

# A muscle-specific enhancer is located at the 3' end of the myosin light-chain 1/3 gene locus

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**Two skeletal myosin light chains, MLC<sub>1</sub> and MLC<sub>3</sub>, are generated from a single gene by transcription from two different promoters and alternate splicing of the pre-mRNAs. To define DNA sequences involved in MLC transcriptional control, we constructed a series of plasmid vectors in which segments of the rat MLC locus were linked to a CAT gene and assayed for expression in muscle and nonmuscle cells. Whereas sequences proximal to the two MLC promoters do not appear to contain tissue-specific regulatory elements, a 0.9-kb DNA segment, located >24 kb downstream of the MLC<sub>1</sub> promoter, dramatically increases CAT gene expression in differentiated myotubes but not in undifferentiated myoblasts or nonmuscle cells. The ability of this segment to activate gene expression to high levels, in a distance-, promoter-, position-, and orientation-independent way, defines it as a strong muscle-specific enhancer element.**

[Key Words: Rat myosin light chain 1/3; CAT assay; enhancer; muscle-specific expression]

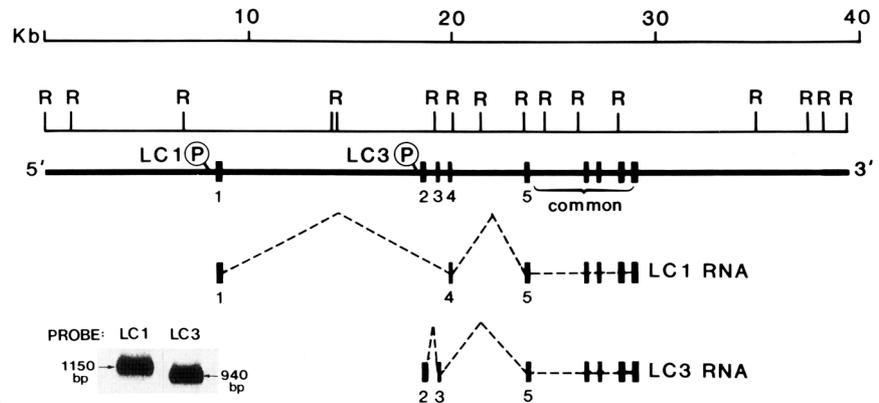
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The specialization of the cells that make up a differentiated tissue depends on their ability to switch genes on and off in response to intracellular or environmental signals. A major goal of current developmental biology is to understand and define regulatory components responsible for the activation of specific sets of genes. This is a necessary prerequisite for understanding the complex regulation underlying developmental programs of gene expression in higher organisms.

Skeletal muscle is an attractive model to study the coordinate expression of developmentally regulated genes. Here we focus on the single genetic locus encoding the myosin alkali light-chain proteins MLC<sub>1</sub> and MLC<sub>3</sub>. An identical exon-intron structure of the genes encoding MLC<sub>1</sub> and MLC<sub>3</sub> has been reported for rat (Periasamy et al. 1984), mouse (Robert et al. 1984), and chicken (Nabeshima et al. 1984). The unique structures of the MLC<sub>1</sub> and MLC<sub>3</sub> proteins are generated by differential transcription from two promoters, accompanied by two alternate splicing pathways. MLC<sub>1</sub> and MLC<sub>3</sub> mRNAs are therefore distinguished by the joining of two unique sets of 5' exons to a common set of 3' exons (see Fig. 1). The splicing pathways resulting in either MLC<sub>1</sub> or MLC<sub>3</sub> mRNAs are obligatory; MLC<sub>1</sub>-specific exons are never linked to MLC<sub>3</sub>-specific exons. The MLC<sub>1</sub> and MLC<sub>3</sub> proteins first appear in skeletal muscle at late embryonic and early postnatal stages, respectively (Gauthier and Lowey 1979; Roy et al. 1979; Whalen et al. 1982)

and persist in the adult as the predominant isoforms (Lowey and Risby 1971).

Genes encoding multiple proteins in the same locus have been described in other eukaryotic viral and cellular systems (for review, see Andreadis et al. 1987). Multiple proteins can be generated from the same gene by selection of different promoters, terminators, or splice junctions. The MLC<sub>1</sub>/MLC<sub>3</sub> locus is unique in that the alternate promoters used to produce the two proteins are active simultaneously in the same adult muscle tissue; therefore, a single tissue-specific *trans*-acting regulatory mechanism cannot be invoked. To analyze the activation of the MLC locus, we used the C<sub>2</sub>C<sub>12</sub> line (Blau et al. 1983), a subclone of the mouse skeletal muscle cell line C2 (Yaffe and Saxel 1977). The morphological changes associated with myogenesis—fusion of mononucleate myoblasts to form multinucleate myotubes—can be induced quickly in these cells by altering media conditions. The C<sub>2</sub>C<sub>12</sub> line has been used successfully as an expression system to study the transcription of other muscle genes and has the unusual capacity to activate muscle-specific genes in nonmuscle cells upon fusion (Blau et al. 1983). Although the time interval between the appearance of MLC<sub>1</sub> and MLC<sub>3</sub> messages is truncated significantly compared to the *in vivo* time scale, the pattern of sequential activation of the two messages is retained in the C<sub>2</sub>C<sub>12</sub> cell culture system (M. Donoghue et al., unpubl.). By transfecting



**Figure 1.** Genomic map of the rat MLC<sub>1/3</sub> locus, with a diagram of MLC<sub>1</sub> and MLC<sub>3</sub> alternate RNA splicing pathways (Periasamy et al. 1984). Heavy vertical bars denote exons. (R) *Eco*RI site; (P) promoter. (Inset) Northern blot analysis of the mature MLC<sub>1</sub> and MLC<sub>3</sub> messages in total RNA from adult rat skeletal muscle hybridized with MLC<sub>1</sub>- or MLC<sub>3</sub>-specific, <sup>32</sup>P-labeled cDNA probes.

## TRANSCRIPTION OF THE MYOSIN LIGHT CHAIN LOCUS

C<sub>2</sub>C<sub>12</sub> cells with expression vectors containing potential MLC regulatory elements, we have identified an enhancer in the MLC locus which is activated in differentiated muscle cells but silent in cells from other tissue types.

