (+); lane 6, pMLC3 CAT (+); lane 7, pMLC3 CAT (-); lane 8, p $\alpha_{\text{C}}\text{A}$ CAT (+); lane 9, p αACh CAT (+). 70 μl of cell extract were used in a 30 min incubation for pRSV CAT and 50 μl in a 120 min incubation for the others.

C) in extracts from non-muscle 3T6 cells, harvested 48 hours after transfection.

Lane 1, pSB1 ; lane 2, pRSV CAT; lane 3, p αACh CAT (+); lane 4, p $\alpha_{\text{C}}\text{A}$ CAT (+); lane 5, pMLC1 CAT 1.2 (+); lane 6, pMLC3 CAT (+). All incubations were done for 150 min with 70 μl of cell extract. N= non acetylated chloramphenicol, M= monoacetylated forms of chloramphenicol .

cultures by the classical technique of co-precipitation with calcium phosphate (15). In all the experiments described here the transient expression of the CAT gene was measured by dosing enzyme activity in cell extracts . Either dividing myoblasts were transfected and then harvested 24 hours later when still proliferating, or confluent cultures were transfected and harvested 48 hours later when already containing many multinucleated fibers. In such primary cultures the percentage of nuclei in myotubes is difficult to estimate . However, in the primary cultures which had been replated after freezing, the cells were grown under different conditions and

differentiated at lower density and we estimate in this case that more than 50% of all nuclei were in myotubes. In order to minimize variations due to differences in transfection efficiency, duplicate dishes were transfected with the same construction in each experiment.

Results of a representative transfection experiment are shown in Figure 2B. As seen on the chromatogram a significant level of CAT activity, indicated by the conversion of the non acetylated form of chloramphenicol (N) into monoacetylated forms of chloramphenicol (M) is seen in differentiated primary cultures transfected with the pMLC1 CAT 1.2 (+) and pMLC3 CAT (+) constructions. In contrast the constructions pMLC1 CAT 1.2 (-) and pMLC3 CAT(-) with the inverse orientation of the MLC fragments show virtually undetectable levels of CAT activity, suggesting that transcriptional initiation is directed by promoter sequences in the MLC1_F and MLC3_F fragments. The pMLC1 CAT 0.47 (+) construction containing a shorter fragment was not transcriptionally active. This may indicate the presence of key positive regulatory elements in the -369 to -1200 region 5' upstream of the MLC1_F Cap site (see Discussion).

Results from a number of experiments are summarized in Table I. Variations in the level of CAT activity for a given vector between primary culture experiments (which may be as much as x 5) have been compensated for by taking the average figure over several experiments. The variability may reflect differences in the extent of differentiation in a culture dish, since this is difficult to control in primary muscle cell cultures. The results shown in Table I demonstrate that both the MLC1_F and MLC3_F promoter constructs are active in differentiated muscle cells. They give CAT activities which are significantly above those of the negative controls. The pSB1 promoterless vector gives a level of CAT activity which is higher than that seen with the inverse orientation MLC promoter constructs. This CAT activity is probably due to readthrough transcripts originating more upstream, in the prokaryotic part of the pSB1 vector (18). Insertion of DNA sequences in any orientation usually lowers this background CAT activity. The presence of the reverse orientation MLC fragment seems to block such "read through". The positive control, with a viral promoter in pRSV CAT, gives a CAT activity in the differentiated cultures which is two orders of magnitude higher than that seen with the pMLC1 CAT 1.2 (+)

	C3H Myoblasts	C3H Myotubes	3T6	CV1
pMLC1 CAT 1.2 (+)	0.004	0.175	0.0002	0.0005
pMLC1 CAT 0.47 (+)	ND	0.001	ND	ND
pMLC1 CAT 1.2 (-)	ND	0.0006	ND	ND
pMLC3 CAT (+)	0.038	0.35	0.0008	0.011
pMLC3 CAT (-)	ND	0.0003	ND	ND
p alpha cA CAT (+)	0.011	0.22	0.017	0.42
p alpha ACh CAT (+)	0.029	0.73	0.0003	0.0015
pSB1	0.028	0.009	0.0006	ND
pRSV CAT	1.66	19.1	0.19	3.1

