Role of methylation in maintenance of positionally restricted transgene expression in developing muscle

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SUMMARY

In transgenic mouse embryos, expression of a muscle-specific reporter, consisting of a chloramphenicol acetyltransferase gene linked to regulatory sequences from the rat myosin light chain 1/3 locus (MLC-CAT), is graded in developing axial muscles along the rostrocaudal axis and in cell cultures derived from these muscles. Here we demonstrate that maintenance of positional differences in MLC-CAT transgene expression cannot be attributed to differences in the transcriptional competence of corresponding muscles. Rather, patterns of transgene expression are reflected in the extent of CpG demethylation of both MLC1 promoter and MLC enhancer sequences. Variations in reporter gene expression can be reconstituted by in vitro methylation of specific CpGs in transfected MLC-CAT DNA. As the MLC-CAT transgene is activated during embryogenesis, demethylation of the MLC1 promoter lags behind that of the downstream MLC enhancer, which appears to be the initial target for epigenetic modification. In developing somites, demethylation of the transgenic MLC enhancer is not graded and therefore does not reflect early regional differences in MLC-CAT transgene expression patterns. These studies implicate selective methylation in the maintenance rather than in the establishment of transcriptional differences in developing muscles.

Key words: muscle, DNA methylation, transgene, enhancer, promoter, LMPCR, mouse

INTRODUCTION

DNA modification by methylation is distinct from other forms of gene regulation, in that methylated CpG dinucleotides are sustained in subsequent cell divisions through the function of maintenance methylases, acting on hemimethylated DNA during replication (Wigler et al., 1981; Bestor and Ingham, 1983; Leonhardt et al., 1992). Epigenetic modifications can therefore be propagated in the DNA of dividing cells, irrespective of the changing composition of trans-acting factors. This feature makes DNA methylation an attractive mechanism for establishing inherited patterns of gene expression in cell lineages during development. The importance of DNA methylation during embryogenesis has recently been underscored by the targeted disruption of the mouse DNA methyltransferase gene through homologous recombination, resulting in a 3-fold reduction of embryonic DNA methylation and lethality at midgestation (Li et al., 1992).

DNA methylation plays an important role in the regulation of tissue-specific gene expression during skeletal muscle differentiation. Indeed, the experimental scheme used to isolate the gene encoding the myogenic factor MyoD (Davis et al., 1987) was based on the elegant work of Jones and colleagues, who first converted 10T1/2 cells into skeletal muscle with the demethylating agent 5-azacytidine (Taylor and Jones, 1979). Furthermore, an in vitro methylated skeletal α-actin promoter-reporter construct stably introduced into cell lines undergoes site- and cell type-specific demethylation concomitant with initiation of reporter gene expression (Yisraeli et al., 1986). Various deletion mutations in the α-actin promoter prevent demethylation and expression, showing that removal of methyl groups is under cis-acting control and may be required for transcriptional activation during muscle differentiation (Paroush et al., 1990).

In the present report, a detailed analysis of CpG methylation of the DNA regulatory elements in the myosin light chain (MLC)1/3 gene was undertaken to determine whether the process of muscle-specific demethylation during development is uniform at distinct transcriptional control sequences in a single locus. Analysis of the complex patterns of MLC1/3 expression, both during embryonic development and in different fiber types in adult muscle, has been facilitated by the generation of transgenic mice carrying the chloramphenicol acetyl transferase (CAT) reporter gene under the control of MLC regulatory sequences. The transgene, an MLC1 promoter-CAT transcription unit linked to the downstream MLC enhancer, reflects the expression profile of endogenous MLC1 transcription in the fast fibers of skeletal muscles.
(Rosenthal et al., 1989; Donoghue et al., 1991b). In addition, an unexpected gradient of MLC-CAT transgene expression in the segmented axial muscles of these animals (Donoghue et al., 1991a) is established during development along the antero-posterior (AP) axis in the embryo (Grieshammer et al., 1992). This suggests that the DNA regulatory elements included in the MLC-CAT transgene construct may constitute a target for regional regulators, which establish positionally restricted patterns of transgene expression during early muscle development. In the adult, the rostrocaudal gradient of transgene expression is retained only in the segmented axial muscles (intercostals and intervertebrales), since certain muscles in the head, and lateral muscles in the trunk and forelimb display high levels of transgene expression irrespective of their anterior position (Donoghue et al., 1991a; Grieshammer et al., 1992). Regional differences in MLC-CAT transgene expression are retained in skeletal muscle cell lines derived from lateral muscles of the transgenic animals (Donoghue et al., 1992a) and correlate with the methylation status of a CpG dinucleotide in the MLC1 promoter (Donoghue et al., 1992b). Taken together, these studies imply that differential methylation may be involved in the maintenance of muscle positional identity.

