Fiber Type- and Position-dependent Expression of a Myosin Light Chain-CAT Transgene Detected with a Novel Histochemical Stain for CAT

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Abstract. We recently generated and characterized transgenic mice in which regulatory sequences from a myosin light chain gene (MLC1f/3f) are linked to the chloramphenicol acetyltransferase (CAT) gene. Transgene expression in these mice is specific to skeletal muscle and graded along the rostrocaudal axis: adult muscles derived from successively more caudal somites express successively higher levels of CAT. To investigate the cellular basis of these patterns of expression, we developed and used a histochemical stain that allows detection of CAT in individual cells. Our main results are as follows: (*a*) Within muscles, CAT is detected only in muscle fibers and not in associated connective tissue, blood vessels, or nerves. Thus, the tissue specificity of transgene expression observed by biochemical assay reflects a cell-type specificity demonstrable histochemically. (*b*) Within individual muscles, CAT levels vary with fiber type. Like the endogenous MLC1f/3f gene, the transgene is expressed at higher levels in fast-twitch (type II) than in slow-twitch (type I) muscle fibers. In addition, CAT levels vary among type II fiber subtypes, in the order IIB > IIX > IIA. (*c*) Among muscles that are similar in fiber type composition, the average level of CAT per fiber varies with rostrocaudal position. This position-dependent variation in CAT level is apparent even when fibers of a single type are compared. From these results, we conclude that fiber type and position affect CAT expression independently. We therefore infer the existence of separate fiber type-specific and positionally graded transcriptional regulators that act together to determine levels of transgene expression.

THE striking diversity of cell types in higher organisms reflects the selective expression of genes in subsets of cells. Therefore, the regulation of cell type-specific gene expression is an important key to understanding how differences among cells are generated and maintained. Muscle cells have been particularly useful in analyses of this process for several reasons: their differentiation from myoblast to myotube is easily monitored, developmental regulation of several abundant muscle-specific proteins has been documented, and both morphological and molecular aspects of differentiation occur in culture under controlled conditions. Accordingly, numerous genes have been isolated that encode muscle-specific proteins, regulatory elements that mediate muscle-specific transcription have been defined in several of these, and several transcription factors that interact with these elements to control myogenesis have themselves been isolated and cloned (Kedes and Stockdale, 1989; Weintraub et al., 1991).

Less well studied, however, are determinants of diversity among muscle fibers. For example, muscle fibers can be divided into several "types" (e.g., slow-twitch or type I and fast-twitch or type II) according to their contractile and metabolic properties, and many proteins are known to be differentially expressed by fibers of different types (Pette and Staron, 1990). Both environmental and lineage derived influences have been implicated in the generation and maintenance of fiber type diversity (Stockdale and Miller, 1987), but analysis of the transcriptional basis of this heterogeneity is in its infancy. Similarly, physiological studies have shown that muscles from different positions along the rostrocaudal axis vary systematically in the synapses they prefer to receive (Wigston and Sanes, 1985), but the developmental origins and molecular bases of this positional information are not known.

Here, we have used sequences from a myosin light chain (MLC)[1] gene to begin to address these issues. This gene, MLC1f/3f, encodes two myosin light chains (MLC1f and 3f), which are generated by alternative splicing from separate promoters (Nabeshima et al., 1984; Robert et al., 1984; Periasamy et al., 1984; Strehler et al., 1985). MLC1f and 3f are preferentially expressed in fast-twitch (type II) muscle fibers, whereas other MLC isoforms (e.g., MLC1s) are more abundant in slow-twitch (type I) fibers (Pette and Schnez,

1. *Abbreviations used in this paper*: CAT, chloramphenicol acetyltransferase; MHC, myosin heavy chain; MLC, myosin light chain.

1977; Young and Davey, 1981; Mabuchi et al., 1982; Mizusawa et al., 1982; Staron and Pette, 1987*a*,*b*; Pette and Staron, 1990). We previously generated and characterized transgenic mice in which the MLC1f promoter and an MLC1f/3f enhancer (Donoghue et al., 1988) direct muscle-specific expression of the reporter gene, chloramphenicol acetyltransferase (CAT) (Rosenthal et al., 1989). In light of the preferential expression of endogenous MLC1f and 3f in type II fibers, it was natural to ask whether the transgene, which we call MLC1-CAT, also displayed a fiber-type specificity, and the first aim of the work reported here was to test this possibility.