Table 1. Normalized CAT activity in undifferentiated myoblasts and differentiated myotubes in primary cultures, and in non-muscle 3T6 and CV1 cells. CAT activity was expressed as the percentage of chloramphenicol acetylated by 10 μ l of cell extract in a 5 min reaction. Values represent the average of 3 to 5 independent experiments (all transfections were performed on duplicate dishes). All CAT measurements were done under conditions in which no diacetylated form of chloramphenicol was detectable on the chromatograms. ND = not determined.

construction in this culture system. The pMLC3 CAT (+) plasmid gives consistently higher CAT activities than pMLC1 CAT 1.2 (+). Similar results were obtained when primary cultures of differentiated chicken myotubes were transfected in the same way : the transient CAT activity obtained with pMLC3 CAT (+) was also consistently higher (at least 4 times) than that obtained with pMLC1 CAT 1.2 (+) (results not shown).

In the mouse fibroblast 3T6 cell line (see figure 2 and Table I) the MLC1_F and MLC3_F promoter regions give low background levels of CAT activity, similar to that with pSB1, which is several hundred fold lower than with the pRSVCAT vector. In another non-muscle cell line CV1, derived from monkey kidney cells, pRSV CAT activity is higher than that obtained in 3T6 cells, suggesting that transfection may be more efficient in this cell line. pMLC1 CAT 1.2 (+) continues to give negligible CAT activity, and pMLC3 CAT(+) promotes a low level of CAT activity in these cells.

From the results of the transfection experiments with differentiated muscle cell cultures, compared with those with non-muscle cell lines, we conclude that the proximal promoter regions of 1.2 Kbp and 438 bp respectively for MLC1_F and MLC3_F contain regulatory elements conferring tissue-specific expression.

The 5' flanking regions of several muscle genes promote developmentally regulated expression in primary muscle cultures.

We next wanted to establish whether these transfected genes are regulated in parallel with the endogenous gene, which is expressed in myotubes, but not in

dividing myoblasts. The CAT constructions were transfected into mononucleated primary cultures and cell extracts were prepared 24 hours later, before formation of any myotubes, when the mononucleated cells were still proliferating. Two other muscle gene promoters were also tested in these experiments: $\text{p}\alpha_{\text{C}}\text{A CAT}(+)$ and $\text{p}\alpha\text{ACh CAT}(+)$. The $\text{p}\alpha_{\text{C}}\text{A CAT}(+)$ vector contains a 439 base pair fragment derived from the 5' end of the mouse α -cardiac actin gene (12), cloned into the Hind III site preceding the CAT gene in pSV0CAT (see Figure 1). This fragment contains 320 base pairs upstream from the Cap site (including the TATA and CAAT boxes), the untranslated first exon (50 base pairs) followed by 69 base pairs of the first intron of the gene. The plasmid $\text{p}\alpha\text{ACh CAT}(+)$ contains 850 base pairs of flanking sequence upstream of the Cap site (including the TATA and CAAT consensus sequences) and 20 base pairs of non-coding mRNA sequence from the first exon of the chicken gene encoding the α -subunit of the AChR (Figure 1, and reference 6). It has been shown previously to promote a high level of CAT activity in differentiated cultures of the mouse muscle cell line C2/7 but not in myoblasts of this cell line nor in non-muscle 3T6 cells (6).

