Rostrocaudal gradient of transgene expression in adult skeletal muscle

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ABSTRACT Transgenic mice were produced in which expression of the reporter gene chloramphenicol acetyltransferase (CAT) is controlled by regulatory elements of a rodent myosin light chain gene. CAT activity was readily detectable in muscles of these mice but negligible in a variety of nonmuscle tissues. Unexpectedly, levels of CAT expression varied 100-fold from muscle to muscle, forming a gradient in which a muscle's position in the rostrocaudal axis was correlated with its level of CAT enzyme activity and abundance of CAT mRNA. Thus, rostral muscles (innervated by cranial nerves) had the lowest levels of CAT, thoracic muscles had intermediate levels, and caudal muscles (innervated through lumbar and sacral roots) had the highest levels. We established that myosin light chain sequences are responsible for the gradient of CAT expression but observed no strong gradient of endogenous myosin light chain expression. We argue that elements that are silent or masked by other sequences in their native context are revealed in the transgene and that the rostrocaudal gradient of gene expression they produce reveals the existence of a positionally graded endogenous regulator of gene expression. These transgenic mice provide evidence that cells in adult mammals retain "positional information" of a sort hitherto studied largely in embryos. The transgene they express may provide a means for determining how such positional values are generated and maintained.

Embryonic cells acquire and maintain information about their relative positions in the embryo, and their differentiation is influenced by this "positional information" (1). Genetic analyses of Drosophila have defined groups of genes that appear to be involved in the generation of positional information, in that mutations of these genes lead to defects in segment number or identity (2, 3). Molecular studies have now shown (4, 5) that many of these genes encode transcription factors that act both sequentially and in combinations to specify the positions of cells along the embryonic axes. Homologs of some of these (e.g., the Hox and Pax genes) have now been found in many vertebrates, including mammals (6, 7). Although their functional analysis has been slowed by the relative inaccessibility of vertebrate embryos to genetic manipulation, there is growing evidence that many of these genes are involved in the specification of positional values in vertebrates, as they are in flies.

In contrast, there are currently few data to indicate whether the cells of higher vertebrates retain positional information in adulthood. One indication that they do comes from electrophysiological experiments that showed that motor and autonomic preganglionic axons from different rostrocaudal levels of the spinal cord preferentially reinnervate targets (muscle fibers or sympathetic neurons) from corresponding levels (8–13). For example, when rat intercostal muscles were transplanted from various levels of the rib cage to a common site in the neck, they were reinnervated by autonomic axons of the cervical sympathetic trunk, and this reinnervation was positionally selective: rostrally derived muscles were preferentially reinnervated by axons with cell bodies in rostral thoracic spinal segments, whereas caudally derived muscles were preferentially reinnervated by caudally derived axons (10, 11). These results suggest the existence of differences among adult muscles that are graded by body position and that bias the efficacy of synapse formation. However, molecular evidence for such differences has been lacking and it has therefore been difficult to learn how they are generated and maintained.

Recently, in the course of analyzing transgenic mice made for other purposes (14–16), we were surprised to discover that expression of a transgene in adult muscles forms a remarkable positional gradient. In this report, we document the existence of this gradient and begin an analysis of the elements responsible for its generation. Our results provide direct evidence for the existence of positional information in adult mammalian muscle and may provide a molecular correlate of the synaptic gradient detected electrophysiologically.

METHODS

Generation of Transgenic Mice. Transgenic mice were generated from the constructs diagrammed in Fig. 1. MLC1–CAT mice are described in ref. 15 and AChRα–CAT mice are described in ref. 16. MLC1–AChRα mice were prepared using a construct in which the mouse acetylcholine receptor (AChR) γ-subunit cDNA (17) was placed under the control of the myosin light chain (MLC) regulatory sequences from MLC1–CAT (D. McKinnon and J.P.M., unpublished results).

Chloramphenicol Acetyltransferase (CAT) Assays. CAT activity was measured as described (15, 16, 18). Protein concentration was measured by the method of Bradford (19). For MLC1–CAT mice, samples of extract containing 170 ng of protein were incubated with substrate for 1 h; for AChRα–CAT mice, samples containing 100 μg of protein were incubated for 4 h. All incubations were at 37°C and autoradiograms were exposed for 12 h.

RNA Assays. RNA levels were determined either by Northern blot hybridization or by an RNase protection assay. RNA was prepared as in ref. 20. Northern blot hybridization was performed as in ref. 15, using 10 μg of total RNA per lane and random-primed probes specific for MLC1 or for both MLC1 and -3 (21). The autoradiograms were exposed for 8 h at room temperature and quantitated by densitometry. The RNase protection assay was performed as in ref. 22, using a 568-base-pair probe generated from the T3 promoter of a HindIII-

Abbreviations: AChR, nicotinic acetylcholine receptor; CAT, chloramphenicol acetyltransferase; MLC, myosin light chain.

