

An axial gradient of transgene methylation in murine skeletal muscle: genomic imprint of rostrocaudal position

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Summary

We previously used mice bearing a myosin light chain-chloramphenicol acetyltransferase (MLC1-CAT) transgene to show that adult muscle cells bear a heritable, cell autonomous memory of their rostrocaudal position. CAT mRNA and protein are expressed in a >100-fold rostrocaudal gradient in skeletal muscles of developing and adult MLC1-CAT mice (Donoghue, M. J., Merlie, J. P., Rosenthal, N. and Sanes, J. R. (1991). *Proc. Natl. Acad. Sci. USA* 88, 5847-5851; Donoghue, M. J., Alvarez, J. D., Merlie, J. P. and Sanes, J. R. (1991). *J. Cell Biol.* 115, 423-434). Moreover, both in primary cultures and in myogenic cell lines prepared from individual muscles of these mice, CAT levels reflect the body position from which the myoblasts were derived (Donoghue, M.J., Morris-Valero, R., Johnson, Y.R., Merlie, J.P. and Sanes, J. R. (1992). *Cell* 69, 67-77). Here, we show that the methylation state of the MLC1-CAT transgene in skeletal muscles is also graded along the rostrocaudal axis: methylation levels decrease and expression levels increase in the order, jaw → neck → chest and forelimb

→ hindlimb. Methylation levels are also approx. 10-fold higher in rostrally derived than in caudally derived myogenic cell lines, which express low and high levels of CAT, respectively. Within each cell line, undifferentiated cells (myoblasts), which do not express the transgene, and differentiated cells (myotubes), which do, are indistinguishable in methylation state. Thus, differentiation-related changes in transgene expression do not affect position-related levels of transgene methylation. On the other hand, treatment of rostrally derived lines with the demethylating agent, 5-azacytidine, decreases methylation and increases expression of the transgene. Thus, perturbation of methylation affects expression. Taken together, these results suggest that methylation provides a genomic imprint of rostrocaudal body position that may serve as a component of the positional memory that mammalian cells retain into adulthood.

Key words: methylation, muscle, myosin light chain, positional information, rostrocaudal gradient, transgene.

Introduction

Early in embryogenesis, spatially regulated patterns of gene expression establish systems of positional information that eventually specify positionally appropriate histogenesis of cells and morphogenesis of tissues (Wolpert, 1989). Subsequently, many of the genes that initially generated the 'positional values' cease to be expressed (Paro, 1990; McGinnis and Krumlauf, 1992). Nonetheless, transplantation and regeneration experiments in both invertebrates and vertebrates reveal that at least some cells retain a 'positional memory' into adulthood (Ursprung and Nothiger, 1972; Purves and Lichtman, 1985; Brockes, 1989). Thus, distinct mechanisms may account for the establishment and the maintenance of positional information. In recent years, dramatic advances have been made in understanding the intercellular and intracellular signals that establish the body plan (Melton, 1991; St. Johnston and Nüsslein-Volhard, 1992; Slack and Tannahill, 1992). In contrast, mechanisms that

account for the maintenance of positional information as the organism matures remain relatively obscure.

We have recently characterized a set of transgenic mice that provides a model system for the study of positional memory in mammals. The transgene consists of regulatory sequences from the rat myosin light chain (MLC) 1f/3f gene, linked to the bacterial chloramphenicol acetyltransferase (CAT) gene. Expression of this MLC1-CAT transgene reflects that of the endogenous MLC1f/3f gene in that it is muscle-specific, muscle fiber type-dependent and developmentally regulated. Unlike the endogenous MLC1f/3f gene, however, the transgene is expressed in a steep rostrocaudal gradient: muscles that arise from successively more caudal somites express successively higher levels of CAT mRNA and protein, with a difference of >100-fold between muscles of the head and tail (Donoghue et al., 1991a,b; Sanes et al., 1992). Position-dependent expression is directed by sequences derived from the MLC1f/3f gene (even though the endogenous gene is

expressed at similar levels in rostral and caudal muscles), and is not integration site-dependent (similar gradients are seen in each of three independently derived lines of transgenic mice). The gradient of expression is established during embryogenesis (Donoghue et al., 1992; Greishammer et al., 1992), and may result from graded morphogens or intercellular interactions. However, the gradient is stably maintained in adults (Donoghue et al., 1991a), when muscles are separated from each other by long distances (several meters, for example, if giraffes use a similar system). Moreover, muscle cells cultured from these mice and even immortalized cells derived from adult myoblasts (satellite cells) express the transgene at levels that reflect their rostrocaudal position of origin (Donoghue et al., 1992). These results show that adult muscle cells retain a cell-autonomous and heritable memory of their body position.

In general terms, this positional memory could reside in the transcriptional apparatus ('*trans*' models) and/or within the transgene itself ('*cis*' models) (Donoghue et al., 1992). In this report, we focus on one particular '*cis*' model: that differential methylation of the transgene serves as an imprint of rostrocaudal position. Methylation of cytosine (C) in the sequence CpG is the predominant covalent modification of mammalian genomic DNA. Patterns of genomic methylation are often stably heritable within somatic cells of an individual, probably due to the actions of a maintenance methylase. This enzyme recognizes the hemimethylated DNA formed during replication and methylates the corresponding sequence on the nascent strand. Numerous examples have been documented in which levels of methylation correlate with levels of gene expression (generally, highly expressed genes are hypomethylated) and, in some of these cases, methylation is known to influence the level of gene expression. For example, methylation has been implicated in some tissue-specific and developmentally regulated patterns of gene expression, in X-chromosome inactivation and in parental imprinting (the preferential expression of one parental allele of certain autosomal genes; reviewed by Holliday et al., 1990; Razin and Cedar, 1991). Moreover, recent studies have shown that some transgenes are parentally imprinted and have suggested that differential methylation of the two alleles constitutes the imprint (Hadchouel et al., 1987; Reik et al., 1987; Sapienza et al., 1987; Swain et al., 1987; Chaillet et al., 1991; Sasaki et al., 1991; Surani, 1991). Here, we have tested the hypothesis that differential methylation underlies position-dependent gene expression as well. To this end, we assayed the methylation state of the MLC1-CAT transgene in individual muscles from developing and adult animals, and in cell lines derived from rostral and caudal muscles. We show that the methylation state of the MLC1-CAT transgene is position-dependent both *in vivo* and *in vitro*. We then used the cell lines to monitor transgene methylation during the differentiation-induced activation of transgene expression and to monitor transgene expression following pharmacologically-induced demethylation. Results of these experiments suggest that methylation state is an important determinant of position-dependent levels of transgene expression.

Materials and methods

Mice

The transgenic mice used in this study have been described previously. MLC1-CAT mice, lines 52 and 63, contain a 1.2 kb MLC1 promoter and a 922 bp enhancer from the MLC1f/3f gene, placed 5' and 3', respectively, of a cassette containing a bacterial CAT gene and an intron and polyadenylation signal from the SV40 T-antigen gene (Rosenthal et al., 1989; Donoghue et al., 1991a,b). AChR⁻-CAT mice, line 15, contain a 850 bp promoter from the chicken skeletal muscle acetylcholine receptor (AChR)⁻ subunit gene, linked to the CAT cassette (Klarsfeld et al., 1987; Merlie and Kornhauser, 1989). α -actin-CAT mice, line 2854B, contain a 2.2 kb promoter from the chicken skeletal muscle α -actin gene, also linked to CAT (Petropoulos et al., 1989).