An example of a transfection experiment with the different muscle promoters is shown in Figure 2. Quantitation of CAT activities averaged over several experiments is shown in Table I. In differentiated cultures, both the $\text{p}\alpha_{\text{C}}\text{A CAT}(+)$ and $\text{p}\alpha\text{ACh CAT}(+)$ constructions give significant levels of CAT activity. Similar results were also obtained with differentiated primary muscle cultures from chicken (results not shown). In myoblasts the different muscle promoters give rise to CAT activities of the same order as that given by the promoterless vector pSB1, pMLC1 CAT 1.2(+) giving a lower average value. The pRSVCAT construction which is expressed in dividing myoblast cultures provides a positive control. However, it is difficult to make a direct comparison of the promoter activities in myoblasts relative to myotubes, because of the rather high background value given by the promoterless pSB1 control. Potential differences in transfection efficiency or CAT stability between these two cell populations also complicate such an analysis. However, the following observations suggest that the transfection efficiency is not very different between undifferentiated and differentiated muscle cells. The number of positive cells, as

assessed by in situ staining for the b-galactosidase activity, is comparable when undifferentiated or differentiated cultures are transfected with the SV40 early promoter linked to the b-galactosidase gene marker (19). Since the transfection efficiency in such transient expression experiments is very low -less than 1 % of the cell population - it is also important to note that myotubes stain positively in the differentiated cultures (S. Alonso, personal communication).

With these comments in mind, table I shows that all muscle promoter CAT constructions tested here rise from background activities in myoblasts to significant activities in myotubes. This represents an up to 45-fold increase (for pMLC1 CAT 1.2(+)) . The viral RSV promoter also appears more active in differentiated primary muscle cell cultures. This may indicate that factors recognized by the viral promoter accumulate in myotubes; some of these factors may be common to the viral and muscle gene promoters (see discussion).

We conclude from these transfection experiments using primary muscle cell cultures, that significant differences in promoter activity between undifferentiated and differentiated cultures are seen with the four muscle gene 5' flanking sequences described, and that these sequences therefore undergo a developmental regulation in this culture system which mimics to some extent that seen with the corresponding endogenous genes.

The activity of the 5' flanking regions of different muscle genes in promoting CAT gene expression in muscle cell lines.

The same plasmid constructions already described (see Figure 1) were used in these experiments. The following established muscle cell lines were used : the C2.7 mouse cell line, which is a subclone of the line C2 originally isolated from regenerating cells in adult mouse (C3H) muscle (20), the T984 clone 10 line which was isolated from a region of skeletal muscle in the embryo body of a mouse (C3H) teratocarcinoma (21), the sol8 cell line isolated from the slow soleus muscle of a 4 week old C3H mouse (C.Pinset, unpublished results), and an avian muscle cell line, Q NY ts 68, which originated from a quail myoblast cell transformed by a thermosensitive Rous sarcoma virus such that the cells are maintained in a transformed and undifferentiated state at 35°C, the permissive temperature of the virus, and differentiate into myotubes at 41°C, a non-permissive temperature for the

Figure 3. Chromatograms of CAT assays after transfection of C2/7 muscle cells. CAT activity was assayed : A) in extracts from C2.7 myoblasts, harvested 24 hours after transfection. Lane 1, pSB1 ; lane 2, pRSV CAT; lane 3, pMLC1 CAT 1.2 (+); lane 4, pMLC3 CAT (+); lane 5, $\text{p}\alpha_{\text{C}}\text{A}$ CAT (+); lane 6, $\text{p}\alpha\text{ACh}$ CAT (+). All incubations were performed during 150 min, using 70 μl of cell extract. B) in extracts from C2.7 myotubes, harvested 48 hours after transfection. Lane 1, pSB1 ; lane 2, pRSV CAT; lane 3, pMLC1 CAT 1.2 (+); lane 4, pMLC1 CAT 1.2 (-); lane 5, pMLC3 CAT (+); lane 6, pMLC3 CAT (-); lane 7, $\text{p}\alpha_{\text{C}}\text{A}$ CAT (+); lane 8, $\text{p}\alpha\text{ACh}$ CAT (+). Incubation was performed for 30 min with 20 μl of cell extract for pRSV CAT and for 180 min with 70 μl of extract for the other plasmids. N = non acetylated chloramphenicol; M = monoacetylated forms of chloramphenicol.

