A highly conserved enhancer downstream of the human MLC1/3 locus is a target for multiple myogenic determination factors

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ABSTRACT

A potent muscle-specific enhancer element, originally described in the rat myosin light chain (MLC) 1/3 locus located downstream of the coding region, is found in an analogous position in the human MLC1/3 gene. When linked to a CAT reporter gene and transfected into muscle or non-muscle cells, the human MLC enhancer directs high levels of muscle-specific gene expression from homologous or heterologous promoters, irrespective of position or orientation relative to the CAT transcription unit. A significant degree of sequence homology (over 85%) in the 3'-flanking regions of the two MLC genes is restricted to a 200 bp sequence which lies approximately 1.5 kb downstream of the polyadenylation site in both species. The human enhancer sequence includes binding sites for human myogenic determination factors containing a common basic helix-loop-helix motif, and it can be trans-activated to varying degrees in non-muscle cells by these factors. This study establishes the MLC enhancer as an evolutionarily conserved, integral component of the MLC1/3 locus which constitutes a novel target for the action of myogenic determination factors.

INTRODUCTION

The unique genetic locus encoding the fast skeletal myosin light chain proteins MLC1 and MLC3 is conserved in rat (1,2), mouse (3), chicken (4) and human (5). In all species, the two MLC transcripts are generated from two different promoters and alternate splicing of the pre-mRNAs. MLC1 and MLC3 expression is activated at late embryonic and early perinatal stages, respectively (6—8), and both persist in the adult as predominant MLC isoforms (9,10). The unusual structure and developmentally regulated expression of the MLC1/3 locus implies a complex mechanism controlling the differential transcription of the two gene products.

Studies focusing on the function of the two promoters in human, rodent, and chick MLC loci have demonstrated that both MLC1 and MLC3 promoter-associated sequences are capable of directing muscle-specific transcription to varying degrees when tested in primary muscle cell cultures (5,11,12). However, the promoters alone do not stimulate reporter gene expression when transfected into established muscle cell lines, despite the high levels of endogenous MLC1 and MLC3 transcription in these cells (5,11,12,13). Although the two MLC promoters and upstream sequences undoubtedly play a modulatory role in regulating relative levels of MLC1 and MLC3 gene transcription, they do not appear to be solely responsible for the high levels of endogenous MLC transcription in differentiated myotubes, presumably because additional cis-acting regulatory elements are involved.

An additional component of MLC1/3 transcriptional regulation was characterized as a strong cis-acting regulatory element, located in the rat MLC locus over 24 kb downstream of the MLC1 promoter. This element dramatically increases CAT gene expression in differentiated myotubes but not in undifferentiated myoblasts or non-muscle cells. The ability of this element to activate gene expression to high levels, in a distance-, promoter-, position- and orientation-independent way, defines it as a muscle-specific enhancer (13). Analysis of transgenic mice carrying multiple copies of an MLC enhancer-driven CAT gene has demonstrated the ability of the MLC enhancer to activate gene expression to high levels, exclusively in skeletal muscle cells of these animals (14). In the present study we have identified and characterized a muscle-specific enhancer in the cognate human MLC1/3 locus. It is located in an analogous position downstream of the MLC1/3 structural sequences. In this 3'-flanking region, there is a high degree of sequence homology between the rat and human loci over an isolated stretch of 220 bp. The remarkable conservation of both structure and function of this distal regulatory element implies that the enhancer is an integral component of the ancestral mammalian MLC1/3 locus, and suggests a critical role for the homologous sequence in the regulation of human MLC1/3 transcription.

Recently a family of regulatory genes has been identified whose expression in multipotential mesodermal stem cells results in the induction of the myogenic phenotype (15—23). These genes belong to a group of related nuclear DNA-binding proteins which

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can form dimers via a conserved basic helix-loop-helix motif (24,25). In muscle cells, these myogenic factors positively autoregulate their own expression, presumably through direct transcriptional activation (15,26,27). A recognition sequence for these factors has been located in the enhancer of the muscle creatine kinase gene (28 – 30), and appears in other muscle-specific regulatory elements (31,32). We report here the identification of sequences in the human MLC enhancer which closely resemble this consensus motif, and which constitute binding sites for four human myogenic factors. In addition, CAT reporter constructs containing the human MLC enhancer can be differentially trans-activated by the co-expression of these myogenic factors in non-muscle cells, suggesting that the MLC1/3 locus is a target for the action of one or more of these factors during muscle cell differentiation.