In the present study, transfection of cell cultures from different muscle groups with MLC-CAT DNA did not reveal any differences in transcriptional competence, further implicating epigenetic modifications in the position-specific expression of the MLC-CAT transgene. Muscle cell cultures were transfected with differentially methylated MLC-CAT constructs to establish a causal relationship between CAT reporter gene expression levels and methylation at specific sites in MLC upstream and downstream regulatory elements. A PCR-based assay (Steigerwald et al., 1990; McGrew and Rosenthal, 1993) was used for a quantitative analysis of methylation levels at CpG dinucleotides in transgenic MLC sequences during development. These experiments indicate that demethylation of the MLC enhancer precedes that of the MLC1 promoter elements during embryogenesis. However, the graded MLC-CAT transgene expression pattern initially established in the segmented axial muscle masses is not accompanied by a corresponding gradient in MLC enhancer methylation, which is established subsequent to transgene activation. Thus methylation appears to be involved in the maintenance rather than in the establishment of gene expression patterns in different muscle groups.

MATERIALS AND METHODS

Transgenic mouse lines and embryonic dissections

Generation and characterization of the three lines of MLC-CAT transgenic mice used in this study (#7, 52 and 63) has been previously described (Rosenthal et al., 1989; Donoghue et al., 1991a). Line 7 was bred to transgene homozygosity for embryonic analyses to ensure that all pups within a litter were transgenic. The morning of vaginal plug formation was designated 0.5 days post coitum (p.c.). Somites from pooled litters of 11.5 days p.c. embryos were dissected mechanically and subdivided into groups of 5-somite pairs, starting with the most rostral somite. Both the neural tube and notochord were included in the dissected material. A small sample was disrupted by sonication and processed for CAT assays and the remaining material was used for genomic isolation. Genomic DNA was isolated from quickly frozen and pulverized tissue from adult transgenic animals or from frozen embryonic tissues (pooled from 4-10 embryos) as described (McGrew and Rosenthal, 1993). CAT activity was determined from a small amount of each pulverized adult tissue.

Primary cultures and transfections

Primary rat myoblasts were prepared from the following neonatal rat muscles: hindleg, masseter, rostral (2,3), mid (5,6,7) and caudal (9,10) intercostal muscles, as previously described (Donoghue et al., 1988). Myoblasts were grown in plating media (DMEM plus 20% fetal calf serum, 2% glutamine, 12.5 units/ml penicillin-streptomycin) and transfected 3 days after plating, at approximately 70% cell density, by calcium phosphate coprecipitation using 10 μg of the pMLC1CAT920 (MLC1-CAT; Rosenthal et al., 1989) or the pMCK4800CAT (MCK-CAT; Sternberg et al., 1988) construct and 3 μg of a pSV2-lacZ control construct (Promega). After 18 hours, the medium was changed to differentiation medium (DMEM plus 10% horse serum, 2% glutamine, and 12.5 units/ml penicillin-streptomycin), containing 0.3 mg/100ml cytosine β-D-arabinofuranoside (AraC, Sigma) to select against proliferating cells. The cultures were harvested for CAT assays 3 days after switching to differentiation medium. At this stage, myoblasts had differentiated into myotubes and most proliferating cells had been eliminated by the AraC treatment.

For primary mouse cultures, one litter of neonatal day 1 line #7 transgenic pups was dissected per experiment to isolate rostral (2,3,4) and caudal (final three) intercostal muscles, masseter (which included superficialis, major and zygomaticus), diaphragm and rear leg (upper thigh). The tissue was mechanically masticated for 5 minutes and a small sample was isolated for Bradford and CAT assays. The remaining tissue was enzymatically dissociated and cultured following the neonatal rat protocol (see above) with the following modifications. Tissues were dissociated by trypsin digestion for 7 minutes at 37°C, triturated, incubated for an additional 6 minutes, and triturated again. The cells were mixed with an equal volume of plating medium containing 2% chick embryo extract, pelleted and preplated for 60 minutes to remove fibroblasts. Cells were transferred to two gelatinized Primaria 35 mm tissue culture plates. 4-5 days later the proliferated myocytes were switched to differentiation medium containing Ara-C to enrich for postmitotic myotubes. Cells were harvested 2 days later for CAT assays. CAT assays were performed according to Seed and Sheen (1988).