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digested plasmid consisting of the Xho II–BamHI fragment of the CAT transcription unit (18, 20) subcloned into Bluescript (Stratagene).

RESULTS

Transgenic mice in which MLC regulatory elements (a promoter and an enhancer) control expression of the bacterial CAT gene have been characterized (15, 16). CAT levels are high in the hindlimb muscles of these mice and negligible in a variety of nonmuscle tissues. As part of an ongoing study of transgene expression in different muscles (see also ref. 14), we assayed CAT activity in several individual muscles. We were struck immediately by the observation that levels of CAT expression varied >100-fold from muscle to muscle (Fig. 2). The present study was undertaken to investigate this variation.

CAT Expression in Axial Muscles. To understand the source of the variation in CAT expression, we determined the levels of CAT activity in 35 muscles that differ in function, position, and fiber-type composition (Table 1). For the 16 axial muscles tested, we found that the strongest predictor of a muscle’s CAT level was its position along the body’s rostrocaudal axis. Fig. 3 a and b shows data from axial muscles of MLC1-CAT mice, arranged by the cranial nerves or spinal segments that innervate them (23–25); segmental innervation was used because it is a correlate of somite of origin (27, 28), which is unknown in many instances, and of body position, which is difficult to specify in a single dimension. A gradient of CAT activity is evident, with the lowest levels in muscles innervated by cranial nerves and the highest levels in muscles innervated through the lumbar and sacral roots; muscles innervated by cervical and thoracic (T) nerves had intermediate CAT levels.

![Diagram of CAT activity in axial muscles](image)

Fig. 2. Assays of CAT activity in digastricus, sternohyoideus, pectoralis major, and fibularis anterior muscles of an MLC1-CAT transgenic mouse (line 52 in ref. 15). CAT activities vary markedly among muscles in an MLC1-CAT transgenic mouse.

![Table 1](image)

Table 1. Muscles used in this study

<table>
<thead>
<tr>
<th>Number</th>
<th>Name with Innervation and Position</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Masseter V (ant)</td>
</tr>
<tr>
<td>2</td>
<td>Anterior digastricus V (post)</td>
</tr>
<tr>
<td>3</td>
<td>Posterior digastricus VII</td>
</tr>
<tr>
<td>4</td>
<td>Sternohyoid C1, C2</td>
</tr>
<tr>
<td>5</td>
<td>Omohyoid C1–C3</td>
</tr>
<tr>
<td>6</td>
<td>Sternomastoid C1–C3</td>
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<tr>
<td>7</td>
<td>Cleidomastoid C2, C3</td>
</tr>
<tr>
<td>8</td>
<td>Clavotrapezius C4–C6</td>
</tr>
<tr>
<td>9</td>
<td>Diaphragm C4–C6</td>
</tr>
<tr>
<td>10</td>
<td>Lattissimus dorsi C6, C7</td>
</tr>
<tr>
<td>11</td>
<td>Spinoidea C5, C6</td>
</tr>
<tr>
<td>12</td>
<td>Pectoralis major C6, C7</td>
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<tr>
<td>13</td>
<td>Pectoralis minor C7, C8</td>
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<tr>
<td>14</td>
<td>Triceps brachii C7, C8</td>
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<tr>
<td>15</td>
<td>Biceps brachii C5, C6</td>
</tr>
<tr>
<td>16</td>
<td>Pronator teres C7*</td>
</tr>
<tr>
<td>17</td>
<td>Extensor digitorum communus C7, C8</td>
</tr>
<tr>
<td>18</td>
<td>Palmaris longus C7, C8</td>
</tr>
<tr>
<td>19</td>
<td>Intercostal T2 T2</td>
</tr>
<tr>
<td>20</td>
<td>Intercostal T4 T4</td>
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<tr>
<td>21</td>
<td>Intercostal T6 T6</td>
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<td>22</td>
<td>Intercostal T8 T8</td>
</tr>
<tr>
<td>23</td>
<td>Intercostal T10 T10</td>
</tr>
<tr>
<td>24</td>
<td>Psoas major C7 L2, L3</td>
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<tr>
<td>25</td>
<td>Gluteus maximus L4, L5</td>
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<tr>
<td>26</td>
<td>Gracilis L2–L4</td>
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<td>27</td>
<td>Vastus lateralis L2–L4</td>
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<tr>
<td>28</td>
<td>Biceps femoris L4–L6*</td>
</tr>
<tr>
<td>29</td>
<td>Plantaris L4, L5</td>
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<tr>
<td>30</td>
<td>Gastrocnemius L4, L5</td>
</tr>
<tr>
<td>31</td>
<td>Tibialis anterior L4</td>
</tr>
<tr>
<td>32</td>
<td>Extensor digitorum longus L4</td>
</tr>
<tr>
<td>33</td>
<td>Soleus L4, L5</td>
</tr>
<tr>
<td>34</td>
<td>Flexor caudalis S1, S2</td>
</tr>
<tr>
<td>35</td>
<td>Sacrococcygeus ventralis S2, S3</td>
</tr>
</tbody>
</table>