MATERIALS AND METHODS

Generation of human MLC genomic subclones, maps and sequences

The human genomic lambda clone L16.1 (5) was analyzed by restriction enzyme digestion, blotting and hybridization to an Ava II-Dele 280 bp subfragment of the rat MLC enhancer (Donoghue et al, 1988). EcoRI subclones #1,2 and 4 (see Figure 1) were generated by ligating an EcoRI digest of L16.1 into the EcoRI site of pUC9. Southern blotting was performed using Zetabind filters (Cuno Inc). Hybridization was performed in 5 x SSC at 50°C, with 32P end-labelled rat MLC enhancer probe, with a 60°C wash in 2 x SSC. Sequencing was performed on a pUC9-based 850 bp EcoRI genomic subclone using a double stranded template and dideoxy chain termination (33). Dideoxy reagents were purchased from Pharmacia, Inc. DNA polymerase (Klenow large fragment) was from New England Biolabs, forward and reverse primers were from Promega Biotechnology, Inc, and internal primers were synthesized by Research Genetics, Inc.

Plasmid constructions

The CAT expression vectors in Figure 3 were constructed using previously described rat MLC promoter-CAT transcription units (13) and inserting human MLC enhancer-containing fragments, EcoRI-HaeIII 850 bp and Rsal-SspI 200 bp from L16.1 (see Figure 1), via BamHI linkers into the downstream BamH1 sites in rMLC1 CAT or rMLC3CAT in either sense (−5) or antisense (−3) orientations. pl5 – 500 contains a 538 bp Ava II-Hind III rat MLC enhancer fragment inserted at a unique BamH1 site downstream of the rMLC1 promoter-CAT transcription unit (13).

The CAT expression plasmids containing the human MLC1/3 promoters were constructed as described previously (5). The human MLC enhancer was inserted on a 400 bp Rsal/EcoRI fragment by blunt end ligation into the ClaI site located downstream of the CAT transcription unit. The two alternative orientations were designated enh-5 and enh-3.

To express the myf factors in eukaryotic cells, the cDNAs myf3 to myf6 were subcloned into the EcoRI site of the pEMSV-scribe vector (kindly supplied by A. Lassar) as described previously (15 – 17). The control plasmids alpha-cardiac actin CAT (35), P1-CAT containing the chicken β-actin promoter (36), and pAT10CAT (34) were described earlier.

To synthesize glutathione S-transferase-myf fusion proteins in E.coli, the pGEX expression vectors (37) were used. The detailed description of the expression vehicles (GS-myf) and the preparation of fusion proteins from E. coli extracts have been described (16).

Cell culture, DNA transfection and CAT assays

Mouse C2C12 cells were a gift from H. Blau and were cultured in Dulbecco's Modified Eagles Medium (DMEM, Gibco) containing 20% fetal calf serum supplemented with 0.5% chick embryo extract (Gibco) to maintain growing myoblasts, or in 2% horse serum to differentiate the cells after transfection of expression vectors. Mouse C3H 10T1/2 and NIH3T3 fibroblasts were obtained from the American Type Culture Collection (ATCC) and cultured in DMEM containing 10% fetal calf serum. Transfections in Figure 3 were performed as previously described (13), using 15 µg of test construct and 5 µg of an MSV-β-galactosidase vector (gift of C. Smith) as an internal standard. Chick primary cultures were prepared from 13 day old chicken breast muscle or skin fibroblasts as described elsewhere (5,38). Cells were transfected on 60 mm plates with 20 µg of highly purified supercoiled plasmid DNA (15 µg of test construct and 5 µg of an RSV-β-galactosidase vector (39) as internal standard). For cotransfection experiments, 10 µg of the CAT reporter-construct was mixed with 2.5 µg of the various myf expression vehicles and precipitated with calcium phosphate as described elsewhere (40). At least four independent transfection experiments were performed with each construct using two or more separate DNA preparations. Cells were harvested after 48 hours in serum-rich medium (10% FCS) and CAT activity was determined as described by Gorman (41) using 1 – 10% of total cellular extracts standardized either to constant protein concentrations, or to constant β-Gal activity from the cotransfected Lac-Z expression vector. The β-actin CAT construct P1-CAT (36) was used as a constitutively expressed control. In some of the studies presented, certain reactions were allowed to exceed 50% conversion in order to visualize differences in the weaker activities; however, the relative strength of each vector in a given series remained constant with shorter incubation times in which no value exceeded 50% (data not shown).