Cells

Muscle cell lines were derived from MLC1-CAT mice (line 63) by infection of cultured adult satellite cells with a recombinant retrovirus bearing a temperature-sensitive mutant SV40 T antigen oncogene (Donoghue et al., 1992; the retrovirus is described in Renfranz et al., 1991). Cells were maintained as myoblasts at 33°C in F10 medium containing 20% fetal calf serum and 6% chick embryo extract (growth medium). To induce fusion, cultures were shifted to 37°C and fed with Dulbecco's modified Eagle's medium containing 2% horse serum (differentiation medium). For the present studies, the populations described previously (Donoghue et al., 1992) were subcloned by limiting dilution. Cell lines are numbered according to the muscle from which they were derived: **1** (1-3A2, 1-3A3, 1-3C2 and 1-4C) from masseter (jaw); **2** (2-1H) from infrahyoid and related muscles of the neck; **5** (5-12A) from tibialis anterior and extensor digitorum longus (anterior crural muscles of hindlimb); and **6** (6-1B2 and 6-1B6) from gastrocnemius and plantaris (posterior crural muscles of hindlimb). (Series 3 and 4, from back and chest muscles, respectively, were not used in this study).

For treatment with 5-azacytidine (5AC), cells were plated at 50% confluence and maintained at 33°C in growth medium. On the following day, they were fed with Dulbecco's modified Eagle's medium containing 20% fetal calf serum plus 0.9 μ M 5AC (Sigma, St Louis, MO; dissolved in water on the day of use). Following an additional 24 hours at 33°C, cells were washed twice, switched to differentiation medium and transferred to 37°C.

Methylation analysis

High molecular mass DNA was prepared from quick-frozen and pulverized tissue or from cultured cells by the method of Davis et al. (1986). Aliquots of 5-15 μ g DNA were incubated with 400 units of *Eco*RI at 37°C for 16 hours. The enzyme was then heat inactivated and each reaction mixture was split into three equal parts. 200 units of *Msp*I were added to one part, 200 units of *Hpa*II to the second part and no additions were made to the third part. Following incubation with *Msp*I or *Hpa*II at 37°C for 16 hours, all the samples were extracted with phenol-chloroform, precipitated with ethanol, resuspended, electrophoresed on a 0.75% Sea-Kem/2.25% Nusieve (FMC; Rockport, ME) gel and transferred to Gene Screen Plus (NEN-Dupont) by the method of Southern (1975). The membranes were probed with DNA that had been labeled to a specific activity of approx. 10^9 cts/minute/ μ g with 32 P-dCTP by random priming, using a kit from Boehringer-Mannheim (Indianapolis, IN). The following probes were used: (1) the 3.7 kb *Acc*I fragment from pMLC1-CAT-920 (Rosenthal et al., 1989); (2-5) fragments of 408, 674, 1104 and 1804 bp, purified from *Msp*I digests of pMLC1-CAT-920 (these are indicated as probes a-d in Fig. 1A); (6) the 1.6 kb *Hind*III-*Bam*HI fragment from pMLC1-CAT-920; (7) the 2.45 kb *Hind*III-*Sac*I fragment

from p ACh-CAT/BS+ (Klarsfeld et al., 1987; Merlie and Kornhauser, 1989); and (8) the 1.2 kb *AccI*-*HindIII* fragment from pMLC1-CAT-920. Hybridization was performed in $5 \times$ SSC, 0.5% SDS, $10 \times$ Denhardt's solution, 10% dextran sulfate at 65°C . After washing (0.1% SSC, 1% SDS, 65°C), membranes were exposed to film and relative signals were quantified by densitometry.

Other assays

CAT activity was assayed as described by Gorman (1985). Protein concentrations were determined by the method of Bradford (1976). Immunohistochemical staining of cultures was performed as described previously (Donoghue et al., 1992), using the following antibodies to myosin heavy chains (MHCs): MY32, which reacts with adult type II and perinatal MHCs (Sigma); BA-D4, SC-71 and BF-F3, which are specific for type I, type IIA and type IIB MHCs, respectively (Schiaffino et al., 1989; Gorza, 1990; provided by Dr Stefano Schiaffino, University of Padova, Italy); and 2B6, which is specific for embryonic MHC (Gambke and Rubinstein, 1984; provided by N. Rubinstein, University of Pennsylvania).

Results

Assessing transgene methylation

We used a pair of restriction enzymes, *MspI* and *HpaII*, to assay the methylation of the MLC1-CAT transgene. Both enzymes recognize the sequence 5'-CCGG-3', which is frequently methylated at the second C in vertebrate DNA. However, *HpaII* cuts only unmethylated sites, whereas *MspI* is insensitive to methylation at the second C. Thus, differences between the fragments generated by digestion with the two enzymes provide a measure of whether or not specific sites are methylated.

The MLC1-CAT transgene contains five *MspI/HpaII* sites: one in the promoter and four in the CAT gene, but none in the SV40-derived intron, the polyadenylation signal, or the downstream enhancer (Fig. 1A). Digestion of an isolated MLC1-CAT construct by *MspI* or *HpaII* generated the fragments predicted from the restriction map (data not shown). However, more complex patterns could result from *HpaII* digestion of MLC1-CAT genomic DNA, because these mice contain approx. 40 tandemly arrayed copies of the transgene, any or all of which might contain methylated sites. To circumvent this problem, we digested the genomic DNA with *EcoRI*, which cuts once within the 3.7 kb transgene, before incubating with *MspI* or *HpaII*. As expected, an intensely labeled 3.7 kb band was observed when *EcoRI*-digested DNA from MLC1-CAT mice was analyzed by Southern blotting using the complete transgene as a probe (Fig. 1B, lane R), whereas 408 bp, 674 bp and 2.5 kb bands were detected in *EcoRI/MspI* digests (Fig. 1B, lane M). The predicted 45 bp and 125 bp *EcoRI/MspI* fragments were not detected, presumably because they did not bind efficiently to the membrane used for blotting. Faint bands, probably derived from the 5- and 3- ends of the tandem array, were also observed, but these were not analyzed in detail.

Digestion of genomic DNA from MLC1-CAT mice with *EcoRI* plus *HpaII* should generate a pattern identical to that of the *EcoRI/MspI* digest if the transgene is completely unmethylated, or identical to that of the *EcoRI* digest if the

transgene is fully methylated. Partial methylation would be expected to generate a mixture of these and intermediate-sized bands. The *EcoRI/HpaII* digestion pattern shown in