### Results

#### *Sequences surrounding the two MLC promoters do not have tissue-specific activity*

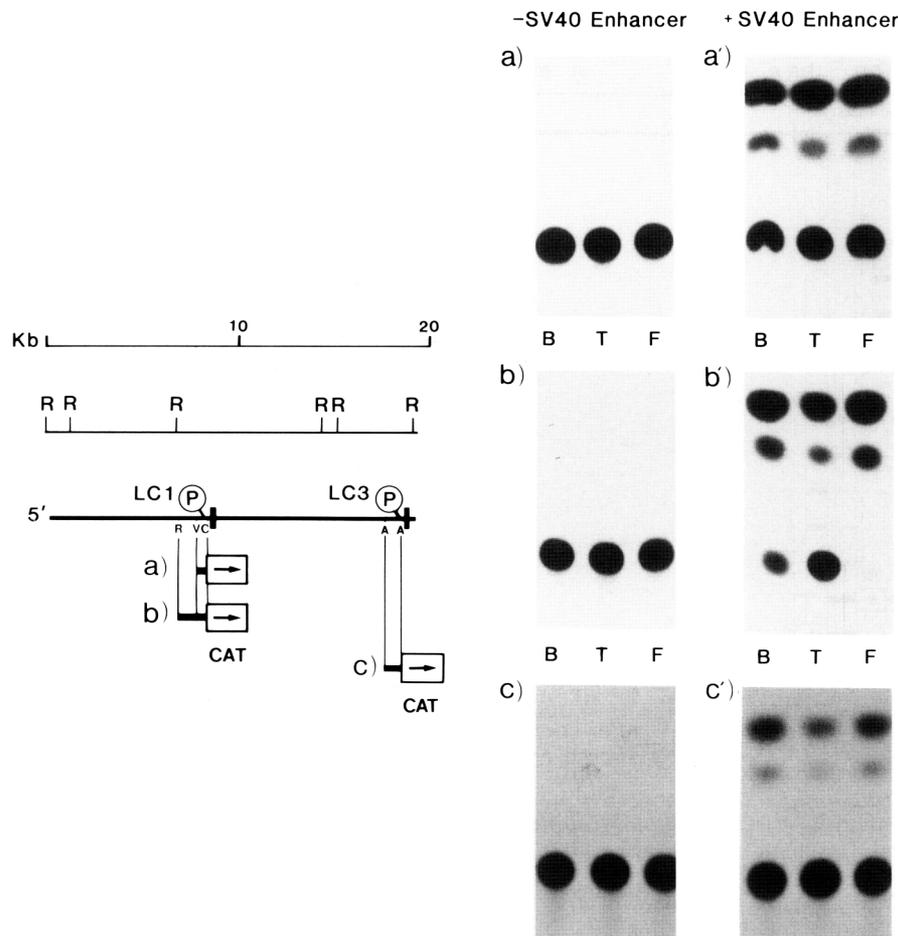
To determine whether sequences associated with either the MLC<sub>1</sub> or MLC<sub>3</sub> promoters are responsible for muscle-specific expression of the MLC locus, we tested the activity of MLC promoter-chloramphenicol acetyltransferase (CAT) constructs with or without an SV40 enhancer element downstream of the CAT transcription unit. As shown in Figure 2, constructs containing either 0.4 kb (a) or 1.5 kb (b) upstream of the MLC<sub>1</sub> TATA box, or 0.55 kb (c) upstream of the MLC<sub>3</sub> TATA box, showed no induction of CAT transcription in differentiated or undifferentiated C<sub>2</sub>C<sub>12</sub> cells or in NIH-3T3 fibroblasts. Insertion of the SV40 enhancer downstream of the CAT transcription unit resulted in high levels of CAT expression in all cell types (Fig. 2a'–c'), indicating that the MLC<sub>1</sub>–CAT and MLC<sub>3</sub>–CAT junctions generate functional messages, and that both MLC promoters can be activated in muscle and nonmuscle cells.

We extended the search for sequences responsible for tissue-specific transcription initiated at the MLC<sub>1</sub> promoter by generating a series of expression vectors in which other genomic segments from the rat MLC locus (Periasamy et al. 1984) were linked to the CAT gene in pUC-based expression vectors (see Materials and methods). We used either the MLC<sub>1</sub> promoter or the SV40 early gene promoter to drive the CAT gene; most MLC fragments were assayed in both orientations relative to CAT transcription (Fig. 3). Altogether, a stretch of ~18 kb (including 8 kb upstream of the MLC<sub>1</sub> transcription start site, 9 kb of the first intron, and 1 kb downstream of the MLC<sub>3</sub> promoter) was screened for tissue-specific activity of CAT transcription by per-

forming transient assays in undifferentiated and differentiated C<sub>2</sub>C<sub>12</sub> cells, and in NIH-3T3 fibroblasts. In all experiments, the activity of a cotransfected expression vector containing the bacterial β-galactosidase gene driven by the murine sarcoma virus transcriptional regulatory elements (MSV-LTR; Laimins et al. 1982) was measured independently, allowing normalization of transfection conditions (see Materials and methods). None of the constructs shown in Figure 3 resulted in CAT activation above background levels in any of the three cell types tested (data not shown). We conclude that within the confines of the C<sub>2</sub>C<sub>12</sub> cell system, it is unlikely that tissue-specific transcription of the MLC locus is activated by sequences in the 5' half of the gene.

#### *A muscle-specific enhancer is located at the 3' end of the MLC gene, >24 kb downstream of the MLC<sub>1</sub> promoter*

After having tested sequences surrounding the two MLC promoters for regulatory activity, we screened the region immediately downstream of the MLC-coding exons for enhancer activity. In series I (Fig. 4), we generated expression vectors with two overlapping subclones (4.2 kb and 3.5 kb) of an *Eco*RI 6.6-kb genomic fragment, including the last MLC intron and the final noncoding exon. A 1.8-kb SV40 promoter–CAT transcription unit was inserted in both orientations into these vectors in a position 5' or 3' to the MLC fragments (Fig. 4a–d). The constructs were tested for CAT activity in transient transfections of undifferentiated or differentiated C<sub>2</sub>C<sub>12</sub> cells, or NIH-3T3 fibroblasts. As seen in Figure 4 (left panel), these constructs were expressed preferentially in C<sub>2</sub>C<sub>12</sub> myotubes where CAT activity was enhanced as much as 40-fold, compared with background levels seen in fibroblasts. We also tested a similar set of MLC vectors in which the SV40 promoter–CAT cassette was replaced by an adenovirus major late (AML) promoter–CAT transcription unit and obtained the same expression profiles (C. Pavlovich, unpubl.). The tissue-specific pattern of activity obtained with these vectors, indepen-



**Figure 2.** Expression of MLC<sub>1</sub> promoter–CAT and MLC<sub>3</sub> promoter–CAT constructs with, or without the SV40 enhancer in C<sub>2</sub>C<sub>12</sub> myoblasts (B), C<sub>2</sub>C<sub>12</sub> myotubes (T), and NIH-3T3 fibroblasts (F). (Left) The CAT transcription unit is represented as a box with an arrow, indicating direction of transcription. (a) Includes 0.4 kb upstream of the MLC<sub>1</sub> TATA box; (b) includes 1.5 kb upstream of the MLC<sub>1</sub> TATA box; (c) includes 0.55 kb upstream of MLC<sub>3</sub> TATA box. (a', b', c') A 200-bp SV40 enhancer fragment has been inserted downstream of the CAT transcription unit. Restriction sites used to construct the vectors: *EcoRI* (R); *PvuII* (P); *HincII* (C); *AvaII* (A). The activities of these vectors was tested in transient CAT assays, as described in Materials and methods. (Right) Autoradiograms of representative assays. In constructs containing the SV40 enhancer, both orientations yielded similar results (M. Donoghue, unpubl.).

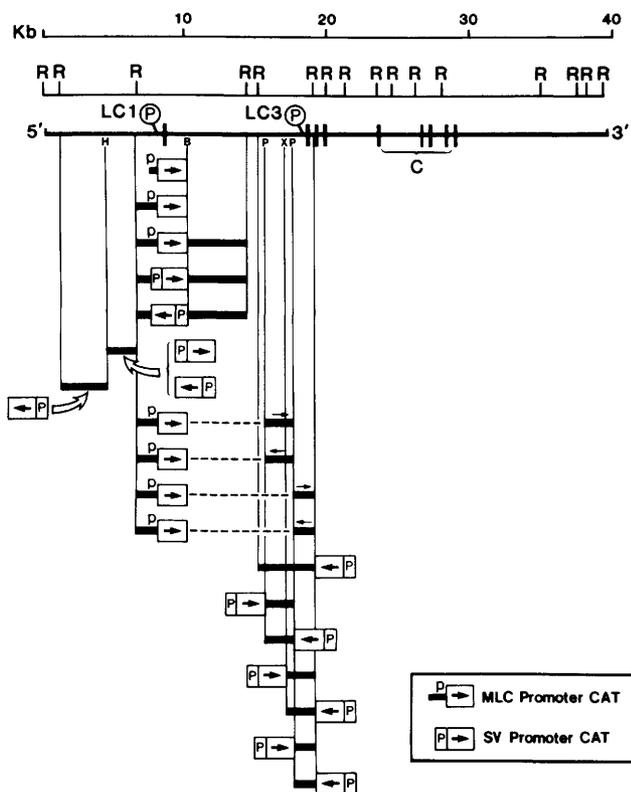
dent of the MLC fragments' position and orientation relative to the CAT transcription unit, suggested the presence of a muscle-specific, enhancer-like regulatory element located within the overlapping portion of the two subclones.