In addition, we recently showed that CAT levels in MLC1-CAT mice are positionally graded along the rostrocaudal axis, such that the specific activity of CAT is >100-fold higher in caudal than in rostral muscles (Donoghue et al., 1991). This gradient does not mimic patterns of endogenous MLC1f or MLC3f expression (i.e., MLC1f/3f mRNAs show no positional gradient; Donoghue et al., 1991) but does provide new evidence for a system of positional information in adult muscle. However, our assays of CAT levels in tissue homogenates were unable to distinguish among several alternative cellular patterns of transgene expression that could underlie the gradient. For example, caudal muscles might contain a higher proportion of CAT-expressing fibers than rostral muscles, or the specific activity of CAT in all fibers could be higher in caudal than in rostral fibers. Because these alternatives imply very different mechanisms for the positional control of transgene expression, the second aim of the present work was to distinguish among them.

Prerequisite to addressing these issues is the ability to assay levels of transgene expression in individual cells. In numerous studies on transgenic animals, this has been accomplished by using *Escherichia coli* β-galactosidase (*lacZ*) as the reporter; a simple and sensitive histochemical stain visualizes sites of lacZ activity at both light and electron microscopic levels (for example see Hiromi et al., 1985; Sanes et al., 1986; Goring et al., 1987; Bunge et al., 1989). In contrast, histochemical stains have not been available for CAT, and we found immunohistochemical methods to be unsatisfactory when applied to tissue sections. Faced with an apparent need to generate and characterize new transgenic mice with an MLC1-*lacZ* transgene, we decided instead to devise a method capable of revealing transgene activity in muscles from MLC1-CAT mice. The last aim of this report, then, is to introduce a novel histochemical stain for CAT that may be generally useful in studies of transgene expression.

Materials and Methods

Preparation of Tissues

Production and characterization of MLC1-CAT transgenic mice has been described previously (Rosenthal et al., 1989; Merlie and Kornhauser, 1989; Donoghue et al., 1991). The transgene contains a 1.2-kb fragment containing the MLC1f promoter and a 0.9-kb fragment containing a downstream MLC1f/3f enhancer; these are placed 5′ and 3′, respectively, of a CAT transcription unit. Mice of lines 52 and 63 were used interchangeably in the studies reported here. Adult transgenic mice, or their wild-type littermates, were killed with an overdose of ether. For immunohistochemical studies, muscles were quick-frozen in isopentane that had been cooled in liquid nitrogen. The muscles were then cross-sectioned at 5–10 μm in a cryostat, and sections were mounted on gelatin-coated glass slides. For histochemical analysis, muscles were fixed by immersion in 2% (wt/vol) paraformaldehyde in PBS (150 mM NaCl, 15 mM sodium phosphate, pH 7.2) for 1 h at 4°C. The fixed muscles were then rinsed in PBS, and cryoprotected by immersion in sucrose (10% sucrose in PBS for 1 h, then 30% sucrose in PBS for 4–16 h, both at 4°C) before being frozen and sectioned as above. To combine histochemical and immunohistochemical analyses, serial sections were cut from unfixed muscles and mounted on separate slides. Slides to be stained for CAT activity were immediately immersed in 2% paraformaldehyde in PBS (pH 7.2) for 5 min at 25°C; alternate slides were stained with antibodies without pretreatment. This compromise protocol was necessary because CAT enzyme is rapidly lost from unfixed sections whereas the antibodies to myosin we used stain fixed tissue poorly.

Histochemical Staining for CAT

CAT activity was revealed with an incubation mixture that contained 0.3 mM acetyl CoA (lithium salt; ICN Biochemicals Inc., Cleveland, OH), 4 mM chloramphenicol (Sigma Chemical Co., St. Louis, MO), 5 mM potassium ferricyanide, 5 mM sodium citrate, 3 mM copper sulfate, and 63 mM sodium maleate, pH 6.0. This reaction mixture was prepared from the following solutions. (A) 8 mM sodium citrate and 5 mM copper sulfate in 100 mM sodium maleate buffer, pH 6.0. (B) 50 mM potassium ferricyanide in water. (C) 16 mM chloramphenicol (prepared by dissolving 5.2 mg chloramphenicol in 125 μl of absolute ethanol, then adding water to 1 ml). (D) 15 mM acetyl coenzyme A (AcCoA) in water. Solutions A and B were stable for over a month at 4°C, but solutions C and D were prepared fresh for each experiment. The staining solution was prepared by mixing solutions A, B, C, and D in the proportions 630:100:250:20. Fixed sections or cultures were covered with staining solution, incubated for 2–24 h at room temperature, then rinsed with PBS or water, and mounted in glycerin for microscopy.