Detection of genomic methylation

For the quantitative analysis of genomic methylation, one (for promoter analysis) or 2 (for enhancer analysis) μg of DNA were digested overnight with a 10-fold unit/μg excess of Thal (BRL) or PvuII (New England Biolabs) restriction enzymes. The DNA was ethanol precipitated and digested overnight with equal amounts of BstXI (NEB) or HhaI (NEB) restriction enzymes followed by ethanol precipitation and ligation-mediated PCR.

Ligation-mediated PCR was performed on the restriction digested genomic DNA samples as originally described (Mueller and Wold, 1989; Garrity and Wold, 1992) and as modified by McGrew and Rosenthal (1993). For quantitation of methylation in the MLC-CAT transgene the labeled DNA samples were run on a 6% 0.4 mm acrylamide sequencing gel. The gel was dried, exposed, and the intensities of the bands in the autoradiograph were quantitated using a scanning densitometer (Molecular Dynamics). The primers used for ligation-mediated PCR had the following sequence:

Promoter analysis

primer 1 5′-TCTTCAGAAGAACAACCTGC-3′
primer 2 5′-AATGACACCTTGGAGAAGACAGTGTGGT-3′
primer 3 5′-CATTGGGAAGACAGTGTGGGGCTC-3′

Enhancer analysis

primer 4 5′-GTGCTTTAATGCTTCCACAC-3′
primer 5 5′-TACACCTCAGCAGACTGTCATGGACAC-3′
primer 6 5′-CAGCCTACGGACGTCATGGGGAAACC-3′
In vitro methylation of plasmid DNA

The MLC-CAT expression cassette (see Fig. 3) was released from the pHMLC1CAT920 construct (Rosenthal et al., 1989) by digestion with AccI (NEB). After heat inactivation of the restriction enzyme and ethanol precipitation the DNA was methylated overnight with SssI methylase, (which methylates every cytosine residue located in a CpG dinucleotide), FnuDIII methylase, HpaII methylase, or HhaI methylase (NEB). For combinations of modifications, the reactions were performed sequentially after heat inactivation and ethanol precipitation, except for the HpaII and HhaI methylases which require the same reaction conditions and were incubated simultaneously. ‘Untreated’ DNA was mock-methylated under HpaII/HhaI conditions by omitting the enzyme from the reaction. Finally, the DNA was phenol/chloroform extracted, ethanol precipitated and resuspended in distilled water.

Transfection of C2C12 muscle cultures by electroporation

C2C12 myoblasts were propagated in plating medium (containing 4\(\times\) penicillin-streptomycin). 5\(\times\)10\(^6\) cells/electroporation were washed twice in ice-cold 1x HBS (140 mM NaCl, 0.77 mM Na\(_2\)HPO\(_4\), and 25 mM Hepes (pH 7.1)), resuspended in 0.5 ml ice-cold 1x HBS, transferred to a precooled cuvette gap 0.4 cm, Bio-Rad), and 10 \(\mu\)g of plasmid DNA in 40 \(\mu\)l H\(_2\)O were added. After incubation for 5 minutes on ice, the cells were mixed by agitating the cuvette and pulsed at 0.24 kV at 960 \(\mu\)F capacitance (time constant approximately 45 mseconds) in a Gene Pulser (Bio-Rad). After incubation for 10 minutes on ice, cells were transferred to 10 ml of growth medium, tritured with a Pasteur pipette to break up the cellular debris, and plated onto 100 mm tissue culture dishes. After 16 hours, the cells were washed with PBS and C2C12 differentiation medium (2% horse serum, 2% glutamine and 50 units/ml penicillin-streptomycin in DMEM) was added. After 48 hours, the fully differentiated cells were harvested and CAT assays were performed on extracts normalized to protein concentration.