In the constructs comprising series II (Fig. 4), we sought to narrow down the region responsible for enhancer activity and to test whether the MLC enhancer can activate the cognate MLC<sub>1</sub> promoter. We purified subfragments from the 3.5-kb active segment and from a 2.5-kb fragment immediately downstream of this region and inserted them at the 3' end of the 0.4-kb MLC<sub>1</sub> promoter–CAT transcription unit (Fig. 4e–m). The resulting constructs were transfected into C<sub>2</sub>C<sub>12</sub> and NIH-3T3 cells, and CAT activity was measured in transient assays. The data in Figure 4 (left panel) localize the muscle-specific enhancer activity to a 0.9-kb *SphI*–*HindIII* segment 2.6 kb downstream of the MLC poly-

adenylation signal (Fig. 4l,m). The same tissue-specific profiles of expression were observed with constructs in which the MLC enhancer fragment was placed downstream of a MLC<sub>3</sub> promoter–CAT transcription unit (M. Donoghue et al., unpubl.), which establishes the ability of the enhancer to activate both MLC promoters. Constructs that included fragments on either side of the enhancer region (Fig. 4f–h) did not activate CAT transcription above background levels in any cell type.

In the previous vectors, the MLC enhancer fragments were in a position ~1.6 kb downstream of the MLC<sub>1</sub> promoter and 3.1 kb 'upstream' of the promoter, separated by plasmid sequences. Yet in the endogenous MLC gene, the active element is >24 kb downstream of the MLC<sub>1</sub> promoter and >14 kb downstream of the MLC<sub>3</sub> promoter. To determine whether this enhancer region can activate gene transcription from a distance, we constructed a set of vectors (Fig. 4, series III) based on the

Donoghue et al.



**Figure 3.** Diagram of the CAT vectors used to test sequences surrounding the two MLC promoters for enhancer activity. All constructs included either the MLC<sub>1</sub> promoter or the SV40 promoter driving the CAT transcription unit (box with arrow). Restriction sites used to construct the vectors: *Hind*III (H); *Bam*HI (B); *Pst*I (P); *Xba*I (X). Horizontal bars represent the MLC sequences included in the constructs. Arrows above bars indicate orientations of MLC sequences relative to CAT gene transcription. Dotted lines represent sequences not included in the constructs. Open arrows mark the position of unique *Bam*HI sites in MLC upstream fragments where a 1.8-kb SV40 promoter-CAT cassette was inserted.

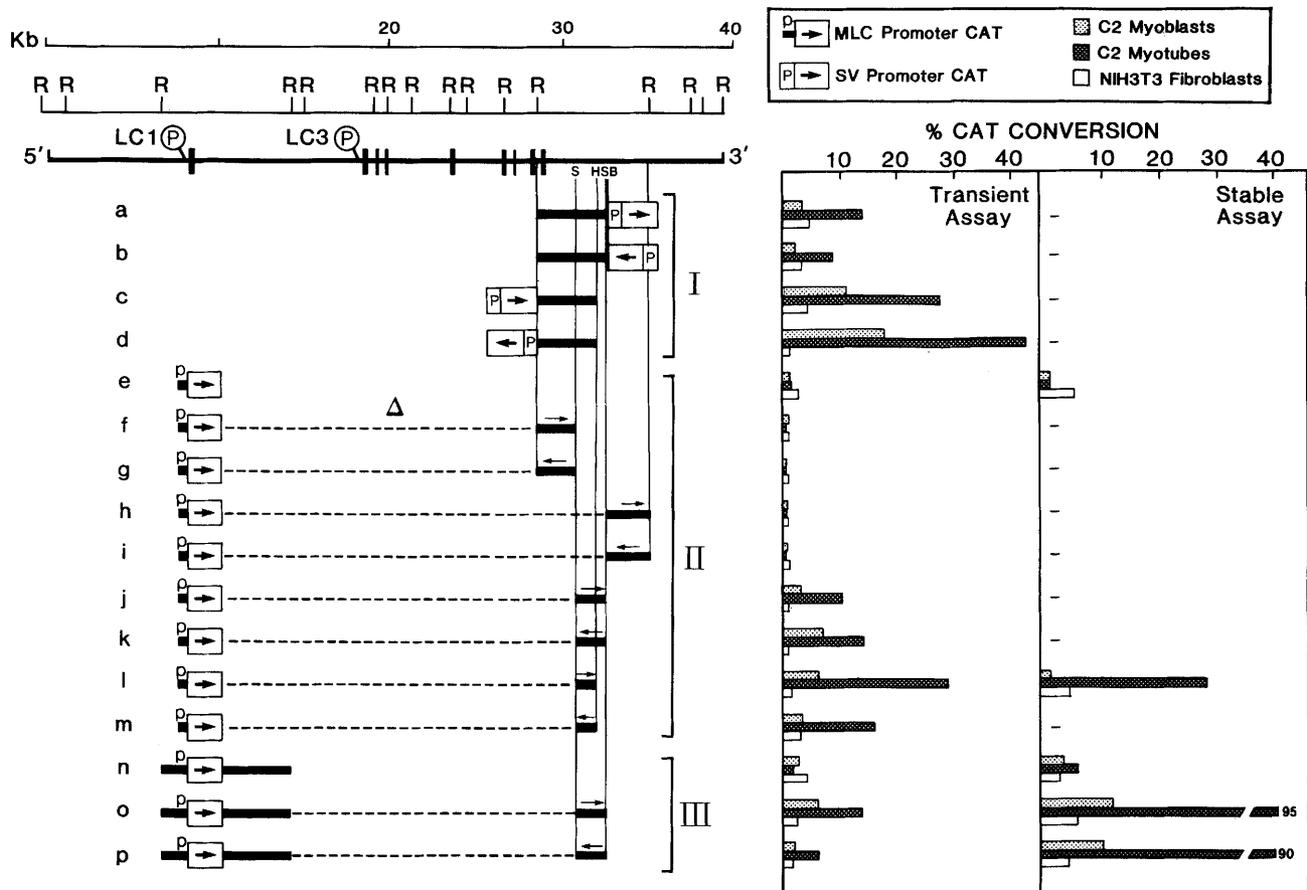
1.5-kb MLC<sub>1</sub>-promoter-CAT construct (Fig. 4n). This vector contains a 4.1-kb 'spacer' fragment from the first MLC intron downstream of the CAT transcription unit. We inserted the 1.5-kb enhancer segment (from Fig. 4j,k) at the 3' end of the spacer (Fig. 4o,p), which placed the enhancer 5.7 kb downstream and 4.2 kb upstream of the MLC<sub>1</sub> promoter around the circular plasmid. In transient CAT assays (Fig. 4), the muscle-specific expression profiles generated by these constructs were not significantly affected by the increased distance between the enhancer and promoter elements, indicating that the MLC enhancer effect is distance-independent.