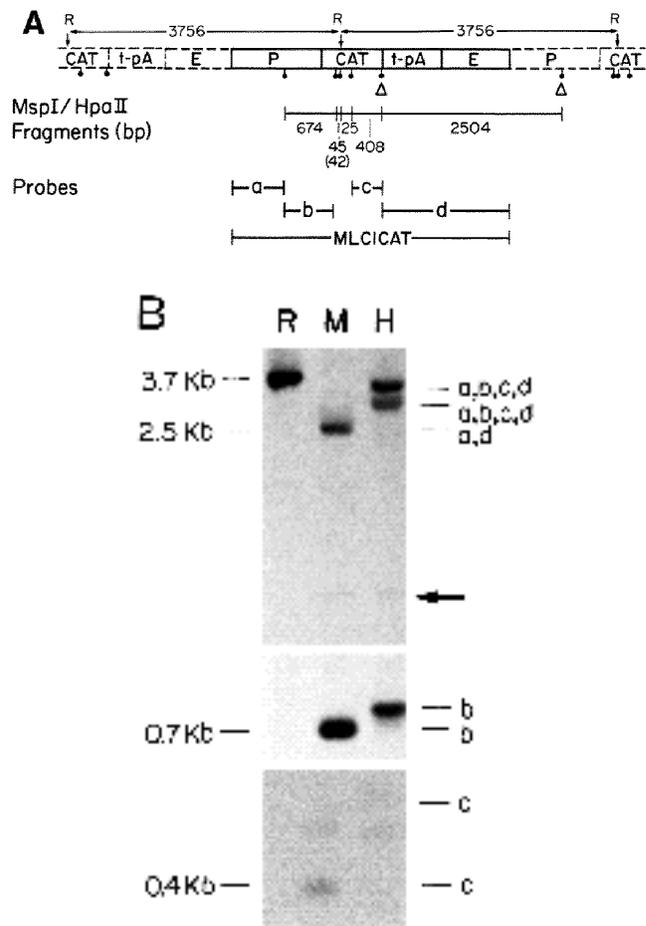
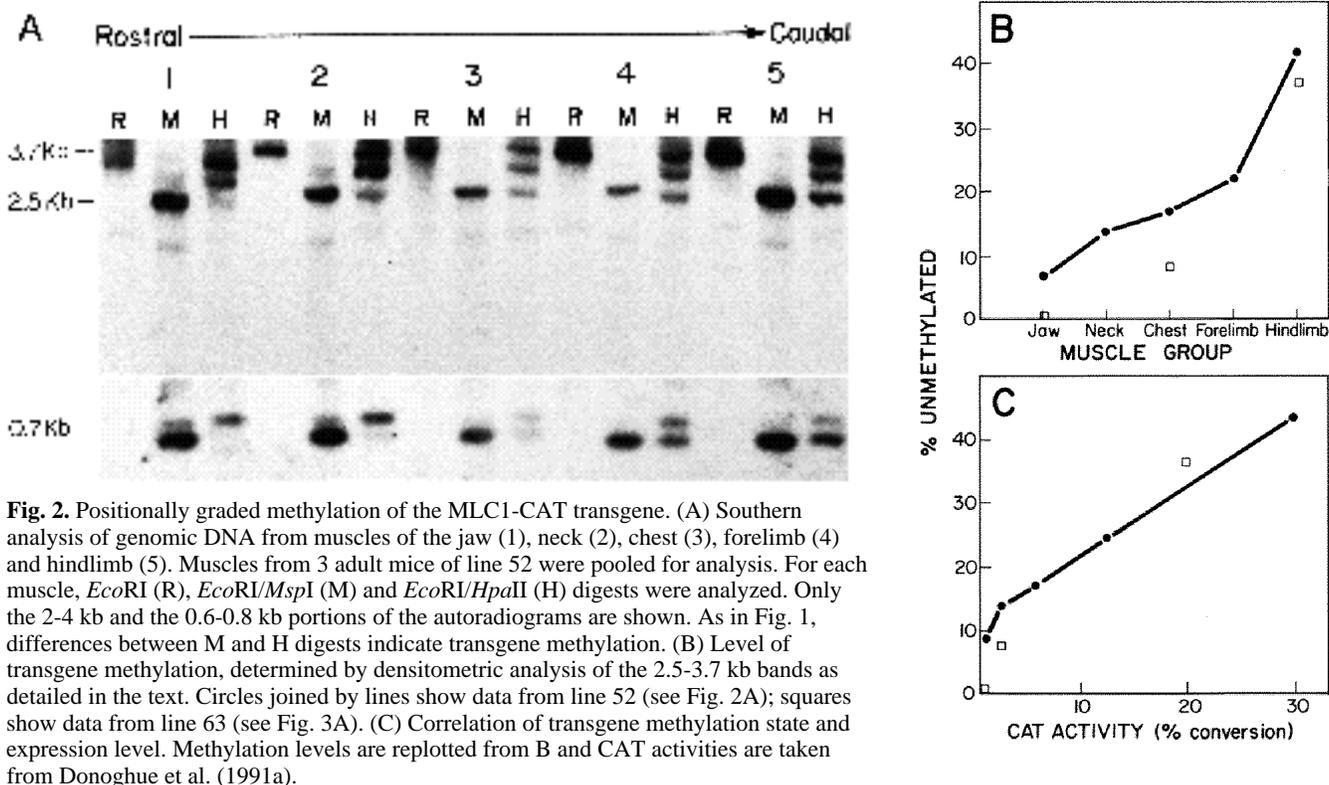


Fig. 1. Strategy for analyzing methylation of the MLC1-CAT transgene. (A) The structure of the transgene is shown at the top. It consists of a CAT gene from *E. Coli* and an intron and polyadenylation signal from SV40 (t-pA) flanked by the MLC1 promoter (P) and an enhancer (E) from the MLC1f/3f gene. Dashes show that multiple copies of the transgene are tandemly arrayed in the genome. Arrows above the gene show the single *EcoRI* (R) site in each copy. Lollipops below the gene mark the *MspI/HpaII* sites. Sizes of *MspI/HpaII* fragments are indicated; note that digestion with *EcoRI* plus *MspI* or *HpaII* reduces the band of 45 bp to 42 bp. Triangles mark the sites that must both be unmethylated for *HpaII* to generate a 2.5 kb band. Probes used for Southern analysis are shown at the bottom. (B) Southern blot of genomic DNA from a muscle cell line digested with *EcoRI* (R), *EcoRI* plus *MspI* (M) or *EcoRI* plus *HpaII* (H) and probed with the full MLC1-CAT transgene. The figure is a montage of three autoradiographic exposures, because smaller bands were fainter than larger ones. Letters at the right indicate bands that were recognized by the probes labeled a-d in A. As explained in the text, the differences between the M and H digests indicate that the transgene is partially methylated in this cell line. The approx. 1.2 kb band (arrowed) was also recognized by probe a and was present in wild-type DNA, indicating that it is derived from endogenous MLC1 promoter. We do not know the origin of the approx. 500 bp band; it was seen consistently in M and H digests of samples from one line of MLC1-CAT mice (line 63), but not in samples from a second line (line 52).



lane H of Fig. 1B is diagnostic of partially methylated DNA. This is seen clearly in the triplet of bands at approx. 2.5, 3.0 and 3.7 kb. The presence of the 3.7 kb band indicates that the two sites denoted by triangles in Fig. 1A are both methylated in some copies of the transgene. The approx. 3.0 kb band (actually a set of poorly resolved bands that range from 2.9 to 3.1 kb) indicates that one or the other but not both of these sites are methylated in other copies of the transgene. The 2.5 kb band (seen more clearly in Figs 2, 3, 8 and 9, below) indicates that both of these sites are unmethylated in yet other copies. To quantify the level of transgene methylation, we determined the intensity of the 2.5 kb band relative to the total intensity of all three bands. This value, which is 2% for the sample in Fig. 1B, represents the fraction of transgene copies in which both of the sites in question are unmethylated. Similar arguments apply to the pairs of bands at 674 and 716 bp and at 408 and 533 bp, but we did not quantify these.

The identity of the hybridizing bands was confirmed by reprobing Southern blots with the fragments of the transgene labeled a-d in Fig. 1A. These probes selectively hybridized to bands indicated on the right in Fig. 1B. In each case, the pattern observed was consistent with the map in Fig. 1A, validating the use of MLC1-CAT-probed Southern blots to assess transgene methylation.