Figure 4. Chromatograms of CAT assays after transfection of sol 8 muscle cells. CAT activity was assayed in extracts from sol 8 undifferentiated myoblasts (A) and differentiated myotubes (B), respectively harvested 24 and 48 hours after transfection. lanes 1, pSB1 ; lanes 2, pRSV CAT; lanes 3, pMLC1 CAT 1.2 (+); lanes 4, pMLC3 CAT (+), lanes 5, $\text{p}\alpha_{\text{C}}\text{A}$ CAT (+); lanes 6, $\text{p}\alpha\text{ACh}$ CAT (+). In A, incubation time was 40 min with pRSV CAT and 150 min with the other constructions, using 70 μl of cell extract, and in B, it was 45 min with pRSV CAT and 165 min with the other constructions, using 70 μl of extract.

virus (22). Differentiated myotubes in all these cell lines express the myosin alkali light chains MLC1_F and MLC3_F, α -cardiac actin and the α -subunit of the AChR, with the exception of Q NY ts 68 which does not express the MLC3_F isoform. These muscle-specific markers are not expressed in the dividing myoblast population (22, 23; Pinset, personal communication). The chromatograms of representative CAT assays, resulting from transfection experiments with C2.7 and sol8 cells, are shown in figures 3 and 4, and the average normalized CAT activities from several experiments (3 to 5) with the different vector constructions are summarized in table 2. As with the primary cultures, dividing myoblast cultures were transfected and cell extracts taken for CAT assays 24 hours later, before any myotubes were detected and while most of the cells were still dividing. This is particularly important with the C2 cell line which differentiates very rapidly. Confluent cultures, already containing myotubes, were transfected and cell extracts prepared 24 or 48 hours later when extensive myotube formation had taken place.

In C2.7 myoblast cultures, CAT activities with all but one muscle promoter construction are similar to the background level given by the promoterless vectors pSB1 and pSV0CAT, or by the reversed MLC constructions pMLC CAT 1.2 (-) and pMLC3 CAT (-). The only exception is the cardiac actin construction p α_c A CAT (+), which gives a significant level of CAT activity in C2.7 myoblasts, only 2-to 5-fold lower than that given by the viral promoter constructions pSV2 CAT and pRSV CAT; this construction is also active in non-muscle cells (see table 1). In differentiated C2.7 cultures, the cardiac actin construction is an order of magnitude more active, as are the two viral constructions. The RSV promoter is consistently more active than the SV40 early promoter and its accompanying enhancer, in muscle cells (see Table 2), as already noted in other cell types (13, 15, 24). In contrast with the other muscle promoter constructions, both MLC1 constructions have CAT activities which are only marginally above background in these differentiated cells. In this subclone C2.7 of the mouse C2 muscle cell line it can be seen that, as already shown (6), the 5' flanking region of the gene of the α -subunit of the chicken AChR promotes a striking (x1000) up-regulation of CAT activity as the muscle cells

	C2.7 Myoblasts	C2.7 Myotubes	sol 8 Myoblasts	sol 8 Myotubes
pMLC1 CAT 1.2 (+)	0.0013	0.006	0.0004	0.0015
pMLC1 CAT 0.47 (+)	0.005	0.009	ND	ND
pMLC1 CAT 1.2 (-)	0.004	0.007	ND	ND
pMLC3 CAT (+)	0.0017	0.06	0.009	0.03
pMLC3 CAT (-)	0.0014	0.0025	ND	ND
p alpha cA CAT (+)	0.041	0.31	0.014	0.01
p alpha ACh CAT (+)	0.0015	1.3	0.04	0.1
pSB1	0.0011	0.0025	ND	0.008
pRSV CAT	0.21	2.9	1	1
pSV2 CAT	0.074	0.63	ND	0.5

Table 2. Normalized CAT activity in undifferentiated myoblasts and differentiated myotubes in C2.7 and sol 8 muscle cell lines. The legend is the same as for table 1.

differentiate. The MLC3_F fragment also promotes a significant level of CAT expression in differentiated myotubes, although it is less active than p α ACh CAT (+).