For densitometric analysis of CAT-staining, sections were viewed with a video camera (Type NC-70L; Dage-MTI, Inc., Michigan City, IN) attached to a microscope, and images were processed with a microcomputer-based image analysis system (Image 1; Universal Imaging Corp., Westchester, PA). Three 10× fields were digitized per muscle, and 20 cross-sectioned muscle fibers were analyzed from each field. To avoid bias in the selection of fibers, a grid was laid over the video monitor, and fibers that lay under predetermined points were chosen for analysis. A square was then drawn within each fiber, and the computer calculated the average gray level of pixels within the square on a 0–256 scale. Neutral density filters were used to generate a calibration curve, which showed that all measurements (generally between gray levels 40 and 220) were within the linear range of the system; this range is similar to that shown by Inoué (1986, see page 332) using the same method and a similar camera.

Immunohistochemical Staining for Myosin

Four monoclonal antibodies to myosin heavy chains, BA-D5, BF-F3, BF-35, and SC-71, were generously provided by Dr. Stefano Schiaffino (University of Padova, Italy). The specificity of these antibodies has been documented previously (Schiaffino et al., 1989; Gorza, 1990), and is discussed further in Results. An additional monoclonal antibody, MY32, that recognizes all fast (type II) myosin heavy chains was purchased from Sigma. All five antibodies to myosin are mouse IgGs, and were supplied as ascites. For staining, unfixed sections were incubated with antibody for 2–3 h at a 1:100–1:500 dilution, washed, reincubated with fluorescein-conjugated goat anti-mouse Ig (Boehringer-Mannheim, Inc., Indianapolis, IN) for 1 h, washed again, mounted in glycerol/para-phenylenediamine and observed with epifluorescent illumination.

Results

A Histochemical Stain for CAT

In previous studies of MLC1-CAT mice, we assayed CAT enzyme activity and CAT RNA levels in tissue homogenates (Rosenthal et al., 1989; Merlie and Kornhauser, 1989; Donoghue et al., 1991). These methods established that expression of the transgene is muscle specific, rostrocaudally graded, and developmentally regulated. To extend this analysis, it was important to assess the cellular distribution of the transgene's product within muscles. To this end, we developed a histochemical stain for CAT.

The histochemical stain for CAT (EC 2.3.1.28) activity is based on previously described stains for choline acetyltransferase (EC 2.3.1.6) and carnitine acetyltransferase (EC 2.3.1.7). All of these enzymes catalyze the transfer of an acetyl group from AcCoA to an acceptor, generating an acetyl ester plus CoA. The free sulfhydryl group on CoA can then combine with heavy metal salts to form a precipitate. In the most widely used protocols, the CoA is precipitated as a colorless lead salt, and the precipitate is rendered visible by substitution in a second step (Burt, 1970; Kása et al., 1970; Kása, 1986). This approach was not useful for CAT, because the enzyme was inhibited by lead (data not shown). More successful was an alternative stain for carnitine acetyltransferase (Higgins and Barnett, 1970; Barnett et al., 1983), which was itself modified from a stain for acetylcholinesterase (Karnovsky and Roots, 1964; Karnovsky, 1964; Butcher, 1983). In this "direct coloring" method, the sulfhydryl group reduces ferricyanide to ferrocyanide, which then combines with copper ions, forming a brown precipitate (called Hatchett's Brown) in a single incubation. Although the composition of the precipitate remains uncertain (Tsuji and Larabi, 1983), this approach provides a simple and sensitive method to demonstrate sites of activity of enzymes that generate a product with a free sulfhydryl group.