Methylation of the MLC1-CAT transgene is positionally graded in adult skeletal muscle

We analyzed the methylation state of the MLC1-CAT transgene in five sets of muscles that spanned the rostrocaudal axis. These were: (1) the masseter and digastricus of the jaw, (2) the infrahyoid complex and related muscles of the

neck, (3) the pectoralis major and minor of the chest, (4) the triceps of the forelimb and (5) the tibialis anterior and extensor digitorum longus of the hindlimb. As shown in Fig. 2A, levels of transgene methylation varied with body position, in muscles from adult MLC1-CAT mice (line 52). The transgene was highly methylated in muscles of the jaw, as can be seen when the *EcoRI/MspI* and *EcoRI/HpaII* digests from this muscle group are compared (Fig. 2A, lanes 1M and 1H). In contrast, the transgene was much less methylated in DNA derived from muscles of the hindlimb (Fig. 2A, lanes 5M and 5H). DNA from neck, chest and forelimb muscles showed intermediate levels of methylation (Fig. 2A, lanes 2-4). Quantitation of methylation level, as detailed above, revealed that the fraction of transgene copies that were unmethylated at the sites in question varied from 7% to 42% in a rostrocaudal gradient (Fig. 2B). In that one of these sites is in the MLC1 promoter and the other is in the CAT gene, it is apparent that both elements contained sites whose methylation level varied with body position. Moreover, levels of transgene methylation and of transgene expression were highly correlated ($r=0.97$; Fig. 2C). Thus, intermuscular differences in transgene methylation are related both to body position and to transgene activity.

Transgene- and tissue-specificity of positionally graded methylation

Based on the results presented above, we performed a series of studies to characterize the phenomenon of position-dependent transgene methylation. First, because levels of transgene methylation have been shown to be integration site-dependent in some cases (Allen et al., 1990; Sasaki et

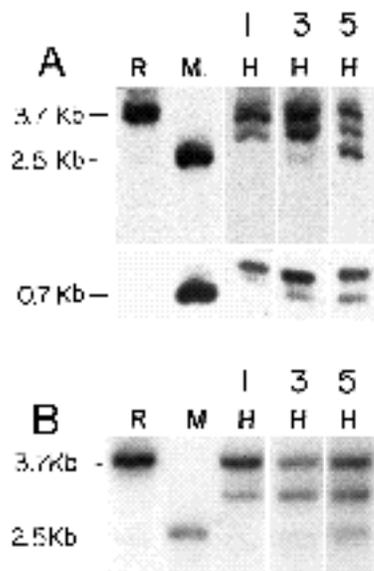


Fig. 3. Positionally graded methylation of the MLC1-CAT transgene is independent of integration site and postnatal age. Methylation analysis of DNA from jaw (1), chest (3) and hindlimb (5) muscles of adult (A) and postnatal day 1 (B) mice of line 63. Only a single *EcoRI* (R) and *EcoRI/MspI* (M) digest is shown from each experiment, to mark positions of the complete transgene (3.7kb) and the completely unmethylated (2.5kb and 674 bp) fragments; these methylation-independent patterns did not vary among samples. *EcoRI/HpaII* (H) digests, which are methylation-dependent showed differences in methylation state among samples.

al., 1991), we assayed methylation in a second, independently derived line of MLC1-CAT mice, line 63. The transgenes in lines 52 and 63 are integrated at distinct chromosomal locations, but both lines bear approx. 40 transgene copies and both express the transgene at similar absolute levels and in a similarly graded fashion (Donoghue et al., 1991a). Southern analysis showed that the methylation state of the MLC1-CAT transgene also varied with rostrocaudal position in line 63 (Figs 2B,C and 3A). Thus, the signals that lead to positionally graded methylation, like those that promote positionally graded expression, appear to be contained within the transgene itself.

Second, we asked whether position-related differences in methylation level appear before adult levels of expression are reached. To this end, we assayed methylation in neonatal muscles, in which CAT expression is already graded, but only approx. 5% of adult levels (Donoghue et al., 1992). In neonates, as in adults, transgene methylation was highest in jaw muscles, lowest in hindlimb muscles and intermediate in chest muscles (Fig. 3B). This result indicates that the methylation pattern of the transgene is established while transgene expression is submaximal.

Third, because the CAT gene as well as the MLC1 promoter contain sites whose methylation state varied with body position, we asked whether MLC1-derived sequences were necessary for position-dependent methylation. For this purpose, we assayed methylation in muscles from two other lines of transgenic mice. CAT was linked to a promoter

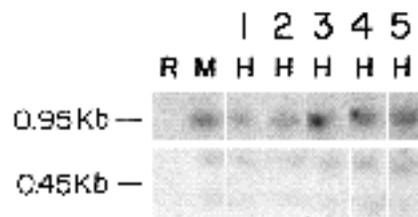


Fig. 4. Methylation of the AChR -CAT transgene is position-independent. DNA from muscle groups 1-5 (see Fig. 2) of AChR -CAT mice were probed with the AChR -CAT construct. The 3 major *MspI/HpaII* fragments detected are shown; all were predominately unmethylated in the muscles tested.

from the AChR -subunit gene in one of these lines (AChR -CAT; Merlie and Kornhauser, 1989) and to a promoter from the skeletal -actin gene in the other (sk-actin-CAT ; Petropoulos et al., 1989). Expression of CAT is muscle-specific in both lines and the AChR -CAT transgene, like the MLC1-CAT transgene, is present at approx. 40 copies/genome (Merlie and Kornhauser, 1989; copy number for sk-actin-CAT mice has not been determined). However, unlike the MLC1-CAT transgene, expression of both the AChR -CAT transgene (Donoghue et al., 1991a) and the sk-actin-CAT transgene (data not shown) varies only a few-fold among muscles and shows no detectable positional gradient. Similarly, Southern analysis revealed no intermuscular differences in methylation state of either the AChR -CAT (Fig. 4) or of the sk-actin-CAT (data not shown) transgene. For example, the 408 bp *MspI/HpaII* fragment contained entirely within the CAT gene (Fig. 1A) was predominantly unmethylated in all muscles from both lines of mice, whereas the methylation state of this fragment was position-dependent in MLC1-CAT mice. Thus, the CAT gene itself does not contain signals sufficient for position-dependent methylation, nor is graded methylation a common feature of transgenes that are expressed in a muscle-specific fashion. Instead, the simplest interpretation of these data is that position-dependent methylation, like position-dependent expression (Donoghue et al., 1991a) is directed by MLC1-derived sequences in the transgene.

Fourth, we asked whether the endogenous MLC1 promoter was methylated in a position-dependent fashion. For this purpose, we probed DNA from adult wild-type and AChR -CAT mice with a fragment containing the MLC1-promoter and transcription initiation site ('P' in Fig. 1A). This probe hybridized to an approx. 1.2 kb band in *EcoRI/MspI* digests (see arrowed band in Fig. 1B), which is presumably 5' to the *MspI* site at nucleotide -100 in the MLC1f promoter (Robert et al., 1984; Daubas et al., 1985; published sequence extends only approx. 100 bp upstream of this site). This band was present in *EcoRI/HpaII* digests of DNA from all five adult muscles assayed (see above), but was absent from *EcoRI/HpaII* digests of liver (data not shown). Thus, the sites that flank this fragment are predominantly unmethylated in muscle but fully methylated in liver. These results are similar to those of Lamson and Stockdale (1989), who showed that two *HhaI* sites in the chicken MLC1f promoter are unmethylated in adult muscle but methylated in liver. More important in the present context, our analysis indicates that the endogenous MLC1 pro-