RESULTS

The gradient in MLC-CAT transgene expression is maintained in segmented muscle primary cultures

The transgenic mice used in this study contained approximately 40 copies of the CAT reporter gene, under the control of the 1200 bp rat MLC1 promoter and the skeletal muscle-specific 920 bp MLC enhancer, which lies over 24 kb downstream of the promoter in the endogenous locus (see schematic representation in Fig. 3). Previous studies have shown that a gradient of transgene expression is established during myogenesis in the developing somites, increasing in an anterior-to-posterior direction in the developing embryo (Grieshammer et al., 1992).

The present study is focused on the intercostal muscles because they maintain the positionally graded MLC-CAT transgene expression profile into adulthood, and because positional variation in CAT expression between anterior and posterior intercostal muscles is not confounded by fiber type-dependent differences in transgene expression (Donoghue et al., 1991b). Adult and neonatal satellite cells isolated from various lateral muscles of MLC-CAT transgenic mice express the CAT transgene in a pattern that reflects their expression in vivo (Donoghue et al., 1992a). To determine whether graded expression of the MLC-CAT transgene persisted in axial muscle cultures, we compared CAT activity in intercostal muscle cells isolated from neonatal animals, assayed directly or differentiated in culture. As a control, we also compared cells isolated from head and lateral muscles (masseter, diaphragm, and leg). The results in Fig. 1 show the cell cultures established patterns of MLC-CAT transgene activity similar to those seen in the excised muscle samples. This suggests that satellite cell populations in neonatal intercostal muscles are already committed to a predetermined gradient of MLC-CAT transgene expression.

Differential MLC-CAT gene expression is not established in transfected muscle primary cultures

To determine whether cells from various muscles differ in their ability to transcribe the MLC-CAT gene, we prepared primary cultures from neonatal rat intercostal and lateral muscles, and measured MLC-CAT expression from transiently transfected DNA. The transfected MLC-CAT plasmid contained the transcription unit used for the generation of the MLC-CAT transgenic mice (Rosenthal et al., 1989). As a control, parallel sets of cultures were transfected with a CAT gene driven by the regulatory sequences from the muscle creatine kinase gene (MCK-CAT) which is not positionally regulated in transgenic mouse muscles (Johnson et al., 1989). As shown in Fig. 2, transfected MLC-CAT activity did not establish the pattern of CAT expression observed for transgenic primary cultures.
Fig. 2. MLC-CAT activity in transfected primary rat muscle cultures. Primary cultures were prepared from neonatal muscles as described in Materials and Methods. Cells were transfected with the MLC-CAT construct or a construct containing the CAT gene driven by the 4800 bp MCK promoter (MCK-CAT). CAT activity was normalized to β-galactosidase activity and shown relative to the highest value obtained for each CAT construct (set to 100). Values (with standard error) are averages of 6-8 independent transfections. The gray shading reflects relative CAT activity (light=low; dark=high) obtained for cultured MLC-CAT transgenic mouse cells isolated from the same neonatal muscles (Fig. 1).

(compare to Fig. 1) but rather resembled the CAT expression profile under the control of unrelated muscle-specific regulatory sequences (MCK-CAT). Therefore, the maintenance of positionally restricted MLC-CAT expression in cultured transgenic muscle cells did not reflect differences in amounts or activities of trans-acting factors, but was likely to depend upon epigenetic modifications of the MLC-CAT transgene during development.

Methylation status of both the transgenic MLC promoter and enhancer correlates with CAT activity in different adult muscles

The results described above suggested that stable modification of MLC regulatory elements may be responsible for the maintenance of the positional variation in MLC-CAT expression in neonatal muscles. This was consistent with the previous finding that differential methylation of a CpG at a distal HpaII site in the transgenic MLC1 promoter correlated with MLC-CAT activity in various lateral muscles and in cell lines derived from adult mice (Donoghue et al., 1992b). We extended this methylation analysis to study additional sites in the MLC-CAT transgene both in the proximal promoter and in the downstream enhancer, and to include segmented axial muscles which retain the rostrocaudal gradient in transgene expression.