Cranial or spinal nerves from which innervation is derived are taken from refs. 23–25. C, cervical; T, thoracic; L, lumbar; S, sacral. Classification of muscles as axial or limb is from refs. 23 and 26.

*Segmental innervation of these muscles has not been determined in rats; values are estimated from feline and human data.

That CAT expression is graded is most clearly seen in the intercostal muscles (arrow in Fig. 3), each of which is derived from a single somite and innervated by a single spinal segment (28, 29). CAT levels, measured in alternate intercostals, increased in the rostrocaudal progression, T2 < T4 < T6 < T8 < T10. The specific activity of CAT increased nearly 2-fold per two segments, or 10- to 15-fold from T2 to T10. Thus, even within a set of structurally and functionally similar muscles, CAT levels form a rostrocaudal gradient.

CAT Expression in Limb Muscles. The high values of CAT activity previously seen in hindlimb muscles of MLC1–CAT mice (14, 15) seemed likely to reflect their caudal position. However, in that programs of myogenic development may be regulated differently in the trunk and limbs of embryos (30, 31), it was important to separate positional effects from differences between axial and limb musculature. To this end, we compared fore- and hindlimb muscles considered to be homologous on functional, structural, and evolutionary grounds (ref. 26; Fig. 4a). Overall, CAT expression was greater in forelimb than in axial muscles at the same segmental level (crosses in Fig. 3), indicating that CAT expression reflects factors in addition to rostrocaudal position. Moreover, no proximodistal effects of CAT expression were
been generated.

some than in hindlimb muscles. Qualitatively similar results were obtained in a second line 52 mouse and in two line 63 mice. Fore- and hindlimb members of each pair, numbered as in Table 1, were as follows: A, 10 and 24; B, 11 and 25; C, 12 and 26; D, 14 and 27; E, 15 and 28; F, 17 and 32; G, 18 and 29. Ref. 26 details criteria for considering these pairs to be homologues.

expression might reflect either post-transcriptional differences among muscles—e.g., variations in the metabolism of CAT mRNA or protein—or they might reflect an underlying transcriptional gradient. (ii) If transcriptionally mediated, the observed pattern could reflect regulation either by chromosomal sequences external to the transgene or by sequences within the transgene itself. (iii) If the gradient results from transcriptional regulation mediated by sequences in the transgene, the relevant elements might derive from the MLC gene, from the CAT gene, or from a combination of the two. We undertook a series of experiments to distinguish these alternatives.

Two experiments tested the possibility that positional differences in CAT activity reflect post-transcriptional differences among muscles in CAT metabolism. (i) We measured CAT RNA levels in five groups of muscles that spanned a nearly 100-fold range of CAT specific activities. (Small groups rather than single muscles were used in this experiment, because of the difficulty of obtaining sufficient RNA from individual muscles with the limited number of transgenic animals available.) The abundance of CAT mRNA was directly proportional to CAT enzyme activity in a set of muscles from MLC1-CAT mice (Fig. 5a), demonstrating that the gradient is not due to translational or post-translational effects. (ii) Because this result does not rule out the possibility that the gradient reflects differences among muscles in CAT mRNA stability, we studied transgenic mice in which transcription of the CAT gene was regulated by the promoter of the AChR α subunit (15). In these mice, which produce the same reporter protein as the MLC1-CAT mice and a nearly identical mRNA (differing only in ~30 bases of the 5′ untranslated region; see Fig. 1), expression of the transgene was as muscle-specific as in the MLC1-CAT mice. However, CAT levels varied little (~5-fold) among muscles in each of three independently generated lines of AChRα-CAT mice. Moreover, CAT levels in these mice did not correlate with

![Fig. 3](image-url)

**Fig. 3.** CAT activities are rostrocaudally graded in axial muscles of MLC1-CAT transgenic mice. CAT activities in axial muscles of MLC1-CAT transgenic mice of lines 52 (a) and 63 (b). For comparison, the range of means from several fore- and hindlimb muscles are presented as crosses (for individual values, see Figs. 4 and 6). Values in each graph are averages from two or three mice. Intercostal muscles T2, T4, T6, T8, and T10 are indicated by arrows. (c) MLC1 RNA levels in muscles of wild-type mice. Muscles are numbered as in Table 1 and arranged by the cranial or spinal nerves that innervate them.