The results obtained with another mouse muscle cell line, sol 8, are illustrated in Figure 4 and summarized in Table 2. In transfection experiments with these cells, little difference was seen between differentiated and undifferentiated cultures. The MLC3_F and α AChR constructions gave only marginally (2.5- to 3-fold) higher CAT activities in sol 8 myotubes, relative to myoblasts; the cardiac actin promoter showed no difference, nor did the viral RSV promoter. The MLC1_F construction gave CAT activities below the pSB1 background in both myoblasts and myotubes, although a slight increase is observed on differentiation. In the mouse T984 clone 10 cell line, rather similar results were obtained, with low CAT activities for viral and muscle gene constructions (results not shown). In the transformed quail muscle cell line, Q NY ts68, there was also little difference in the behaviour of transfected muscle or viral constructions between myoblasts and myotubes; the viral and muscle promoters constructions, with the exception of pMLC1 CAT 0.47(+) (pMLC1 CAT 1.2(+)) was not tested), gave very high activities (results not shown), perhaps reflecting a more efficient transfection of this virally transformed cell line. Although the endogenous gene is not expressed, pMLC3 CAT(+) gave a high level of activity in these cells. In experiments with both sol 8 and the Q NY ts68 cell lines, extensive muscle fiber formation was observed in the differentiated cultures. It would appear that, in these two lines, although some expression may be seen with the transfected muscle promoter constructions, it does not follow the regulation of the endogenous gene. In the C2.7 cell line, this depends on the muscle gene considered.

DISCUSSION

In the experiments reported here, we show that the regions immediately upstream of the Cap sites of a number of muscle genes promote the activation of a CAT indicator gene in primary muscle cells, and that this promoter activity is developmentally regulated between undifferentiated and differentiated cultures. Other sequences within or around the gene may, of course, be important for optimal expression *in vivo*. An intronic sequence is implicated in muscle troponin I expression for example (25), and our own results on a mutant cardiac actin gene locus in BALB/c mice (12) may point to the importance of a regulatory sequence which is located at more than 6 Kb upstream of the cardiac actin gene. However we conclude that at least part of the DNA sequence information which confers developmental specificity is present in the proximal promoter region of the genes we have tested. Tissue specificity is also conferred by this region, with the exception of the α -cardiac actin promoter which shows a significantly higher level of expression in the 3T6 and CV1 non muscle cell lines than do the other muscle promoters tested and the promoterless pSB1 vector. Similar results have been reported for the chicken (7) and human (8, 26) cardiac actin promoters.

The myosin alkali light chain gene encoding MLC1_F and MLC3_F : functional characterization of promoter sequences.

The results of transfection experiments in primary muscle cultures demonstrate that the myosin alkali light chain gene has two functional promoters. For the MLC1_F promoter, a sequence of 1.2 Kb located 5' to the Cap site of exon 1 gives a CAT activity in differentiated cultures which is comparable to that seen with the α -cardiac actin gene promoter for example. This level of CAT activity is more than a hundred fold higher than that given by the control construction with the MLC1_F promoter in the reverse orientation. No activity is seen in myoblasts or in non-muscle cells, indicating that this region contains sequence elements which confer tissue and developmental specificity of expression. A shorter 5' region of 369 base pairs is inactive in all cultures tested, although it contains the TATA and CAAT boxes and other potentially significant conserved sequences (4). The implication is that key regulatory elements are present between -369 and -1200 base pairs upstream of the MLC1_F Cap site. A second internal promoter region is present immediately upstream of exon 2 which

encodes the 5' end of MLC3_F mRNA. In this case, 438 base pairs are sufficient to promote CAT activity in differentiated cultures. This sequence lacks canonical TATA and CAAT boxes, but includes regions which are conserved between the avian and mammalian genes (4). Little or no activity is seen in undifferentiated primary myoblasts and in 3T6 or CV1 non muscle cells. The fact that both MLC1_F and MLC3_F promoters showed transcriptional activity *in vitro* in run off experiments using Hela cell extracts (5) probably reflects permissiveness in the regulation of exogenous promoters in the latter system.