In the transient assays described above, a low percentage of cells in the C<sub>2</sub>C<sub>12</sub> myoblast cultures differentiated spontaneously during the 48- to 60-hr incubation post-transfection, presumably because dividing cells reached confluence. The CAT activity levels above background in undifferentiated myoblast cultures transfected with vectors containing the MLC enhancer element (see Fig. 4, left panel) therefore may have arisen

from these committed or differentiated cells. Alternatively, these results might indicate that the MLC enhancer is muscle lineage specific rather than differentiation specific. To resolve this issue, we established cell lines in which MLC<sub>1</sub>-CAT constructs were stably integrated into the host cell genome, allowing steady-state levels of CAT expression to be compared in pure myoblasts and fully differentiated myotubes without the time constraints of a transient assay. Stable cultures were generated by cotransfection of C<sub>2</sub>C<sub>12</sub> and NIH-3T3 cells with representative MLC<sub>1</sub>-CAT constructs (see Fig. 4) and the neo<sup>r</sup> expression vector, pSV2neo, followed by selection for chromosomal integration in the presence of G418 (Southern and Berg 1982). Mass neo<sup>r</sup> cultures of C<sub>2</sub>C<sub>12</sub> cells, harvested as myoblasts or differentiated myotubes, and of NIH-3T3 fibroblasts were assayed for CAT activity. The results (Fig. 4, right panel) confirmed that vectors containing the MLC<sub>1</sub> promoter fragments alone (Fig. 4e,n) are inactive, whereas insertion of sequences including the MLC enhancer downstream of the CAT transcription unit (Fig. 4l,o,p) resulted in a high level of CAT activity in C<sub>2</sub>C<sub>12</sub> differentiated myotubes. The same constructs were far less active (10-fold) in C<sub>2</sub>C<sub>12</sub> undifferentiated myoblasts and essentially inactive in NIH-3T3 fibroblast cultures. Similar profiles of expression were obtained with several individual subclones of the stable lines (M. Donoghue, unpubl.). The absolute levels of CAT activity in these stable cultures varied between different constructs and between different individual subclones, presumably due to the unavoidable variation in the site of chromosomal integration during the selection process. Nevertheless, the CAT expression profiles presented in Figure 4 suggest that the MLC enhancer activates gene expression in a differentiation-specific manner.

#### *The MLC enhancer is a strong regulatory element*

The original definition of enhancers as regulatory elements that activate transcription in a position- and orientation-independent way was derived from studies of viral enhancers, which characteristically have dramatic effects on gene expression. One example is the SV40 enhancer, which routinely increases transcription by at least two orders of magnitude in many cell types (for review, see Khoury and Gruss 1983), including the cells used in this study (see Fig. 2). We therefore chose the SV40 enhancer as a standard against which to compare the strength of the MLC enhancer. For this purpose, a new CAT expression vector was constructed from vector n (see Fig. 4), in which the MLC enhancer was replaced by the SV40 enhancer. Stable C<sub>2</sub>C<sub>12</sub> and NIH-3T3 cultures were established in parallel for this vector and for vectors o and p from Figure 4 by cotransfection with pSV2neo DNA, followed by G418 selection. Mass cultures of stable C<sub>2</sub>C<sub>12</sub> myoblasts, myotubes, and NIH-3T3 transformants were harvested and assayed for CAT activity. Figure 5 shows a comparative kinetic analysis of CAT enzyme activity for the three expression vectors described above. As seen in Figure 5B, in C<sub>2</sub>C<sub>12</sub> myotubes, the MLC enhancer is ~10-fold more active than



**Figure 4.** Diagram of the CAT vectors used to test sequences downstream of the MLC gene for enhancer activity and results obtained with these vectors in transient and stable CAT assays. (Series I) The SV40 promoter-CAT transcription unit is linked to MLC fragments in both 5' and 3' positions; (series II) various downstream genomic fragments are linked in both orientations to the 3' end of the 0.4-kb MLC<sub>1</sub> promoter-CAT transcription unit; (series III) the 1.5-kb downstream fragment from constructs *j* and *k* was linked in both orientations 4.1 kb from the 3' end of an MLC<sub>1</sub> 1.5 kb promoter-CAT transcription unit. Restriction sites used to construct the vectors: *Sph*I (S); *Hind*III (H); *Bam*HI (B). Histograms of CAT activity show values from representative CAT assays, performed as described in Materials and methods. (Left) Results from transient transfections; (right) results from stable cultures. Dashes indicate constructs not tested in stably transformed cells.

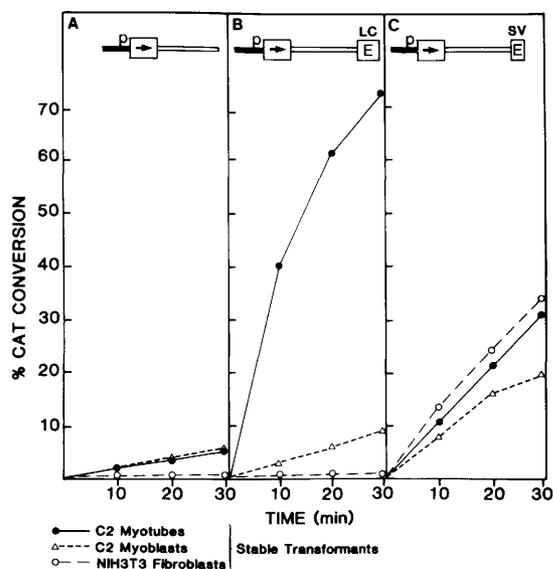
in myoblasts and 100-fold more active than in fibroblasts. Consistent with the data in Figure 2, the SV40 enhancer is effective in activating CAT transcription from the MLC<sub>1</sub> promoter-CAT transcription unit in both C<sub>2</sub>C<sub>12</sub> and NIH-3T3 cells, even at a considerable distance (Fig. 5C). From the comparison of the two enhancers in these lines, we conclude that the MLC enhancer is at least as strong as the SV40 enhancer in differentiated muscle cells.

#### *The MLC enhancer is active in primary muscle cells*

In this study the MLC enhancer was initially defined in differentiated cultures of C<sub>2</sub>C<sub>12</sub> satellite cells, a permanently immortalized line derived from mouse adult skeletal muscle. We wished to rule out the possibility that the observed activity of the enhancer sequences was an artifact of the C<sub>2</sub>C<sub>12</sub> cell culture system by testing MLC enhancer activity in primary muscle cells. Fetal rats were sacrificed at 18 days of gestation, and a single cell suspension was made from hindlimb muscles. Primary

fibroblasts were isolated by a rapid preabsorption step, and the remaining myoblasts were cultured separately. Both cell types were transfected with three constructs: the MLC<sub>1</sub> promoter-CAT transcription unit alone (see Fig. 4e), and the same construct with either the MLC enhancer (see Fig. 4l) or the SV40 enhancer (see Fig. 2a'), immediately downstream from the CAT transcription unit. The cells were harvested 60 hr post-transfection; at this time, all primary muscle cells were terminally differentiated, contractile myotubes.

Figure 6 shows a comparative kinetic analysis of CAT enzyme activity generated by the three constructs in either primary muscle or fibroblast cells. Although the MLC<sub>1</sub> promoter-CAT construct alone was inactive in both cell types (Fig. 6A), addition of the MLC enhancer increased CAT activity levels >20-fold in myotubes but had no effect in fibroblasts (Fig. 6B). In contrast, the SV40 enhancer-containing construct was active in both cell types (20-fold over background; Fig. 6C). This result extends the tissue-specific activity of the MLC enhancer



**Figure 5.** Kinetic analysis of CAT activity in extracts of mass cultures stably transformed with MLC-CAT expression vectors as shown. (A) Construct *n* (Fig. 4). (B) Construct *p* (Fig. 4) identical to *n*, with the addition of the 1.5-kb MLC enhancer fragment (LC) at the 3' end of the intron spacer. (C) Construct contains the SV40 enhancer (SV) in place of the MLC enhancer at the 3' end of the intron spacer. Constructs with the MLC enhancer or the SV40 enhancer in alternate orientations produced results similar to those shown.

to primary cells while confirming that the C<sub>2</sub>C<sub>12</sub> line is an appropriate model system for studying MLC gene expression.