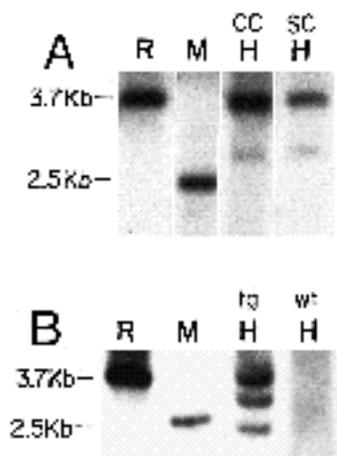


Fig. 5. Position-independent methylation of the MLC1-CAT transgene in cerebral cortex (CC) and spinal cord (SC) (A). B shows DNA from hindlimb muscles of MLC1-CAT (tg) and wild-type (wt) mice, for comparison. The transgene was highly methylated in both rostrally and caudally derived non-muscle tissues. Other abbreviations as in Figs 2 and 3.

motor is not methylated in a position-dependent fashion. Thus, position-dependent methylation and position-dependent expression are characteristics of the transgene that are not shared by the endogenous gene. As discussed previously (Donoghue et al., 1991a, 1992), we do not yet know why the transgene faithfully mirrors the behavior of the endogenous gene in several respects but differs strikingly in others. (See Sasaki et al. (1991) for a related discussion of differences in parental imprinting between transgenes and their endogenous counterparts.)

Finally, we asked whether position-dependent methylation was confined to skeletal muscle, the only tissue that expresses the transgene, or whether it also occurred in non-expressing tissues. Genomic DNA was isolated from pairs of tissues that differ in body position but are similar in cellular composition. These were: the ear and tail (containing predominantly epidermal and dermal cells); brachial and lumbar plexi (Schwann cells and fibroblasts); and cerebral cortex and spinal cord (neurons and glia). We also tested cells that had been dissociated from ear or tail and grown in primary culture for two weeks. We found that the transgene was highly methylated in all of these samples (e.g., Fig. 5); no significant differences were observed between rostrally and caudally derived tissues. Thus, position-dependent methylation and position-dependent expression of the MLC1-CAT transgene are both muscle-specific.

Position-dependent transgene expression in muscle cell lines

The parallels between the methylation state and expression level of the MLC1-CAT transgene raised the question of whether methylation state is a determinant or a consequence of expression. A strategy for addressing this issue is to manipulate either expression or methylation, and then to assay the effect of the manipulation on the other parameter. Because such manipulations are best performed *in vitro*, we cultured myoblasts from individual muscles of MLC1-CAT mice and immortalized them by retrovirus-mediated

transfer of a temperature-sensitive oncogene. We previously showed that such immortalized populations express CAT at levels that depend on their position of origin and we suggested that they would be useful for analyzing how such expression is regulated (Donoghue et al., 1992).

For the present studies, we characterized several cell lines derived from transformed populations of caudal (hindlimb) and rostral (jaw and neck) myoblasts. Cells in all of the lines grew as myoblasts with a doubling time of approx. 1 day in serum-rich medium at 33°C, the permissive temperature for the mutant oncogene (Fig. 6A,C,E,G). Shifting the cells to 37°C and refeeding with serum-poor medium (see Methods) caused them to withdraw from mitosis and fuse to form multinucleated myotubes (Fig. 6B,D,F,H). Little fusion occurred if only the temperature or the medium was switched (data not shown), indicating that inactivation of the oncogene and serum deprivation were both required for full differentiation. Levels of CAT activity were negligible in proliferating myoblasts, but increased rapidly with myotube formation, peaking approx. 3-6 days after the medium was switched (Fig. 7). This differentiation-dependent increase in transgene expression is typical of numerous muscle-specific genes, including the endogenous MLC1f/3f gene (Caravatti et al., 1982; Cox et al., 1990). Most important in the present context, peak CAT levels were approx. 100-fold higher (range 30- to 1000-fold) in the caudally derived than in the rostrally derived lines.

Because MLC1-CAT expression is fiber-type-dependent as well as positionally graded *in vivo* (Donoghue et al., 1991b), it was important to ask whether cell lines that expressed different levels of CAT varied in fiber type as well as in position of origin. Accordingly, we stained the myotubes with a panel of monoclonal antibodies to myosin heavy chains (MHCs), the expression of which is a generally accepted criterion for fiber type (Pette and Staron, 1990). Myotubes in all lines were stained strongly by an antibody specific for embryonic MHC; as expected, myoblasts were unstained (Fig. 6I,K). Some myotubes were stained by antibodies specific for MHCsI, IIA and IIB in all lines; however this staining was weak and occurred with roughly equal frequency in rostrally and caudally derived lines (not shown). Myotubes in all lines stained intensely with antibody MY32, which recognizes perinatal and adult type II MHCs but not embryonic MHC (Fig. 6J,L). Because levels of MHC IIA and IIB are low, we presume that the myotubes express perinatal MHC. The expression of high levels of embryonic and perinatal MHC but low levels of adult MHCs is a common characteristic of mammalian muscle cell lines and primary cultures (Dusterhoft et al., 1990; Miller, 1990). Finally, electrophoresis of cellular proteins on one-dimensional SDS polyacrylamide gels revealed no substantial differences among cell lines (not shown). Based on these results, we conclude that our cell lines are similar in morphology, development, protein composition and MHC expression, and are therefore suitable for analyzing position-dependent differences in gene expression.

Position-dependent transgene methylation in muscle cell lines

We assessed the methylation state of the MLC1-CAT transgene in myotubes from eight muscle cell lines, five derived

from jaw and neck muscles, and three from hindlimb muscles. As shown in Fig. 8, the transgene is more highly methylated in all of the rostral cell lines than it is in any

of the caudal cell lines. Thus, the relationship of methylation state to body position that is established *in vivo* is retained *in vitro*.