Electrophoretic mobility-shift assay (EMSA) and methylation interference footprinting

Binding and reaction conditions used for EMSA and methylation interference footprinting have been described in Braun et al. (42). Approximately 400 ng of purified fusion protein was used for the band shift experiments in a reaction volume of 25 µl. For methylation interference footprinting the assay was scaled up five times. The following oligonucleotides were synthesized:
For EMSA, double stranded oligonucleotides were labelled and purified as described (43); for footprinting single stranded oligonucleotides were end-labelled prior to the annealing reaction.

**RESULTS**

**Identification of homologous sequences downstream of the rat and human MLC1/3 gene**

To search for a regulatory element in the human MLC1/3 locus analogous to the enhancer in the rat gene (13), we analyzed subfragments of the human genomic clone L16.1 (5) for sequences that would hybridize to a rat MLC enhancer probe. The L16.1 clone represents the region downstream of the MLC1/3 gene and includes the last noncoding exon plus approximately 10 kb of sequence downstream of the polyadenylation site (Figure 1). EcoRI restriction fragments of L16.1 were subcloned and hybridized with the 32P-labelled 280 bp fragment from the rat MLC enhancer (13). As shown in Figure 1, the 2 kb EcoRI fragment (subclone #4), located immediately downstream of the last human MLC exon, hybridized to the rat enhancer probe even under highly stringent conditions. An 850 bp Hae III-EcoRI subfragment from clone #4 (Figure 1) was isolated and its DNA sequence was determined on both strands. This human sequence was aligned by matrix analysis to the rat enhancer element (data not shown) to locate sequence similarity between the two species. An internal 250 bp region of the human sequence (Figure 2, upper strand, positions 160 to 410) was over 80% identical to the rat enhancer (Figure 2, lower strand), with significantly less sequence similarity on either side. Although the rat and human MLC1/3 coding regions are highly conserved, it appears that considerable sequence divergence has occurred downstream of the genes during mammalian evolution with the exception of this small segment, located approximately 1.5 kb downstream of the last exon.

The conserved human sequence is a muscle-specific enhancer.

To investigate whether the DNA sequence conserved in the human MLC1/3 locus is involved in MLC transcriptional control, we constructed a series of vectors in which successively smaller hybridizing fragments from genomic clone L 16.1 were inserted and hybridized to the rat MLC enhancer with the 850 bp human genomic fragment, which included almost all of the homologous sequences described in Figure 2, conferred high activity in differentiated muscle cells in either orientation (designated 200-5 or 200-3 in Figure 3), suggesting that the entire functional MLC enhancer element is contained within this genomic segment.

To determine whether the human enhancer element could also stimulate transcription from the human MLC1 and MLC3 promoters, we constructed a second set of expression vectors by inserting a 400 bp RsaI/EcoRI MLC enhancer fragment (see Figure 1) downstream of the CAT transcription unit in either orientation. Since it has been shown that in several species the MLC1 and MLC3 promoters alone were substantially more active in primary muscle cells than in cell lines (5,11,12), we performed transfection assays with the human MLC (LC) CAT expression vectors in primary muscle cell and fibroblast cultures. As shown in Figure 4a, the resulting CAT activities indicated that the MLC1 promoter alone was essentially inactive in primary fibroblasts...
Figure 4. CAT activity directed by the human MLC1 (a) or MLC3 (b) promoters and the human MLC enhancer in primary cultures of chick breast muscle cells and skin fibroblasts. The CAT constructs LC1-CAT and LC3-CAT carrying the human MLC1 and MLC3 promoters, respectively, constructs containing the MLC enhancer in sense (enh5) or antisense (enh3) orientation, as well as control plasmids containing the rat MLC enhancer (15-500), the chicken alpha-cardiac actin promoter (card.actin CAT) or the chicken beta-actin promoter (P1-CAT), were transfected into chicken primary breast muscle cells (M) or skin fibroblasts (F) and CAT activity was determined according to standard procedures. The numbers below indicate the percentage of chloramphenicol conversion into the acetylated CM derivatives.