#### *The MLC enhancer is inactive in other differentiated cell types*

To establish more firmly that the MLC enhancer is a tissue-specific element, we assayed its activity in two other cell lines: HepG2, a human hepatoma-derived liver cell line (Knowles et al. 1980), and Caco-2, a human colon carcinoma cell line (Pinto et al. 1983). These lines have been shown previously to display highly tissue-specific phenotypes. Figure 7 compares the activity of MLC enhancer- and SV40 enhancer-driven CAT constructs (see Fig. 6) in four different cell types. Although the SV40 enhancer functioned in all cells tested, the MLC enhancer was active only in muscle cells and inactive in the fibroblast, liver, and intestinal cultures. These data establish the MLC enhancer as a regulatory element whose activity is tightly restricted to differentiated muscle tissue.

#### *The MLC enhancer induces muscle-specific transcription from the correct initiation sites in the MLC promoters and in a heterologous $\beta$ -globin promoter*

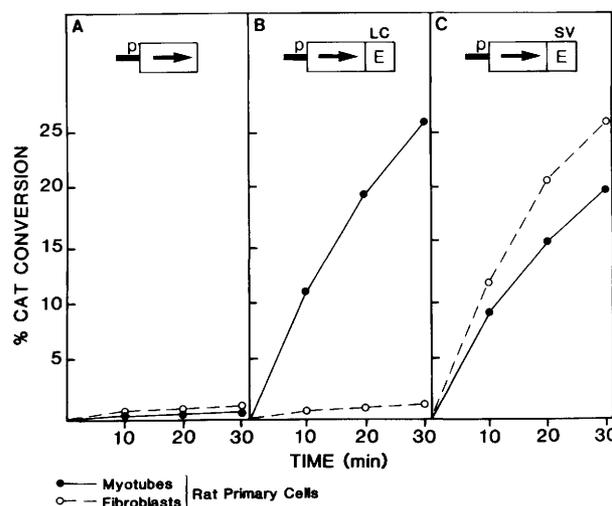
To confirm that correctly initiated transcription is induced by the MLC enhancer from the two MLC pro-

motors, either a 400-bp MLC<sub>1</sub> promoter fragment or a 550-bp MLC<sub>3</sub> promoter fragment was linked to a herpes simplex virus thymidine kinase (HSV-tk) gene, with the MLC enhancer placed downstream of the thymidine kinase transcription unit. Stably transformed C<sub>2</sub>C<sub>12</sub> mass cultures were generated using each vector, and RNA was harvested from differentiated myotubes. RNase protection analysis of these messages (Fig. 8A) confirmed that transcription of the linked thymidine kinase genes was initiated at the previously mapped MLC<sub>1</sub> and MLC<sub>3</sub> start sites (Strehler et al. 1985).

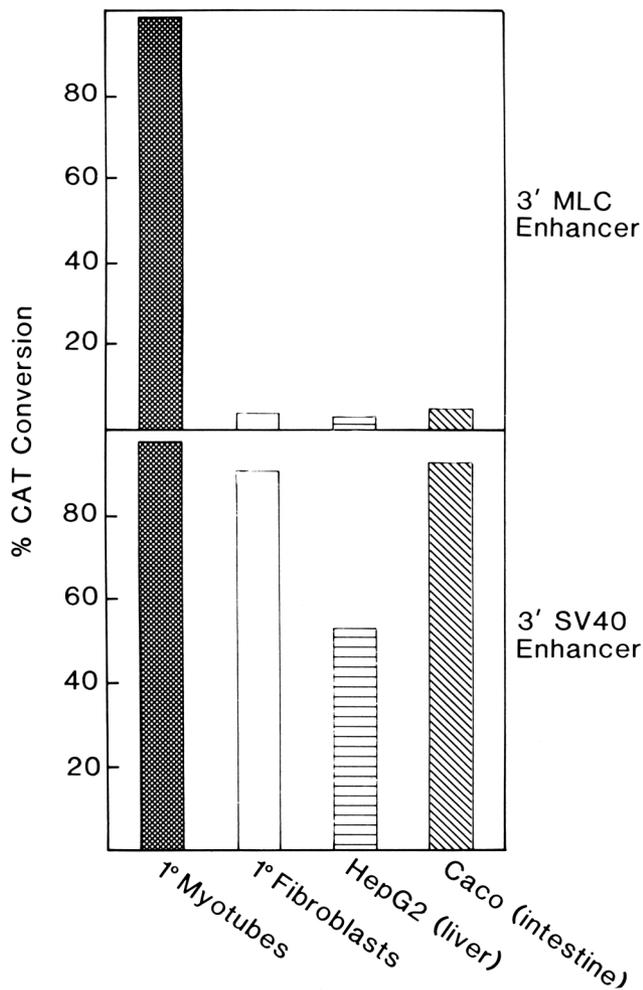
In addition, a vector was constructed in which the MLC enhancer was placed downstream of an intact human  $\beta$ -globin gene (Treisman et al. 1983), effectively replacing the native 3'  $\beta$ -globin enhancer. C<sub>2</sub>C<sub>12</sub> mass cultures stably transformed with this vector were assayed for the presence of  $\beta$ -globin RNA. Correctly initiated  $\beta$ -globin RNA was detected in myotubes but not in myoblasts with RNase protection assays (Fig. 8B). This establishes the ability of the MLC enhancer to induce differentiation-specific transcription of a heterologous gene locus that is normally silent in this cell type.

#### *The MLC 0.9-kb fragment includes sequences homologous to the muscle creatine kinase enhancer and viral enhancers*

To determine the DNA sequence of the MLC enhancer element, the 0.9-kb fragment described above was introduced into phage M13mp19 and sequenced using the dideoxynucleotide chain termination method (Sanger et al. 1977). The complete sequence of the fragment is shown in Figure 9. A computer-assisted scan of the se-



**Figure 6.** Kinetic analysis of CAT activity in extracts of muscle and fibroblast primary cells transiently transfected with MLC promoter-CAT vectors, all of which include the 0.4-kb MLC promoter fragment linked to the CAT transcription unit, with or without added enhancer elements inserted downstream of the CAT transcription unit. (A) Construct *e* (Fig. 4), no added enhancer; (B) construct *m* (Fig. 4), plus the 0.9-kb MLC enhancer (LC); (C) construct *a'* (Fig. 3), plus the SV40 enhancer (SV).



**Figure 7.** Comparison of CAT activity in various cell types transfected with the constructs described in Fig. 6. Histograms show CAT enzyme activity from a representative transient assay.

quence for known muscle gene regulatory motifs revealed no significant homology with any of the known muscle-specific promoter elements (Baldwin et al. 1985; Miwa and Kedes 1987). However, several striking similarities were found between sequences in the MLC enhancer and a muscle-specific enhancer recently identified upstream of the mouse muscle creatine kinase (MCK) gene (Jaynes et al. 1986; Sternberg et al. 1988). Specifically, two motifs in the MLC enhancer CACCTGCTGC (position 445, noncoding strand) and CACATGCTCA (position 804, coding strand) share 10 out of 10 bp and 7 out of 10 bp, respectively, with a sequence at position -1159 in the MCK enhancer. In addition, an 11-bp sequence on the noncoding strand near the distal end of the enhancer fragment, CATGTG-GAAAG, bears significant homology to viral enhancer core sequences in SV40 (TGTGGAAAG; Weiher et al. 1983) and BK (CATGGTTTG; Rosenthal et al. 1983). Another 8-bp stretch farther upstream on the same strand (GGATGAGA) is identical, with the exception of

1 base mismatch, to a regulatory sequence in the long terminal repeat of the mouse mammary tumor virus (GGATGTGA; Hearing and Shenk 1983). Experiments to determine the functional significance of these motifs are currently under way.