To obtain quantitative estimates of transgene methylation in different muscle tissues we employed a sensitive, PCR-based assay (Steigerwald et al., 1990; McGrew and Rosenthal, 1993). This method combines the use of methylation-sensitive restriction endonucleases with ligation-mediated PCR to generate two amplified products whose ratio correlates with the level of methylation at the site under investigation. The primer-linker scheme used in this study is shown in Fig. 3. The methylation status of a Thal site in the MLC1 promoter, 60 bp upstream of the transcription start site, was analyzed in various tissues of adult MLC-CAT transgenic mice. As seen in Fig. 4B, DNA obtained from non-skeletal muscle tissues was highly methylated, as illustrated by the heart in which the site was demethylated only to a level of 2.5% (Fig. 4B, lane 1). Thal site demethylation varied from 4% to 43% between lateral muscles (lanes 2-6, 8), consistent with data obtained for the more distal HpaII site in the promoter (Donoghue et al., 1992b), and varied from 6% to 16% between rostral and caudal intercostal muscles (lanes 9-12), roughly correlating with differences in CAT activity (compare Fig. 4A,B). The soleus, a predominantly slow twitch muscle, (lane 7), expressed relatively low levels of the MLC-CAT transgene, yet the Thal site was demethylated to the same extent as that of fast twitch muscles which transcribed high levels of the transgene (e.g. EDL, lane 8). Thus, MLC1 promoter methylation levels reflected positional but not fiber-specific differences between various muscle groups.

Methylation of the distal MLC enhancer element was analyzed in the same tissues. A HhaI site is situated centrally in the MLC enhancer core but does not lie within any known myogenic factor binding sites (Wentworth et al., 1991; Rosenthal et al., 1992a,b). This site was highly methylated in...
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DNA isolated from the heart (3% demethylation, Fig. 4C, lane 1). Unexpectedly, the distal enhancer element was more highly demethylated than the proximal promoter site in skeletal muscle. The degree of MLC enhancer demethylation reflected levels of transgene expression in different lateral muscles, varying from 27% to 60% (lanes 2-6, 8). Like the MLC1 promoter, the MLC enhancer was highly demethylated in the soleus (lane 7), despite the low level of CAT transgene expression, presumably because the predominantly slow fibers in this muscle limit expression of fast fiber-specific genes through other mechanisms. Also, relatively high levels of enhancer demethylation in the masseter (lane 2), a facial muscle arising from the cranial paraxial mesoderm, did not correlate either with its relatively low level of CAT activity or with its anterior position. However, enhancer demethylation correlated closely with levels of transgene expression in segmented axial muscles, ranging from 31% to 68% in the intercostal muscles (lanes 9-12).

This data is supported by genomic sequencing which revealed that three additional CpG sites in the enhancer mimicked the methylation status of the HhaI site in skeletal muscle versus non-muscle tissue (data not shown). This analysis suggests that the differential methylation of multiple CpG dinucleotides in the MLC enhancer may be involved in the control of MLC-CAT transgene expression.

### In vitro methylation of specific sites in the MLC1 promoter and MLC enhancer represses the transcriptional activity of transfected constructs

The results above showed that the spatial variability in MLC-CAT transgene expression correlated with the level of methylation in the MLC1 promoter and the distal MLC enhancer element. We next determined whether in vitro methylation of specific sites in the MLC-CAT construct would repress its activity in transient transfections of the C2C12 muscle cell line. Methylation of the CAT coding sequences was unlikely to influence expression of the MLC-CAT transgene, since an independent study demonstrated that methylation of the HpaII sites in the CAT gene did not have any effect on the activity of a linked SV40 promoter (Kruczek and Doerfler, 1983). However, since the vector sequences contain many CpG dinucleotides, whose methylation might influence the transcriptional activity of the reporter gene, the MLC-CAT cassette was first excised from the plasmid before electroporation into C2C12 myoblast cultures (see Materials and Methods).

The results in Fig. 5 show that methylation of all CpG dinucleotides completely abrogated the muscle-specific transcriptional activity of the reporter construct (compare lanes 1 and 9). Although the MLC1 promoter by itself is incapable of driving reporter gene expression in transfection assays (Donoghue et al., 1988; Rosenthal et al., 1990), modification of the proximal ThaI site or the distal HpaII site in the promoter repressed CAT activity by 50% and 65%, respectively, and their combined methylation had an additive effect (compare lanes 3, 5, and 7 to lane 1). The modification of the proximal promoter site, in comparison to the distal site, had a smaller effect on CAT expression than expected. This is probably because in vitro methylation efficiency of ThaI was not as complete as with other methylases (data not shown). Methylation of the HhaI site in the enhancer alone had a small but significant effect on CAT activity (Fig. 5, lane 2), and further weakened CAT expression when combined with a partially methylated promoter (compare lanes 3 and 4; and lanes 5 and 6). These results demonstrate that the methylation of specific sites within both the MLC1 promoter and distal MLC enhancer can affect linked CAT gene expression. Such a study can only approximate the situation in vivo, where multiple CpG dinucleotides are methylated to varying extents in both MLC1 promoter and MLC enhancer sequences (see Fig. 4). Nevertheless, these results suggest that different combinations of
specific modifications could account for varying levels of MLC-CAT transgene expression in vivo.