Different results are obtained with the same promoter in different muscle culture systems.

Muscle gene promoters in primary muscle cell cultures.

We conclude from the results presented here that primary muscle cell cultures provide a transfection assay system for muscle gene regulatory sequences which is more generally applicable than that provided by the permanent cell lines tested. All four muscle 5' sequences examined, derived from the mouse MLC1_F/MLC3_F gene, the mouse α -cardiac actin gene and the chicken gene encoding the α -subunit of the AChR, gave little or no CAT activity in dividing myoblast cultures and significantly higher CAT activities in differentiated cultures. This is in keeping with the observation on the chicken α -skeletal actin promoter in primary cultures from chicken (10) where developmental regulation was also demonstrated. The extent of developmental regulation in the experiments reported here is difficult to quantitate because myoblasts and myotubes are essentially different cell types, so that parameters such as transfection efficiency or CAT stability may vary between these two populations (see Results section). In the primary myoblast cultures, the basal level of CAT activity seen with the promoterless vector is relatively high and the figures for the muscle promoter constructs probably represent background; the fold increase between myoblasts and myotubes may thus be much higher than would appear from a comparison of the two sets of figures for a given promoter. In the case of the viral promoter on the other hand, the CAT activity in myoblasts is very significantly above background, so that the fold increase (x16) here can be appreciated. This increase probably reflects, at least in part, a real difference in the regulation of the viral promoter. It is not seen between myoblasts and myotubes of

the sol8 cell line, in which exogenous muscle genes also show very little developmental regulation. A similar difference in the activity of the SV40 promoter has been reported by other authors (27, 28). It is also striking that the polyoma virus is transcribed and replicates efficiently only in differentiated and not undifferentiated muscle cultures (29); studies with mutant viruses indicate that this is due to a particular region of the viral early promoter, namely the "A" box, and hence suggests that cell factors interacting with this enhancer are accumulated in differentiated muscle cell cultures. The implication is that this factor(s) is also involved in the activation of muscle structural genes (4, 30).

Muscle gene promoters in established muscle cell lines.

In contrast, with the cell lines, variable results were obtained. For the MLC1_F constructs, with either 369 bp or 1200 bp of flanking sequence, no promoter activity was detected with any one of four muscle cell lines tested, although the endogenous gene was expressed. Given that a small number of myotubes receive a large number of plasmid copies in these transfection experiments (see results section), this may indicate that in the cell lines low levels of the transcriptional factor(s) necessary for the activation of the MLC1_F promoter region are present. The promoter of the chicken gene encoding the α -subunit of the AChR, on the other hand, as already reported (6), shows a high degree of developmental regulation in the C2.7 cell line, but not in sol8 cells, although again the endogenous gene is similarly expressed in both differentiated cultures. Indeed this cell line, like the transformed quail cell line Q NY ts68, shows virtually no significant developmental regulation with any exogenous gene promoter tested. In the case of the α -cardiac actin promoter, a relatively high level of CAT expression was detected in C2.7 myoblasts, compared with the activity of other muscle gene promoters in this cell system, whereas this was not the case in primary cultures. A similar result has been reported for the chicken (7) and human (28) α -cardiac actin gene in C2 myoblasts, and it has been suggested that this reflects higher levels of certain muscle transcriptional factors in these cells; the endogenous α -cardiac actin gene, which is not expressed, is presumably inaccessible at this stage (28). The indication therefore is that muscle cell lines may contain abnormally high or low levels of regulatory factors which will affect the apparent expression of transfected sequences, although the endogenous gene is

developmentally regulated. We conclude that muscle cell lines are not always a reliable assay system for functional regulation of promoter activity.

From the results presented here, it is clear that not all muscle promoter constructs respond similarly when transfected into a given muscle cell environment. One 5' flanking region will promote CAT activity only in primary differentiated cultures (e.g. MLC1F) whereas others will promote activity to different degrees in muscle cell lines. This implies that different transcription factors or different concentrations of them are required to activate each proximal promoter sequence.

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