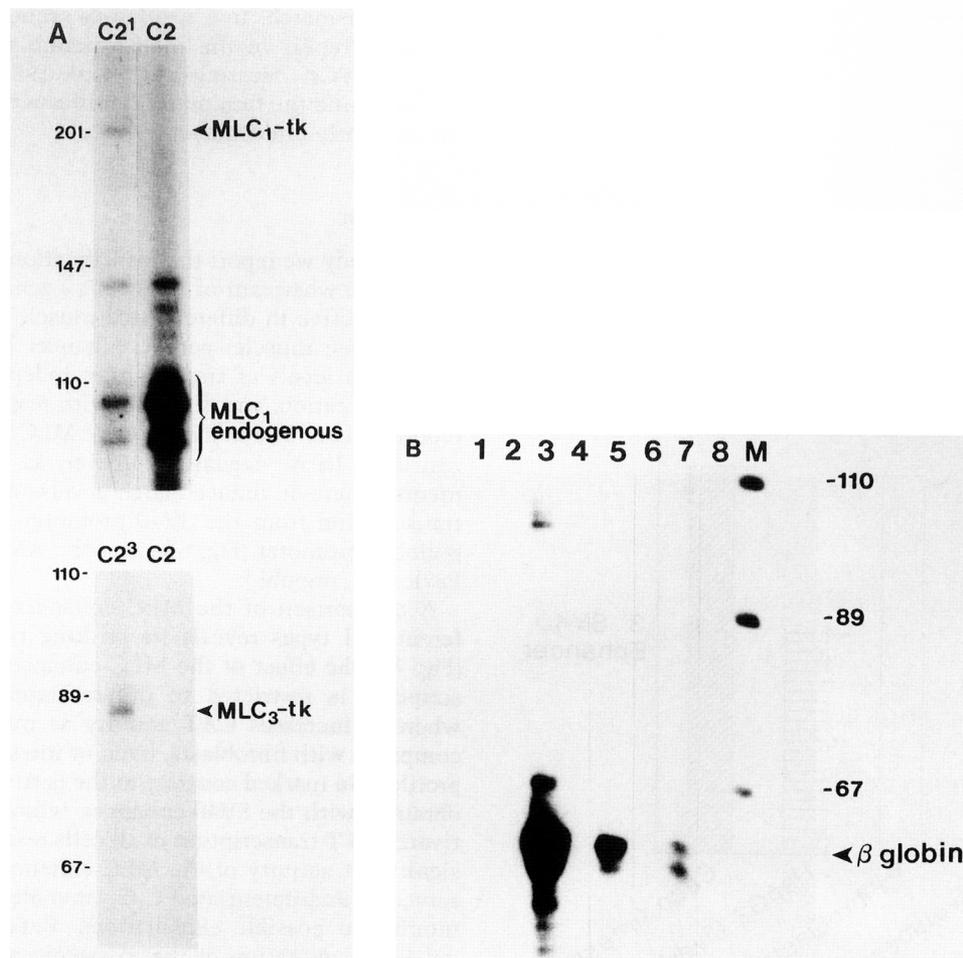
## Discussion

In this study we report the identification of an enhancer element downstream of the  $MLC_{1/3}$  gene, which is specifically active in differentiated muscle cells. It constitutes a true muscle-specific enhancer because it activates high levels of transcription independent of location, orientation, and distance, with respect to the CAT reporter gene. The activity of the MLC enhancer is not restricted to its cognate promoter, as seen in experiments where it induced high levels of tissue-specific transcription from the SV40 promoter (Fig. 4a-d), the  $\beta$ -globin promoter (Fig. 8B), and the AML promoter (C. Pavlovich, unpubl.).

A comparison of the MLC enhancer activity in different cell types reveals its striking tissue specificity (Fig. 7). The effect of the MLC enhancer on CAT transcription is restricted to differentiated muscle cells, where it increases CAT activity as much as 100-fold compared with fibroblasts, liver, or intestinal cells. This profile is in marked contrast to the pattern of expression obtained with the SV40 enhancer, which effectively activates CAT transcription in all cells tested. The low but significant activity of the MLC enhancer routinely observed in undifferentiated  $C_2C_{12}$  myoblasts (Fig. 4) has a number of possible explanations. Putative factors involved in activation of the myogenic program may already be present in low amounts in  $C_2C_{12}$  myoblasts, which could also explain the relatively rapid onset of both morphological and biochemical changes associated with  $C_2C_{12}$  myogenesis upon fusion induction. Alternatively, cultures of  $C_2C_{12}$  myoblasts may include a small percentage of 'committed cells' that have already initiated the myogenic program (Minty et al. 1986). Finally, the transfected CAT constructs may be under less stringent differentiation-specific control than the endogenous muscle-specific genes. In any case, the 10-fold increase in MLC enhancer activity in  $C_2C_{12}$  cells upon fusion of stably transformed cultures (Figs. 4 and 5) implies that the MLC enhancer preferentially activates gene expression in differentiated muscle cells.

By testing the rat MLC enhancer in mouse adult satellite  $C_2C_{12}$  cells and in rat fetal primary muscle cells, we were able to compare its activity in two skeletal muscle cell types that are different with regard to species and developmental stage. The fact that we obtained the same results with both cell types implies that *trans*-acting signals recognizing the MLC enhancer are functionally identical in rat and mouse and are present from late fetal stages throughout adulthood. Furthermore, these data suggest that the same myogenic program responsible for the activation of the MLC locus during early development may regulate its expression during adult muscle regeneration from satellite cells.

Donoghue et al.



**Figure 8.** (A) RNase mapping of MLC<sub>1</sub> and MLC<sub>3</sub> transcript initiation in thymidine kinase vectors carrying the MLC enhancer. Total RNA from differentiated C<sub>2</sub>C<sub>12</sub> cultures (C2) and from parallel differentiated cultures stably transformed with either a MLC<sub>1</sub>-tk (C2<sup>1</sup>) or a MLC<sub>3</sub>-tk (C2<sup>3</sup>) vector was hybridized with complementary RNA probes of MLC promoter–thymidine kinase junction fragments. The MLC<sub>1</sub>-tk probe protects both a 200-base fragment, resulting from hybridization to MLC<sub>1</sub>-tk RNA, and several bands below 110 bases, resulting from cross hybridization of the rat probe with endogenous mouse MLC<sub>1</sub> transcripts. Other bands present in both lanes are due to artifactual probe self-hybridization. The MLC<sub>3</sub>-tk probe protects an 86-base fragment resulting from hybridization with MLC<sub>3</sub>-tk RNA (hybridization to endogenous MLC<sub>3</sub> fragments produces a 15-base protected fragment not resolved on these gels). (B) RNase mapping of transcript initiation in a human  $\beta$ -globin vector carrying the MLC enhancer. Total RNAs from undifferentiated and differentiated C<sub>2</sub>C<sub>12</sub> cultures stably transformed with a human  $\beta$ -globin gene linked to the MLC enhancer were hybridized with Sp6-generated complementary probes spanning the expected transcript initiation site. A doublet of 50 bases represents protection of correctly initiated  $\beta$ -globin transcripts. (Lane 1) No RNA; (lane 2) yeast RNA; (lane 3) human reticulocyte RNA; (lane 4) untransfected C<sub>2</sub>C<sub>12</sub> myotube RNA; (lane 5) lighter exposure of lane 3; (lane 6)  $\beta$ -globin-transfected C<sub>2</sub>C<sub>12</sub> myoblast RNA; (lane 7)  $\beta$ -globin-transfected C<sub>2</sub>C<sub>12</sub> myotube RNA; (lane 8) undigested Sp6 probe.