and only weakly active in myotubes. The addition of the 400 bp fragment representing the human MLC enhancer sequence in either orientation (enh 5 or enh 3) stimulated muscle-specific CAT expression from the MLC1 promoter 20-30 fold. By contrast, the MLC3 promoter alone (Figure 4b) was highly active in muscle cells compared to its negligible activity in non-muscle cells. When linked to the MLC3 promoter-CAT transcription unit, the 400 bp MLC enhancer fragment either had no effect or was only mildly stimulatory (Figure 4b). These observations suggest that the MLC3 promoter may contain an additional regulatory element which stimulates expression in primary muscle cultures without a major influence by the MLC enhancer.

In summary, the homologous sequence located at the 3' end of the human MLC locus is capable of activating tissue-specific gene expression from the MLC promoters as well as from the heterologous SV40 promoter (data not shown), irrespective of position and orientation. It therefore exhibits all the characteristics of a muscle-specific enhancer element. The MLC3 promoter seems to contain an additional tissue-specific regulatory element which renders it clearly less responsive to stimulation by the enhancer sequences.

The MLC enhancer forms complexes in vitro with several myogenic factors

Among the sequences that are most highly conserved between the rat and human MLC enhancers are several related motifs with the consensus CANNTG (underlined in Figure 2). Three of these motifs, termed A and C (CAGGTG), or B (CAGCTG), are similar in both their consensus and surrounding sequences to a motif in the muscle creatine kinase (MCK) enhancer, another muscle-specific regulatory element (21,43,44,45). In the MCK enhancer, a 10 bp motif (GCAGCAGGTG) with an identical CANNTG consensus sequence to sites A and C, constitutes a binding site in vitro for the myogenic determination factors MyoD and myogenin (28,30). In intact muscle nuclei, this site in the MCK enhancer is occupied exclusively in differentiated myotubes by a protein complex which creates a DNA footprint overlapping the pattern produced in vitro by a MyoD bacterial fusion protein (46).

To determine to what extent the sequences in the human MLC enhancer could interact with different myogenic determination proteins, oligonucleotides representing the CANNTG consensus motifs were tested in gel mobility-shift assays for their ability to bind myogenic proteins produced by fusing Glutathione-S-transferase (GS) with myf3, myf4, myf5 or myf6 coding sequences (15,16). The proteins myf3, myf4 and myf6 are the human analogs to mouse MyoD (18), mouse and rat myogenin.
(21,23) and rat MRF4 (20) or mouse herculin (19), respectively. An oligonucleotide including the upstream CANNTG consensus sequence at position 195 (Figure 2) did not bind to any myogenic factor (data not shown), presumably because the surrounding sequences substantially diverge from the 10 bp motif as defined in the MCK enhancer (28,30). As shown in Figure 5, the two oligonucleotides including motifs A (GATCAAGTAA CAG CAGGTCCAAAATAAAAGT) and B (GATCCATCTA CAC CAGCTGGCAAAAATGAC) (consensus sequence underlined) produced band shifts after incubation of the labelled MLC enhancer-derived oligonucleotides with purified preparations of myf3, myf4, and myf5, but interacted only weakly with myf6. Oligonucleotide B exhibited strong binding to myf4 and weak interactions with myf3, myf5, and myf6. No band shifts were obtained with the GS protein alone or with control extracts from bacteria lacking the fusion vector (data not shown). The binding of oligonucleotides A and B to the myogenic fusion proteins was effectively competed by an excess of identical unlabelled oligonucleotides, but not by oligonucleotides containing altered core consensus sequences (see Figure 7). This indicates that the interactions between sites A and B in the MLC enhancer were sequence-specific.