![Fig. 4](image-url)

**Fig. 4.** CAT activities in limb muscles of an MLC1-CAT transgenic mouse: CAT levels are higher in each hindlimb muscle than in its forelimb serial homologue. (a) Schematic diagram of a limb showing muscle groups from which fore- and hindlimb homologues were taken. (b) CAT activities in muscles from a single line 52 mouse. Qualitatively similar results were obtained in a second line 52 mouse and in two line 63 mice. Fore- and hindlimb members of each pair, numbered as in Table 1, were as follows: A, 10 and 24; B, 11 and 25; C, 12 and 26; D, 14 and 27; E, 15 and 28; F, 17 and 32; G, 18 and 29. Ref. 26 details criteria for considering these pairs to be homologues.

The gradient of CAT activity we observed might have been generated in any of several ways. (i) Patterns of CAT

**Role of MLC Regulatory Elements in Graded CAT Expression.** The gradient of CAT activity we observed might have been generated in any of several ways. (i) Patterns of CAT

Moreover, CAT levels in these mice did not correlate with
CAT levels in the MLC1-CAT transgenic mice (r = 0.07) and were not rostrocaudally graded (Fig. 6). This result provides further evidence that muscles do not vary systematically in their ability to process CAT mRNA or protein. Thus, graded levels of CAT activity are likely to reflect graded transcriptional activation of the transgene.

One way in which transcription of transgenes can be regulated is by endogenous chromosomal sequences near their sites of integration in the genome. Such integration site-dependent patterns of expression are not infrequently observed in transgenic mice (32, 33). To test this possibility, we measured CAT activity in muscles from three separately generated lines of MLC1-CAT mice (lines 7, 52, and 63; described in ref. 14), each of which has a distinct site of integration as assessed by Southern blot hybridization (ref. 14 and J.P.M., unpublished results). Gradients of the same polarity and similar magnitude were observed in at least three mice from each of the three lines (for example, compare Fig. 3a and b). These results provide strong evidence that the relevant regulatory elements are contained within the transgene itself.

Finally, we asked whether the transgene regulatory sequences responsible for graded CAT expression were within the CAT gene, rather than in the MLC promoter or enhancer. One line of evidence against this alternative is that CAT activity shows no gradient in the AChRα-CAT mice described above (Fig. 6). However, this result does not exclude the possibility that a combination of MLC and CAT sequences are required for graded expression. We therefore assayed muscles from new transgenic mice in which the same MLC regulatory sequences used in the MLC1-CAT transgene control expression of the AChR γ-subunit gene instead of CAT. RNase protection assays showed that transgene-encoded mRNA displayed a rostrocaudal gradient of abundance in these mice (data not shown). Thus, sequences in the MLC promoter and/or enhancer are evidently responsible for positionally graded CAT expression in muscles from MLC1-CAT transgenic mice.

Patterns of Endogenous MLC Expression. Having determined that elements derived from an MLC gene can lead to positionally graded gene expression, it became necessary to consider the possibility that the patterns we observed reflected hitherto undetected differences in endogenous MLC expression. To this end, we assayed endogenous MLC RNA levels in a set of 10 muscles that spanned the rostrocaudal axis. The MLC1/3 gene, from which the transgene is derived, uses two promoters and alternative splicing to produce two transcripts; one encodes MLC1 and the other encodes MLC3 (34, 35). The promoter in the transgene is the MLC1 promoter but the enhancer may regulate transcription of either mRNA (18). We therefore performed Northern blot analysis using a probe specific for MLC1 RNA and a probe that hybridizes to both MLC1 and MLC3 RNA. As shown in Fig. 3c, MLC1 RNA abundance varied only a fewfold from muscle to muscle, compared to the >100-fold variation in CAT activity, and MLC1 RNA levels were correlated weakly if at all (r = 0.40 between Fig. 3a and c) with CAT levels. Similarly, levels of MLC3 RNA were correlated poorly if at all with CAT levels or with rostrocaudal position (data not shown).