The tissue-specific regulation of other genes encoding contractile proteins such as skeletal  $\alpha$ -actin in rat (Melloul et al. 1984) and chicken (Nudel et al. 1985; Bergsma et al. 1986), human cardiac  $\alpha$ -actin (Minty and Kedes 1986), rat embryonic myosin heavy chain (Bouvagnet et al. 1988), rat MLC<sub>2</sub> (Nudel et al. 1986), and mouse M-creatine kinase (Jaynes et al. 1986) has been associated with sequences upstream of their promoters. In the case of the quail troponin I gene, regulation of muscle-specific expression is associated with both the promoter region and a relatively weak enhancer-like element in the

first intron (Konieczny and Emerson 1987). From the experiments presented here, we conclude that the MLC locus is regulated differently. Yet a comparison of the MLC<sub>1</sub> and MLC<sub>3</sub> promoter sequences from rat, mouse, and chicken reveals a striking degree of conservation in the MLC<sub>1</sub> promoter region for  $\sim$ 170 bp upstream of the cap site and significant stretches of homology in the 250 bp preceding the MLC<sub>3</sub> promoter (Daubas et al. 1985; Strehler et al. 1985). The role of these conserved sequences and their relationship to other muscle-specific promoter elements is presently unclear. Because the

same pattern of sequential MLC promoter activation is seen in all three species, it is possible that the common promoter-associated sequence motifs may play a role in the timing of MLC<sub>1</sub> and MLC<sub>3</sub> expression during development. We are currently testing this hypothesis.

It is possible that the downstream enhancer represents only one of several regulatory elements in the MLC locus. There are no additional enhancer elements internal to the gene as far downstream as exon 5 (M. Gallego, pers. comm.). This leaves a 4-kb genomic segment, including exons 6–8, untested. The existence of a far upstream regulatory element, located >8 kb, 5' to the MLC<sub>1</sub> promoter, also cannot be ruled out. Far upstream enhancers have been identified in both the lysozyme gene (–6.1 kb; Theisen et al. 1986) and the albumin gene (–10 kb; Pinkert et al. 1987). Currently, we are scanning sequences far upstream of the MLC locus for additional regulatory elements.

The formal possibility that the primary role of the MLC enhancer is to activate a downstream muscle-specific locus cannot be ruled out, as nothing is known about gene linkage near the MLC locus. Preliminary Northern blot analysis of muscle or liver RNA, using genomic probes downstream of the MLC enhancer, shows no transcript hybridization for at least 2.5 kb, 3' to the enhancer (H. Ernst, unpubl.).

The location of an enhancer downstream of the gene it

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10      20      30      40      50      60      70
GGATCCGCTA TTAATCCAG AGOCCITGGA AGOCAGAGGA AGATGTATCT CTGAGTTTGA GGCCTACCTAC
80      90      100     110     120     130     140
TCTACAGAAA GAGTCCAGG ACAGACATTA CAOGAGAAGC CCTGCCCCCT CTCTAAAATA AAAGTATTTT
150     160     170     180     190     200     210
CAGAAGCATA AAGGTCACAG TGTAGAGAAA ATGACTGCTA CACGTAGTCT TAATATAGA GGGCTCTTTT
220     230     240     250     260     270     280
TTTTTTTTTT TTGATCTGTG GTGTAGATGT CTTTACATTT TTTTCAAGAT AGAAAAGCAT GATGTCGTGTG
290     300     310     320     330     340     350
GGGTATAAAT TGTGTGTTTT GAGCCTTGTG TATAAGCCTT TCCCTCAAG ATTTTATAAT AGTGGTTTAA
360     370     380     390     400     410     420
CTGTCCCGAC GGGCTAACTT CAGCAGACTG TCATGGGAGC TAACTTATT AAATTAACCAT GTGTGAAOCG
430     440     450     460     470     480     490
CTCATTAATC AAGTCCAGC AGGTGCAAAA ATGGAGCTGC GCAGGCAGAA GAGTGAATCGT GATTTTTAAA
500     510     520     530     540     550     560
ATCCCCACCA GCTGGCGAAG CAACAGCTGC CTAATTCCTC ATCTTTTAAA AATAACTTTT GAAAAGCCTG
570     580     590     600     610     620     630
TGCTGTATAA GCAAATATTT TCAAGTTTGT TTTTAAACCA TCTTGAAGTT ACCTTCCTCA GAAAATACAT
640     650     660     670     680     690     700
TATGTGCTGA TTTTTTTGTC TCAAAATGAC ATTGGAAGTC TAAGGATATA AAAATTTAAT TCTTTTTAGA
710     720     730     740     750     760     770
AAITGAAATA TTATTTAACT GGAGACTTAA ATTGTGCTT AACTCTTGCT CTTCCCTTTT TCCCTTTTGT
780     790     800     810     820     830     840
TCCCTTCTCT CCGCACTCC CTTCCCTTCT CTTGAGATGC TCATGGGGGG CTTCTCTCTT TCGTACTCTT
850     860     870     880     890     900     910
CTTCTTCTCT TCATCCCTCT CCCTTGTCTT GTCTTTGAC TAAACCTTTC CACATGGAAA AAATAAATTTG
920
TATCTTAAAG CTGGATCC
856     849     MLC
OCTACTCT
GGATGAGA
GGATGTGA MMTV-LTR
896     886     MLC
GTACAACCTTC
CATGTGGAAAG
TGTTGGAAG SV40
CAT GGTITG BKV

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**Figure 9.** Sequence of the 0.9-kb MLC enhancer element. *Bam*HI linkers added during subcloning are included. Oligonucleotides homologous to known viral enhancer core motifs are underlined.

activates is unusual but not unique. In recently published studies, an erythroid-specific enhancer was located in a region between 100 and 500 bp downstream of the chicken adult  $\beta$ -globin gene (Choi and Engel 1986; Hesse et al. 1986; Emerson et al. 1987). Because the  $\beta$ -globin gene lies in a cluster of tandemly transcribed genes, it has been postulated that the  $\beta$ -globin enhancer, which is situated between two very similar members of the cluster (adult  $\beta$  and embryonic  $\epsilon$  genes), may either have a role in the activation of both genes or may silence the 3'  $\epsilon$  gene while enhancing the 5'  $\beta$  gene in adult erythrocytes. Another erythroid-specific enhancer with sequence homology to the  $\beta$ -globin enhancer has been located downstream of the chicken histone H5 gene (Trainor et al. 1987).

If the downstream enhancer identified in this study proves to be the primary transcriptional regulatory sequence in the MLC locus, activation of the distal MLC<sub>1</sub> and MLC<sub>3</sub> promoters must involve an unusual mechanism. In initial experiments defining the mode of enhancer action on several linked promoters, the promoter proximal to the enhancer was activated preferentially (de Villiers et al. 1982; Kadesch and Berg 1983). Another study has documented simultaneous transcription from two widely spaced promoters in the immunoglobulin heavy chain locus, both presumably under the control of a single enhancer in the intron of the gene, 17 kb downstream of the 5' promoter (Wang and Calame 1985). In the MLC locus, the position of the enhancer downstream of the coding sequences places it >24 kb away from the MLC<sub>1</sub> promoter and >14 kb away from the MLC<sub>3</sub> promoter. Although the phenomenon of enhancer action at a distance is well documented, the mechanical details of this type of gene regulation remain to be elucidated.

## Materials and methods

### Tissue culture and preparation of primary cells

The C<sub>2</sub>C<sub>12</sub> subclone of the original C2 line (Yaffe and Saxel 1977) was a gift from H. Blau. Cells were propagated in growth medium (DMEM + 20% fetal calf serum and 0.5% chick embryo extract) at subconfluent densities. Differentiation was induced by switching cells to fusion medium (DMEM + 10% horse serum or 5% calf serum). Fusion was visible after ~10 hr; cells were maximally fused after 48–60 hr. NIH-3T3, HepG2 (Knowles et al. 1980), and Caco-2 (Pinto et al. 1983) cells were propagated in DMEM + 10% fetal calf serum.