**Demethylation of the MLC enhancer reflects activation of MLC-CAT transgene expression during embryogenesis**

To investigate if the graded methylation pattern in adult intercostal muscles was established during somitogenesis, we determined the DNA methylation status of the MLC transgenic sequences at an embryonic stage when the gradient in MLC-CAT expression is being generated in the somites (Grieshammer et al., 1992). Successive sets of five somites dissected from transgenic 11.5 day p.c. embryos were analyzed for CAT activity or were used to prepare genomic DNA. As observed previously (Grieshammer et al., 1992), a gradient of CAT activity was present in the maturing rostral somite sections of 11.5 day p.c. embryos, increasing in a rostral to caudal direction (Fig. 6A). Analysis of the DNA from these samples revealed that the \( \text{ThaI} \) site in the MLC promoter was highly methylated in all somite sections (Fig. 6B) whereas the \( \text{HhaI} \) site in the MLC enhancer was uniformly demethylated to a level of 30% in the more mature somite sections (Fig. 6C). The less mature, more caudal somite sections (Fig. 6C) were slightly more methylated at the \( \text{HhaI} \) site than the more rostral somites, consistent with their developmental lag in maturation (compare to CAT activity in Fig. 6A). The initial activation of MLC-CAT transgene expression in the somites is therefore reflected in the demethylation of MLC enhancer sequences, but the gradient of CAT transgene expression does not initially correlate with enhancer methylation levels. Later, enhancer methylation becomes graded in the adult segmented axial muscles arising directly from myotomal precursors in the somites. Thus, levels of demethylation of the MLC enhancer and promoter appear to be involved in the maintenance rather than in the establishment of the rostrocaudal gradient in MLC-CAT transgene expression.

**DISCUSSION**

**Maintenance of MLC-CAT transgene expression patterns in skeletal muscles by methylation**

This study presents multiple lines of evidence which implicate CpG methylation of both proximal and distal control elements in the positional restriction of MLC-CAT transgene expression. First, differences in transgene expression levels in different adult skeletal muscles are generally reflected by the extent of demethylation at CpG dinucleotides in the MLC promoter and in the downstream MLC enhancer. The persistence of positional variation in transgene expression appears to be mediated by the modification of MLC regulatory sequences in the context of the MLC-CAT transgene. Transgene methylation patterns are not influenced by the site of integration into the genome, as three independently generated transgenic lines display the same modification patterns. Third, MLC-CAT expression can be regulated by DNA methylation, as demonstrated by muscle cell transfections which showed that site-specific in vitro methylation of the MLC regulatory sequences inhibited CAT reporter gene expression from transiently transfected plasmid DNA. Similar studies have previously demonstrated that in vitro methylation of promoter elements can inhibit gene expression after transient or stable transfer into other mammalian cell types (reviewed in Cedar and Razin, 1990).

The present study establishes an unprecedented correlation between methylation in a downstream enhancer element and the regulation of tissue-specific gene expression. The correlation between MLC enhancer methylation and tissue restriction of expression extends to the endogenous locus as well: the rat MLC enhancer is demethylated in skeletal muscles compared to non-muscle tissues (McGrew and Rosenthal, 1993). That epigenetic modifications of distal regulatory elements may function to control transcription has been suggested by studies...
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of the H19 gene. Recently, differential hypersensitivity to enzymatic digestion of enhancer sequences located downstream of this gene was correlated with the tissue specificity of its expression (Bartolomei et al., 1993). It remains to be seen if the H19 downstream enhancer sequences are differentially methylated as well.