Although endogenous MLC expression showed no strong positional gradient in wild-type mice, it remained possible that the endogenous and inserted genes exhibited a graded expression in the MLC1-CAT transgenic mice that we had shown to span a 40-fold range of CAT RNA abundance (Fig. 5a). As shown in Fig. 5b, MLC1 RNA levels varied only ~2-fold among these groups and showed no correlation with CAT levels (r = 0.03). Because we have measured RNA levels rather than rates of transcription, we cannot formally exclude the possibility that muscles vary systematically in their ability to process MLC mRNA. However, there are currently no data suggesting the existence of the large-scale (~100-fold) variations in MLC RNA stability that would be required to mask a corresponding variation in transcription rates. Thus, the rostrocaudal gradient of CAT expression apparently reflects a specificity that is not evident in the expression of the endogenous MLC gene.

Finally, because endogenous MLC1 and MLC3 levels vary with fiber type and are selectively expressed in fast-twitch fibers (36), we considered the possibility that differences in fiber type composition could account for differences in CAT level among muscles. In fact, the one predominantly slow muscle we tested, the soleus (37), had a particularly low CAT level, given its position (Fig. 6). On the other hand, reference to published work (37) shows that most of the other muscles that we studied vary far too little in fiber type composition to account for the >100-fold variations in CAT level described here. For example, the intercostal muscles vary only a fewfold in fiber type composition from segment to segment.
but vary >10-fold in CAT level (Fig. 3). Additionally, our own determination of CAT expression as a function of fiber type (M.J.D. J.P.M. and J.R.S., unpublished data) have conclusively demonstrated that fiber type differences cannot account for the rostrocaudal gradient of CAT expression that we have documented.

**DISCUSSION**

We have documented a >100-fold rostrocaudal gradient of gene expression in the skeletal muscles of adult transgenic mice. Similarly graded expression was observed in three independently derived lines of MLC1–CAT mice, as well as in one line of MLC1–AChR mice. The gradient is particularly striking in axial muscles, where caudal muscles have the highest levels of CAT activity, thoracic muscles have intermediate levels, and rostral muscles have the lowest levels. In contrast, systematic gradients are not evident within either limb, and limb muscles generally have higher CAT levels than axial muscles from corresponding levels. However, in each of seven pairs of homologous muscles, the hindlimb muscle of the pair has a higher level of CAT activity than its forelimb serial homologue. Thus, limb as well as axial muscles provide evidence for positionally regulated expression of the transgene.

Our interest in the graded expression of CAT was heightened by previous studies of positionally selective reinnervation of adult muscles (10–13). This work led us to predict the existence of molecular differences among motor neurons and among muscles that correlate with body position and that favor synapse formation between positionally matched partners. Such factors might well be important in the establishment of patterns of synaptic connectivity in the embryo; whether their persistence in adults helps to maintain these patterns or whether it is merely a vestige of embryonic processes remains a matter of speculation. Suzue et al. (39) generated a monoclonal antibody (ROCA1) that recognizes a positionally graded antigen on glial cells in adult intercostal nerves. However, molecular markers of axial position have not heretofore been reported in adult motor neurons or muscle fibers.

Our analysis indicates that sequences in the MLC1/3 gene mediate the graded expression of CAT in the MLC1–CAT mice. In that endogenous MLC expression is not obviously graded, the normal role of these sequences is unclear; they may be silent or masked in their native context, or overshadowed by other elements that are present in the endogenous gene but absent from the transgene. These uncertainties do not, however, affect our interpretation of the results, which is that the graded distribution of CAT activity is likely to result from the graded distribution of an endogenous regulator of gene expression. This regulator is present in adult animals (but might also be present in embryos), is active in muscle fibers (but might also be active in other tissues), and describes a gradient that spans the body. All of these properties are distinctive in that descriptions of putative "positional" genes in mammalian development focused on expression in embryos, expression in tissues other than muscle, and expression in limited segmental domains (1–7, 31). However, these properties are those predicted for factors that favor synapse formation between axons and muscle fibers from corresponding levels of the neuraxis (10–13). We therefore speculate that the molecules responsible for graded expression of the transgene normally regulate expression of a set of endogenous genes and that genes whose products promote positionally selective synapse formation are members of this set. If this interpretation is correct, the transgene we studied may be useful for learning how the positional gradient is regulated and, eventually, for seeking the endogenous genes that establish it.

Experiments reported here were performed in the laboratory of J.P.M. and J.R.S. while M.J.D. was a visitor at Washington University. M.J.D. was supported by a training grant from Boston University and a grant to N.R. from the Council for Tobacco Research until August, 1990; she is now supported by a grant from the National Institutes of Health to J.P.M. and J.R.S. We thank J. Mudd and T. Hanley for assistance and J. Lichtman for comments. This work was supported by research grants from the National Institutes of Health and the Muscular Dystrophy Association to J.P.M. and J.R.S.