Primary myoblasts were prepared from 18-day fetal rat hind limbs by dissecting and mincing, followed by dissociation in 0.25% trypsin at 37°C for 30 min. Cells were pelleted in plating media (DMEM + 20% fetal calf serum) at 200g, resuspended in plating media, and filtered to produce a single cell suspension. Fibroblasts were preabsorbed on collagen-coated plates for 30 min, and supernatant containing myoblasts was transferred to a second plate. Myoblasts were transfected 1–2 days after plating. Fibroblasts were passaged twice after the initial plating to clear cultures of contaminating muscle cells.

Donoghue et al.

*CAT expression vectors*

MLC<sub>1</sub> promoter–CAT vectors were constructed with 0.4-kb *PvuII*–*HincII* or 1.5-kb *EcoRI*–*HincII* MLC<sub>1</sub> promoter fragments from the genomic subclone  $\lambda$  LCR20 (Periasamy et al. 1984), both including the cap site and spanning 105 bp of untranslated sequences, excluding the MLC<sub>1</sub> translational initiation codon. MLC<sub>3</sub> promoter–CAT vectors were constructed with a 0.55-kb *AvaII* MLC<sub>3</sub> promoter fragment from genomic subclone  $\lambda$  LCH14 (Periasamy et al. 1984), including the cap site and spanning 15 bp of untranslated sequence. These fragments were linked with *HindIII* linkers to a 1.6-kb *HindIII*–*BamHI* subclone of pA10CAT2 (Laimins et al. 1982), consisting of the CAT-coding sequence and the small intron and polyadenylation site of SV40 t antigen. MLC promoter–CAT junctions were confirmed by DNA sequencing. To test other parts of the MLC locus for enhancer activity, intragenic segments between the two MLC promoters or extragenic segments downstream of the MLC-coding sequences were inserted 3' to the CAT gene. SV40 promoter–CAT expression vectors were constructed by inserting genomic subfragments of MLC clones  $\lambda$  LCR20, LCH14, and LCH12a (Periasamy et al. 1984) into a pUC9 or pUC18 background containing an SV40 1.8-kb *BglIII*–*BamHI* promoter–CAT transcription unit from PA10CAT2 (Laimins et al. 1982). Vectors containing the SV40 enhancer were prepared by inserting a 200-bp *PvuII*–*NcoI* genomic fragment, containing the 72-bp and 21-bp repeats (see Khoury and Gruss 1983), into a unique *BamHI* site downstream of the CAT transcription unit.

*Transfections and stable cell lines*

Introduction of expression vectors into all cells was performed by CaPO<sub>4</sub> coprecipitation (Graham and Van der Eb 1973), using 15  $\mu$ g of a CAT construct and 5  $\mu$ g of an MSV- $\beta$ -galactosidase construct (gift of C. Smith). For stable transfections, 2  $\mu$ g of the pSV2neo plasmid (Southern and Berg 1982) was included. Cells were kept in growth media for 4–12 hr post-transfection, glycerol-shocked, and harvested 36–48 hr later. C<sub>2</sub>C<sub>12</sub> and primary muscle cell differentiation was induced by switching to fusion media after the glycerol shock. Stably transformed lines of neo<sup>r</sup> C<sub>2</sub>C<sub>12</sub> and NIH-3T3 cells were selected by addition of 600  $\mu$ g/ml G418 to the growth media 3 days post-transfection. After 10 days in G418-containing media, neo<sup>r</sup> clones were harvested as mass cultures or subcloned from individual colonies.

*CAT assays*

CAT assays were performed with 10–30% of crude extract from a single 100-mm plate of confluent cells. Because transfection efficiencies and the number of cells per plate differed between cell types and between undifferentiated and differentiated C<sub>2</sub>C<sub>12</sub> myotubes,  $\beta$ -galactosidase assays (Herbomel et al. 1984; Lee et al. 1984; Edlund et al. 1985) were first performed with 20% of the extract to provide values of relative transfection efficiency. The resulting values were used to normalize the amount of extract added to the subsequent CAT assays (variations in  $\beta$ -galactosidase activity did not exceed a threefold difference). CAT activity was determined by measuring the amount of [<sup>14</sup>C]chloramphenicol (NEN) converted to its acetylated form during an incubation at 37°C for 1 hr (unless otherwise indicated) in the presence of 0.4 mM acetyl CoA. The products were separated by thin-layer chromatography, and spots were cut out and counted. All constructs were tested in at least three separate assays, with at least two different plasmid preparations. Representative values from single assays are shown, but variability between assays normalized to  $\beta$ -galactosidase expression was less than twofold.

*Northern blot analysis*

Adult rat skeletal muscle RNA used in Figure 1 was a gift from S. Izumo. For Northern blot analysis, 10  $\mu$ g per lane of total RNA was electrophoresed on a 1% agarose–formaldehyde gel, blotted onto a nylon membrane (GeneScreen, Dupont), and hybridized with probes <sup>32</sup>P labeled by the random oligomer elongation method (Feinberg and Vogelstein 1983). Probes specific for MLC<sub>1</sub> RNA were prepared from a fragment subcloned from the MLC<sub>1</sub> cDNA clone c91 (Periasamy et al. 1984), encoding exons 1 and 4; probes specific for MLC<sub>3</sub> RNA were prepared from a fragment subcloned from the MLC<sub>3</sub> cDNA clone c35 (Periasamy et al. 1984), encoding exons 2 and 3.

*RNase protection assays*

To map the transcription start sites of MLC<sub>1</sub> and MLC<sub>3</sub> promoters, promoter fragments used in Figure 2 were linked to an HSV-tk gene (gift of S. McKnight). These fragments include 105 bp and 15 bp of MLC<sub>1</sub> and MLC<sub>3</sub> untranslated sequence, respectively. An MLC enhancer element was inserted 3' to the HSV-tk gene in both constructs. Vectors were cotransfected into C<sub>2</sub>C<sub>12</sub> myoblasts, together with a pSV2neo selectable marker. RNA was isolated from mass cultures of neo<sup>r</sup> stably transformed, differentiated myotubes. Total RNA from C<sub>2</sub>C<sub>12</sub> untransfected myotubes served as a negative control. Antisense RNA probes labeled with <sup>32</sup>P were generated by T7 or Sp6 polymerase-mediated in vitro transcription of either a 210-bp *SphI*–*RsaI* MLC<sub>1</sub>-tk or a 230-bp *RsaI* MLC<sub>3</sub>-tk junction fragment. RNase protection assays were performed with 10 mg of RNA according to the protocol of Zinn et al. (1983). After hybridization of RNAs to labeled probes, RNase-protected fragments were analyzed on a denaturing 6% acrylamide gel.

To map the start site of the transcription from the human  $\beta$ -globin-MLC enhancer vector, total RNA from mass cultures of stably transformed C<sub>2</sub>C<sub>12</sub> myoblasts or myotubes was prepared. Human reticulocyte RNA (gift of S. Orkin) was hybridized to the same probe as a positive control. Total RNA from C<sub>2</sub>C<sub>12</sub> untransfected myotubes served as a negative control. Ten micrograms of RNA was hybridized to labeled Sp6 polymerase-generated complementary probe of a *HindIII*–*NcoI* fragment spanning the human  $\beta$ -globin transcription initiation site. RNase-protected fragments were analyzed on a denaturing 6% acrylamide gel.

*DNA sequencing*

The 0.9-kb MLC genomic enhancer fragment was purified and subcloned into M13mp19 (Norlander et al. 1983). Clones carrying the insert in both orientations were selected. Nested sets of overlapping deletions were produced (Dale et al. 1985) and complete sequences of both strands were generated by the method of Sanger and colleagues (1977) with deoxyadenosine 5' [ $\alpha$ -<sup>35</sup>S]thiotriphosphate (Amersham). Sequence analysis was performed using the Intelligenetics program.

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## A muscle-specific enhancer is located at the 3' end of the myosin light-chain 1/3 gene locus.

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