Interestingly, CpG methylation does not interfere with nuclear factor binding to the MLC enhancer core (U. Grieshammer, unpublished observations), presumably because none of the CpG dinucleotides present in this element is contained within known protein binding sites. This is consistent with another study in which the DNA-binding capacity of a transcription factor was inhibited by methylation only if a CpG in its recognition site was modified (Watt and Molloy, 1988). The precise mechanism of the methylation-mediated inhibition of MLC-CAT transcription in vivo is therefore more likely to involve changes in local chromatin configuration (reviewed in Cedar and Razin 1990; Bird, 1992) with concomitant modification of regulatory protein-DNA interactions. From the present study it appears that the regulatory sequences included in the MLC-CAT transgene comprise a target for a subset of factors which impose novel methylation patterns when isolated from the context of the endogenous locus. It will now be possible to identify these elements by further deletions or mutations, and to establish whether these elements are conserved in other mammalian MLC1/3 loci.

MLC-CAT transgene expression in different myogenic cell lineages

The graded expression pattern of the MLC-CAT transgene in segmented intercostal and intervertebral muscles arises early in the developing myotomal muscle masses, and is not evident in the overlying body wall muscles or in limb musculature (Grieshammer et al., 1992). The significance of these distinctions remains to be determined, but it is likely that differences in transgene expression seen in these muscle groups may reflect differences in their embryonic origins. In developing somites, multiple myogenic lineages arise during embryogenesis that can be distinguished by their location in the somite (Or Dahl and LeDouarin, 1992), by their migratory behavior and by their dependence upon axial structures, such as the neural tube and notocord, for myogenic differentiation (Rong et al., 1992; Pourquie et al., 1993). Additional genetic evidence from the analysis of Splotch mouse embryos (carrying a homozygous mutation in the developmental regulatory Pax3 gene) confirms that the formation of limb muscles is dependent on Pax3 function whereas that of the axial, facial and body wall muscles is not (Bober et al., 1994). Taken together, these studies indicate that different regulatory pathways may be responsible for the formation of specific myogenic lineages.

The results of the present study suggest that these differences may be ultimately maintained by the establishment of distinct epigenetic modifications controlling regional gene regulation. Specifically, the methylation pattern of the MLC enhancer element, located distal to the transcription start site, directly reflects the rostrocaudal gradient of MLC-CAT transgene expression in the intercostal muscles. The expression and corresponding methylation of the MLC-CAT transgene may therefore serve as a marker for the establishment of different myogenic lineages in the embryo. It remains to be determined whether variations in methylation of endogenous loci also mark the subtle distinctions which give rise to specific muscle groups.

Generation of methylation patterns in the embryo

During mammalian development the genome undergoes global changes in methylation levels (Monk et al., 1987; Chaillet et al., 1991; Kafri et al., 1992) which are subsequently modified at individual loci. The establishment of tissue-specific methylation patterns has been shown to occur before, concomitant
with, or after the transcriptional activation of genes during embryogenesis (reviewed in Razin and Cedar, 1994). In developing avian muscles, asynchronous demethylation of the two promoters in the MLC1/3 locus occurs such that transcriptional activation of the MLC1 promoter precedes its demethylation, whereas in the MLC3 promoter demethylation of a CpG dinucleotide and transcriptional activation occur concomitantly (Lamson and Stockdale, 1989). From the present analysis it appears that in mammals, demethylation of the downstream MLC enhancer element may be a necessary prerequisite for initiation of transcription from the MLC1 promoter, both in the context of the transgene and in the endogenous locus. This model is supported by recent work which showed that an intrinsic enhancer was necessary and sufficient for tissue-specific demethylation and activation of an immunoglobulin gene locus (Lichtenstein et al., 1994).

It is unlikely that methylation is the primary determinant of positionally restricted MLC-CAT transgene expression in the developing myotome, however. The relatively uniform methylation of transgenic MLC enhancer sequences in embryonic somites from different axial levels suggests that the variations in MLC enhancer methylation seen in neonatal muscles are established at a later time during development. Subsequently, levels of gene expression in different muscles would be maintained by local chromatin configuration, reflected in selective CpG methylation of regulatory sequences.

Alternatively, regional regulators directly establish a differential methylation pattern using a subset of CpG dinucleotides which has not yet been analyzed. Such sites could serve as an initial mark for the final graded methylation pattern, which would be established during later developmental stages. This mechanism would be analogous to a current model of genomic imprinting, in which a chromosomal mark on imprinted alleles is acquired after fertilization but prior to gene inactivation (Latham et al., 1994). Characterization of the processes acting upon the discrete set of MLC regulatory sequences included in the MLC-CAT transgene may uncover similar molecular pathways for establishing positional information in developing muscles of vertebrate embryos.

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