The precise interaction of these factors with the A and B binding motifs in the MLC enhancer was determined by methylation interference footprinting. As seen in Figure 6, methylation of one guanine residue on the coding strand and three guanine residues on the non-coding strand markedly interfered with binding of either myf3, myf4, or myf5 to the A motif. Since two complexes were found for the myf4 fusion protein (see Figure 4), both were footprinted (B1 and B2) with no noticeable differences in the protected G residues. The same pattern was obtained for binding of myf4 to the B motif (data not shown). Interaction of myf6 with oligonucleotides A and B as well as binding of myf3 and myf5 to oligonucleotide B were too weak to generate reproducible methylation interference patterns. Similar analysis of MyoD (myf3) binding to an enhancer fragment encompassing motif C, which has the same consensus sequence as motif A (CAGGTG) gave essentially the same methylation interference pattern as seen for motif A (data not shown).

The contact points for each of the human myogenic factors found in the three MLC enhancer motifs notably overlap the interference pattern previously observed for the interaction of a similar sequence in the mouse MCK enhancer with a MyoD GS-fusion protein (30) and with myogenin-E12 heterodimers produced in vitro (28). These observations suggest that the 10 bp in the A, B and C motifs, found in both the MLC and MCK enhancers, are sufficient to form a common target for related myogenic factors from several species.

The specific interactions of human myogenic factors with the MLC enhancer in vitro were further tested by generating the mutant Mut A (GATCAAGTAA GGAACCTTGCAAAAATAGT) and Mut B (GATCCATCTA GGAACCTTGCAAAAATGAC) in which the central nucleotides of the binding sites were altered (bold face). These mutant oligonucleotides were used as probes for binding of selected myogenic factors in a gel shift assay. As shown in Figure 7, mutations in either the A or B motif significantly reduced the binding of GS-MyoD (the mouse anal og to myf3), indicating that the interaction of this factor with the MLC enhancer is dependent on the intact sequence motifs represented by the A and B oligonucleotides. Binding of myf6 was very weak and did not appear strictly related to the consensus sequence. This result confirms our previous findings that under the conditions used in vitro, the myf6 fusion protein exhibits different binding characteristics from the other myf factors (16). From these results we conclude that multiple myogenic factors form similar specific protein-DNA interactions with several related sequences in the MLC enhancer. As shown in Figure 2, these sequences are highly conserved between rat and human. In addition they closely resemble corresponding binding sites for
myogenic factors in the enhancer element associated with the unrelated muscle-specific gene, MCK (28–30). This suggests that one or more myogenic factors may be directly responsible for the coordinate activation of MLC and MCK transcription during myogenic differentiation.

The human MLC enhancer can be trans-activated by multiple myogenic determination factors

The interaction of human myogenic determination factors with the human MLC enhancer in vitro (Figures 5/6 and 7) suggested that these factors might be directly involved with enhancer activation during myogenesis. To investigate possible differences in the trans-activating capacities of the four human myogenic factors, a series of cotransfection experiments was performed in C3H 10T1/2 fibroblasts with selected human MLC promoter-CAT vectors and low concentrations (2.5 /ig/plate) of the expression vectors pEMSV-myf3, 4, 5, and 6. In pilot studies we have observed that limiting the amount of cotransfected myogenic expression vector produced reproducible differences in the trans-activation capacity of the four myogenic factors, whereas higher concentrations obliterated these differences.

As shown in Fig. 8, an MLC1 promoter-CAT vector without the human MLC enhancer was not activated by the pEMSV-scribe construct alone nor by any of the myf expression constructs. Addition of the MLC enhancer downstream of the CAT transcription unit resulted in approximately 100 fold stimulation by myf3 but very little by the other three myf constructs. In contrast, an enhancerless MLC3 promoter-CAT vector was strongly activated by the myf3 factor but only weakly by the other myf factors. Linkage of the MLC enhancer to the MLC3-CAT vector had little or no additive effect. Whereas the unrelated TK-promoter used as a control was slightly activated (data not shown), the pAT10CAT control was not activated by either pEMSV-scribe or any of the other myf constructs.

In summary, the results shown in Figures 8 indicate that the human MLC enhancer can mediate gene expression in non-muscle cells, by stimulating transcription from the MLC1 promoter. This activation is dependent on the coexpression of myogenic factors. At low concentrations, myf3 exhibits a particularly strong effect. The differential responsiveness of the two MLC promoters to activation in primary myocytes, and to trans-activation in fibroblasts, suggests that MLC3 transcription is not necessarily dependent upon the MLC enhancer since the MLC3 promoter alone is responsive.