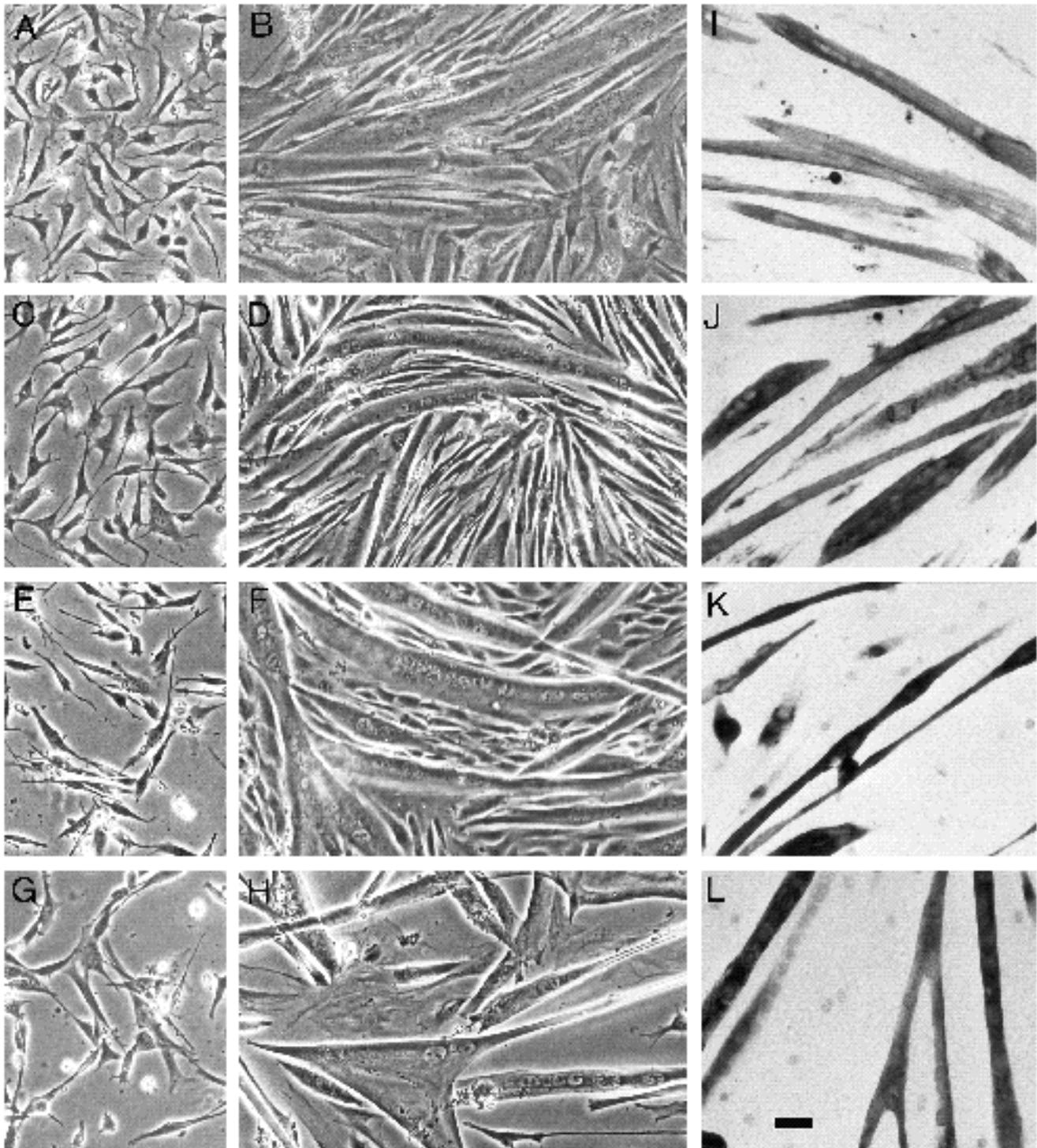


Fig. 6. Myogenic cell lines derived from rostral and caudal muscles. Micrographs of 4 representative lines are shown: 5-12A, from anterior crural muscles of hindlimbs (A,B,I,J); 6-1B2, from posterior crural muscles of hindlimbs (C,D); 1-3A3 from jaw (E,F,K,L); and 2-1H from neck (G,H). All lines grow as myoblasts in serum-rich medium at 33°C (A,C,E,G), fuse to form myotubes in serum-poor medium at 37°C (B,D,F,H) and express perinatal (I,K; stained with antibody MY32) and embryonic (J,L; stained with antibody 2B6) MHCs following differentiation. Comparison of phase-contrast (B,F) and bright-field (I-L) micrographs shows that MHCs accumulate in myotubes but not in adjacent myoblasts. Bar (in L), 50 μ m.

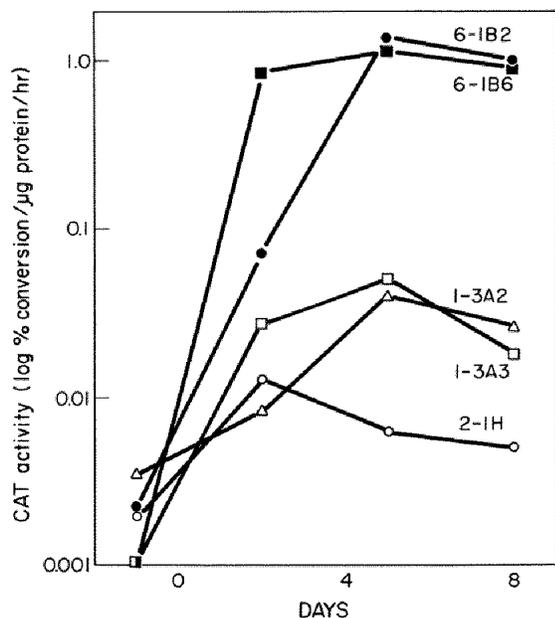


Fig. 7. Transgene expression is developmentally regulated in cell lines from MLC1-CAT mice. Cells were assayed as myoblasts or at indicated times following induction of fusion. Rostral and caudal cell lines express low and equivalent levels of CAT as myoblasts, then accumulate higher and position-dependent levels of CAT on fusion. In this experiment, specific activities of CAT were 30- to 500-fold higher in caudal than rostral lines; in most experiments, differences were >100-fold.

These results are noteworthy in three respects. First, they demonstrate that methylation patterns are stable and heritable: we estimate that the satellite cells from which these lines were derived underwent at least 35 divisions *in vitro* before producing the cultures from which the data in Fig. 8 were generated, yet methylation state remained related to the progenitor's position of origin. Second, the cell lines dissociate methylation state from fiber type: our cell lines, like others that have been characterized, do not express differentiated phenotypes of any specific adult fiber type (see above). In light of the previous demonstration that position and fiber type are independent determinants of MLC1-CAT transgene expression *in vivo* (Donoghue et al., 1991b), the present study suggests that methylation reflects position rather than fiber type. Third, the partial methylation of the transgene in caudally derived cell lines helps to distinguish between two explanations for the partial methylation of transgene DNA in caudal muscles *in vivo*. In that each cell line represents the clonal progeny of a single myoblast, our results favor (but do not prove) the idea that some copies of the transgene are methylated and others unmethylated within individual cells. Less likely is the alternative explanation, that partial methylation of tissue-derived DNA reflects a mixture of some cells with highly methylated transgenes and others with unmethylated transgenes.

Next, we used the cell lines to ask whether methylation state reflects the extant level of transgene expression, or whether it remains stable when expression changes. As detailed above, transgene expression is myotube-specific: proliferating myoblasts in rostral and caudal lines express

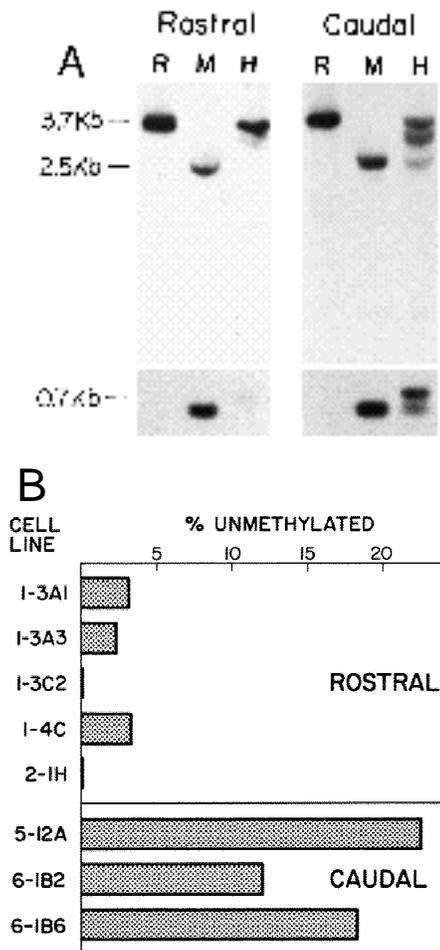


Fig. 8. Position-dependent transgene methylation in muscle cell lines. (A) Southern analysis of DNA from differentiated cultures of a representative rostral and caudal cell line. (B) Percentage unmethylated DNA in each of 8 cell lines. Abbreviations and method of analysis are as in Fig. 2.

low and equivalent levels of CAT whereas myotubes express the transgene at levels which are many-fold higher and position-dependent (Fig. 7). We therefore compared transgene methylation in subconfluent myoblasts and differentiated myotubes of the rostral and caudal cell lines. As shown in Fig. 9, the methylation state of myoblasts and myotubes was identical in each of the lines. This result indicates that methylation state maintains a genomic memory of a cell's potential for transgene expression even when the cell is not currently expressing the transgene. Another implication of this result is that differentiation-dependent and position-dependent expression of the transgene are regulated in different ways, with methylation playing distinct roles in the two processes (see also Lamson and Stockdale, 1989).

Finally, we asked whether we could alter transgene expression by manipulating transgene methylation. For this purpose, we used the DNA demethylating agent, 5AC. In numerous previous studies, treatment of cultured cells with 5AC has been shown to result in demethylation of genomic sequences, probably by inhibiting methylation of nascent

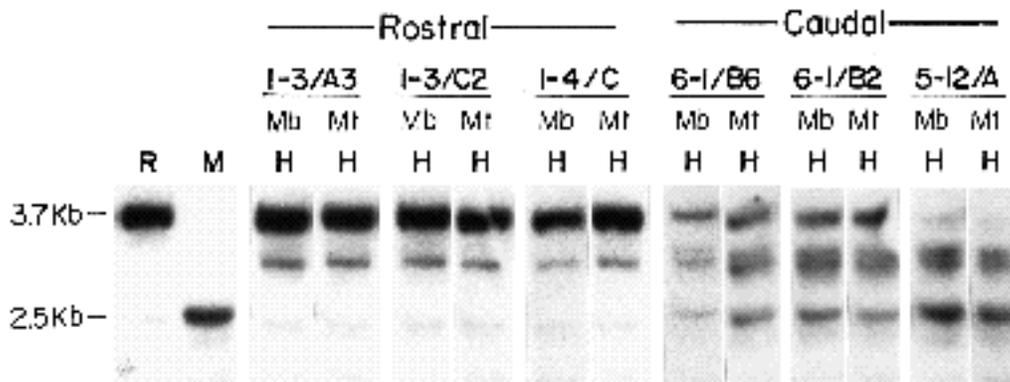


Fig. 9. Transgene methylation remains constant as transgene expression increases during differentiation. DNA was analyzed from cultures of myoblasts (Mb; grown at 33°C), which express little CAT, and from cultures of myotubes (Mt; grown at 37°C), which express maximal levels of CAT (see Fig. 7). For each of 8 lines analyzed (2-1H and 1-3A2, in addition to those shown here), methylation state of myoblasts and myotubes was identical.

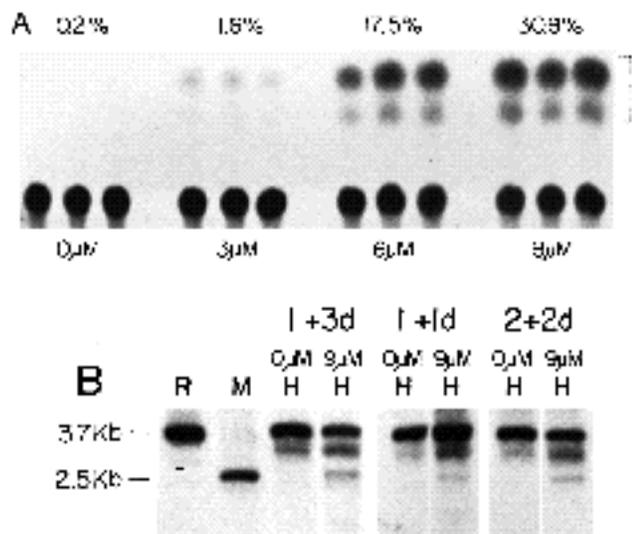


Fig. 10. The demethylating agent, 5AC, induces CAT expression and demethylation in rostrally derived cell lines. (A) Myoblasts of line 1-3A3 were treated for 24 hours with the indicated concentration of 5AC. The medium was then changed and the cultures switched to 37°C; CAT activity was assayed 3 days later, when fusion was complete. Results from three separate cultures at each dose are shown; values are average CAT activity (% conversion/25 μ g protein/hour incubation) for the triplicates. Bracket shows position of reaction products. (B) Methylation analysis of untreated (0 μ M) and 5AC-treated (9 μ M) cultures, 1 and 3 days after the end of 5AC treatment. 5AC led to demethylation of the transgene in myotubes (1+3d) and demethylation preceded myotube formation (1+1d). Similar results were obtained from cells treated with 5AC for 2 days and assayed 2 days later (2+2d).

DNA strands during replication; in many of these cases, demethylation led to expression of an initially silent gene (Jones, 1985; Razin and Cedar, 1991). We therefore treated myoblasts of a rostral cell line, 1-3A3, for 24 hours (1 cell division) with 5AC, then changed the medium to remove the drug and induce myotube formation. 3 days later, the cultures were assayed for CAT activity. As shown in Fig. 10A, 5AC induced a dose-dependent increase in CAT

expression; the increase was approx. 160-fold at 9 μ M. Similar results were obtained with a second rostral cell line, 2-1H (20-, 67- and 91-fold induction at 3, 6 and 9 μ M 5AC respectively). Thus, CAT levels in 5AC-treated rostral cells were comparable to those observed in untreated caudal cells (see Fig. 7). Moreover, Southern analysis revealed that the MLC1-CAT transgene was partially demethylated following treatment with 5AC (Fig. 10B) and the demethylation was apparent by 1 day after drug treatment, before significant myotube formation had occurred. Thus, 5AC treatment results in both decreased methylation and increased expression of the MLC1-CAT transgene, suggesting that methylation is a component of the positional memory that influences this gene's expression.

Discussion

Adult mammalian muscle cells bear a stable memory of their position along the body's rostrocaudal axis. The existence of this system of positional information was initially revealed by electrophysiological experiments which showed that skeletal muscles from particular rostrocaudal positions are preferentially innervated and reinnervated by axons that arise from corresponding levels of the neuraxis (Wigston and Sanes 1982, 1985; Laskowski and Sanes 1987, 1988). More recently, we showed that expression of the MLC1-CAT transgene is graded with rostrocaudal position in developing and adult skeletal muscle, thus providing the first molecular evidence for positional differences among muscles (Donoghue et al., 1991a,b; Sanes et al., 1992). Using CAT activity as a marker, we then showed that position-related intermuscular differences in transgene expression are maintained in primary cultures established from individual adult muscles and in myogenic cell lines generated from the cultured myoblasts (Donoghue et al., 1992). These results demonstrated that muscle cells bear a cell autonomous and heritable positional memory. Here, to seek intracellular mechanisms that might account for this memory, we examined the methylation of the MLC1-CAT transgene. We found that its degree of methylation varies with rostrocaudal position in developing and adult muscle

and in myogenic cell lines. Our results implicate methylation as a genomic imprint of body position.

In evaluating these results, it is crucial to consider how position-dependent methylation and position-dependent expression might be related. On the one hand, the level of transgene methylation could be co-regulated with, or even determined by, levels of transgene expression. On the other hand, methylation state might reflect rostrocaudal position more directly and be a determinant of transgene expression. Three results favor the latter alternative. First, transgene methylation is positionally graded by postnatal day 1, at which time levels of CAT activity are only approx. 5% of their adult values. Thus, a gradient of methylation is established prior to adult levels of expression. Second, transgene methylation was identical in myoblasts and myotubes for each of 8 cell lines examined, even though the transgene was expressed at significant levels only in myotubes. Thus, methylation state is more closely tied to body position than to transgene expression per se. (This result also indicates that differentiation and position affect expression via different mechanisms.) Third, treatment of rostrally derived cells with a demethylating agent, 5AC, led to increased expression of the transgene. Thus, whereas developmental variations in transgene expression do not affect transgene methylation, experimental manipulation of methylation state does influence expression. Taken together, these results support the notion that position-dependent methylation could serve as a determinant of position-dependent gene expression.