DISCUSSION

In this study we report the identification and characterization of a muscle-specific regulatory element downstream of the human MLC1/3 locus. The human MLC enhancer, like the corresponding rat element, stimulates linked gene expression to maximal levels in differentiated muscle cells, is far less active in dividing myoblasts and is essentially inactive in non-muscle cells.

The downstream location of the human MLC enhancer relative to the MLC1/3 coding region, and the similarity of its sequence to the rat MLC1/3 enhancer (13) is suggestive for a highly conserved regulatory mechanism controlling mammalian MLC1/3 transcription.

Here we show that certain sequence motifs in the human MLC enhancer can bind four distinct human myogenic determination factors, myf3, myf4, myf5, and to a lesser degree myf6. These motifs do not represent high affinity binding sites for every myogenic factor, however. Specifically, myf3, 4 and 5 bind relatively strongly to motif A, yet only myf4 displays strong affinity for motif B. The inability of myf3 and 5 to interact effectively with motif B is intriguing, since all myf factors share an internal basic plus helix-loop-helix (bHLH) motif which is common to a large family of DNA-binding proteins (16,24,25,47). Presumably the sequence variations between the A and B consensus CANNTG motifs contribute to the differences in the affinities of the myf3 and myf5 proteins for the two sequences. The binding affinity of bHLH-containing myogenic factors for their DNA targets has been shown to be affected by their interaction with other proteins from the bHLH family (25,28). It is therefore possible that in various tissues or developmental stages, motif B may be a target for heterodimers, formed between myf3 or myf5 and other bHLH proteins. Besides the ubiquitous E12 (24), which has already been shown to bind as heterodimer with MyoD to muscle-specific regulatory elements (25), ITF1...
and ITF2 have recently been characterized as additional potential candidates (47). The inability of myf6 to bind effectively to either the A or B motif in the MLC enhancer is consistent with our previously reported results (16), and again raises the possibility that a high-affinity interaction of myf6 with its target DNA may require heterodimerization with other factors.

The data presented in this and previous studies (16,17) supports the notion that myf factors may regulate differentiation-specific transcription, in addition to their developmental role in the determination of the myogenic program. By cotransfecting MLC enhancer-containing CAT plasmids with limiting concentrations of all four myf expression vectors into non-muscle cells, we have demonstrated dramatic differences in the ability of these factors to trans-activate MLC enhancer-driven gene expression. In particular, myf3 is capable of effectively trans-activating the MLC enhancer, despite the ability of other myogenic factors to bind to the enhancer in vitro. These expression profiles differ substantially from our previously published results cotransfecting higher levels of myogenic expression vectors. Use of these more standard conditions resulted in high levels of MLC enhancer trans-activation with myf4 and myf5 as well as with myf3 (16), suggesting that overexpression of myogenic proteins may obscure differences in their potential roles as transcription activators. Limiting the concentration of these factors in co-transfections of non-muscle cells may therefore provide a more realistic assessment of their natural function in differentiated muscle tissues.

The ability of multiple regulatory elements in the MLC1/3 locus to respond to myf3 underscores the complexity of the pathways involved in myosin light chain gene activation during development. Specifically, in addition to its role in MLC enhancer trans-activation, myf3 also induces transcription of an MLC2 promoter (42), and in the MCK enhancer, where it appears at position 3205335 in both the rat and human enhancers (48). It is likely that other sequence motifs in the rat and human MLC2 promoter (42), and in the MCK enhancer, where it constitutes a binding site for at least two protein complexes, one of which, MEF2, is found only in differentiated muscle cultures (48). It is likely that other sequence motifs in the rat and human MLC enhancers will constitute additional binding sites for novel regulatory factors, as yet uncharacterized in other systems. These factors can now be identified using the conserved motifs in the human and rat MLC enhancers as targets. The elucidation of mammalian MLC1/3 gene regulation during myogenesis therefore promises to provide insight into more general mechanisms underlying muscle development and differentiation.

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