If methylation regulates position-dependent expression, how might it do so? At one extreme, one or a few sites in the transgene, perhaps in its promoter, might need to be demethylated for maximal transcription to occur. Methylation of these sites could either block binding of transcriptional activators (e.g., see Watt and Malloy, 1988; Bednarik et al., 1991) or permit binding of 'methyl CpG binding proteins' that in turn inhibit transcription (e.g., see Levine et al., 1991; Boyes and Bird, 1992). Alternatively, expression might be inversely proportional to the total number of methylated sites in the transgene, with no specific methylated sequence being crucial. The five *MspI/HpaII* sites that we have assayed are only a subset of the >50 CpG dinucleotides that each copy of the transgene contains (11 in the promoter, 32 in the CAT gene and 8 in the enhancer). Variations in the density of methylation could alter chromatin structure (Keshet et al., 1986) and thereby affect transcriptional competence indirectly (Weintraub, 1985; Felsenfeld, 1992). These alternatives, which apply to methylated genes generally (Razin and Cedar, 1991), may be amenable to analysis in the MLC1-CAT transgene, because the position-dependent differences in methylation and expression of this gene are maintained *in vitro*, can be manipulated independently (by 5AC and serum deprivation, respectively) and are manifest in a single, well-studied cell type. One drawback, however, is that the lines of mice we have used bear approx. 40 copies of the MLC1-CAT transgene per genome. We do not know how many of these copies are transcriptionally active, whether the number of active copies varies with position, or whether the demethylated copies are the active ones. To circumvent this limitation, we have now generated new MLC1-CAT mice which con-

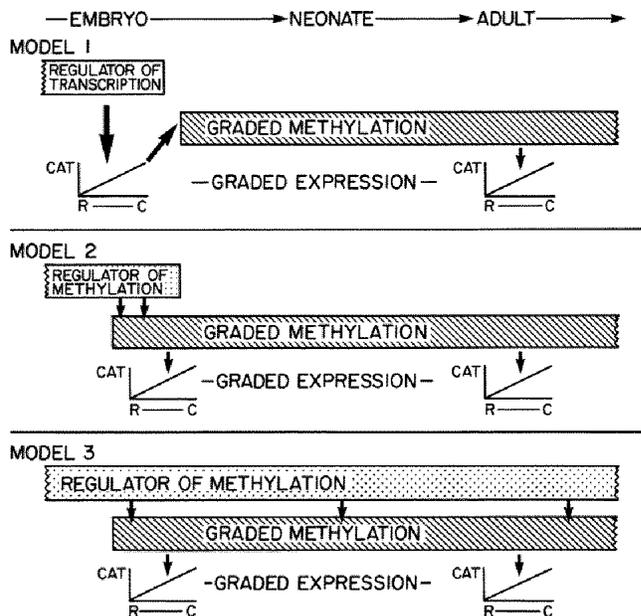


Fig. 11. Models of the relationship of transgene methylation to transgene expression. In model 1, position-dependent transcriptional activation of the transgene in the embryo leads to position-dependent methylation (or demethylation), which in turn maintains position-dependent expression at later stages. In models 2 and 3, position-dependent methylation is imprinted onto the transgene independent of prior transcriptional activation. The difference between these two models is that methylation state is the sole repository of positional memory in Model 2, but is subject to continuous regulation in Model 3. See Discussion for details.

tain only a few copies of the transgene per genome, and have found that CAT expression and transgene methylation are position-dependent in these mice (M. J. D., J. R. S. and J. P. M., unpublished data). These mice can now be used to distinguish the alternatives presented above.

Although we do not yet understand the molecular mechanisms by which methylation regulates transcription in this system, we can consider in general terms how methylation-encoded signals might act during development. Three possibilities are diagrammed in Fig. 11. In the first model, position-dependent expression of transcriptional regulators leads to graded expression of the transgene early in development. Methylation then occurs in inverse proportion to expression - that is, DNA becomes hypomethylated in caudal muscles and hypermethylated in rostral muscles (and nonmuscle tissues). Patterns of methylation would then become 'locked in' to form a stable, heritable positional memory that could influence expression after the original positional regulators had been replaced by more widely expressed transcription factors such as the myogenic factor, MyoD (Weintraub et al., 1991), which is present in all muscles and is known to activate the MLC enhancer (Braun et al., 1990). A precedent for this model is provided by studies on the methylation and expression of retroviral genomes: The Moloney murine leukemia virus can infect early embryonic cells but its promoter is inactive in these cells and subsequently becomes methylated. Thereafter, the proviral genome remains inactive, even at stages when newly introduced viral genomes are highly expressed

(Jähner et al., 1982; Niwa et al., 1983). Moreover, treatment of early-infected cells with 5AC at later stages reactivates the silent proviral genome (Jaenisch et al., 1985; Niwa et al., 1983). Thus, in this system a transient inability to express a gene is rendered permanent by methylation.

In the second model, a position-dependent system might establish patterns of methylation early in development and methylation state would then regulate expression in both embryo and adults. In this model, methylation serves as both the initial regulator of graded expression and the positional memory, and no intermuscular variations in *trans*-acting factors are required at any stage. Possible precedents here are parental imprinting and X-chromosome inactivation: in both of these cases, the two alleles within a single cell are differentially expressed despite sharing a common environment, and differential methylation of the two alleles is thought to be a crucial determinant of their differential expression (Holliday et al., 1990; Surani, 1991; Chaillet et al., 1991).

Finally, in the third model, factors responsible for graded methylation might be present throughout development. In this case, even though methylation state would be a determinant of expression, it would not be a completely autonomous source of positional memory. Instead, methylation would serve as an intermediate in an actively maintained signalling pathway, consistent with the notion that the differentiated state is not passively maintained, but instead requires continuous regulation (Blau and Baltimore, 1991). Recent studies have suggested that control of tissue-specific gene expression by methylation state can function in this way. For example, methylated sequences from a skeletal muscle-specific actin gene become demethylated and activated when transfected into myoblasts but not when transfected into fibroblasts, and demethylation can occur in the absence of replication (Yisraeli et al., 1986; Paroush et al., 1990). Thus, muscle cells may control the expression of this actin gene by controlling its state of methylation.

In summary, we have provided evidence that methylation serves as an imprint of rostrocaudal position, and plays a role in the position-dependent expression of a transgene. At present, we do not know whether positional variations in the activity of transcriptional factors also regulate the transgene's expression, how patterns of methylation are initially determined, or whether methylation state is passively or actively maintained once it is established. It will be important to address these issues, both to learn how positional memory is established and maintained, and to provide means of identifying endogenous genes that we hypothesize are positionally co-regulated with the transgene. As has been the case for parentally imprinted transgenes (references in Introduction), it is our hope that further analysis of the positionally imprinted MLC1-CAT transgene will provide insights into normal developmental processes.

We thank C. Petropoulos and S. Hughes for the gift of β -skeletal actin-CAT transgenic mice, N. Rubinstein and S. Schiaffino for antibodies to myosin, and J. Cunningham and R. Morris-Valero for assistance. This work was supported by grants from the National Institutes of Health and the Muscular Dystrophy Association of America to J. P. M. and J. R. S.

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(Accepted 31 August 1992)