The staining protocol was initially developed, and its specificity assessed, using a fibroblast cell line (QT6; Moscovici et al., 1977) that had been transfected with a Rous sarcoma virus promoter-CAT plasmid (generously provided by I. Ghattas and J. Majors, Department of Biochemistry and Molecular Biophysics, Washington University, St. Louis, MO). When fixed and stained as described in Materials and Methods, many cells in the transfected population were filled with a granular brown precipitate (Fig. 1, *a* and *a'*). No staining was observed when either AcCoA (Fig. 1, *b* and *b'*) or chloramphenicol (Fig. 1, *c* and *c'*) was omitted from the incubation mixture, even when incubation was extended to 24 h. Thus, staining is dependent on an exogenous supply of both substrates of CAT, and does not proceed appreciably when only endogenous sulfhydryl donors or acetyl acceptors are present. Furthermore, no staining was observed when untransfected QT6 cells were incubated with the complete staining solution for 24 h (Fig. 1, *d* and *d'*). Thus, deposition of reaction product in the cytoplasm depends on the presence of CAT, and is not catalyzed appreciably by other enzymes or nonenzymatic mechanisms in wild type cells. Together, these control experiments established the specificity of the staining protocol for CAT.

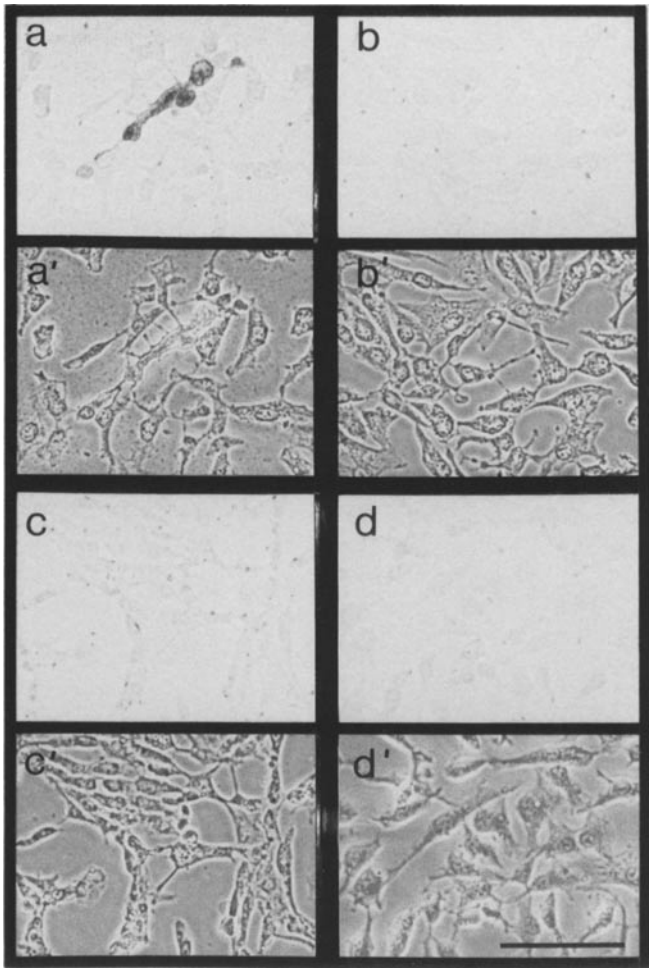

Figure 1. A histochemical stain for CAT activity: demonstration of its specificity in cultured cells. QT6 fibroblasts transfected with a CAT-encoding expression vector (*a–c*), or nontransfected QT6 cells (*d*), were fixed and incubated in the complete staining mix (*a* and *d*), or in a mixture lacking chloramphenicol (*b*) or lacking AcCoA (*c*). The plates were then washed and photographed with bright field (*a–d*) and phase contrast optics (*a'–d'*). Staining is observed only in cells transfected with the CAT expression vector and incubated with the complete staining mix. Bar, 100 μm.

Cellular Localization of CAT in MLC1-CAT Mice

Fig. 2, *a* and *b* show CAT-stained sections of tibialis anterior and gastrocnemius muscles, respectively, from a MLC1-CAT mouse. Reaction product is detectable within most muscle fibers in both muscles. In addition, some nuclei were stained, both within and between muscle fibers. Control experiments similar to those used for cultured cells tested the specificity of the histochemical stain on sections. First, staining was absent when either chloramphenicol (Fig. 2 *e*) or AcCoA (Fig. 2 *f*) was omitted from the incubation. Second, no muscle fibers were stained when muscles from wild type litter-mates of the transgenic mice were incubated with the complete staining mixture (Fig. 2 *g*). Finally, no cytoplasmic staining was observed in sections of liver from MLC1-CAT mice (not shown); liver does not express CAT at detectable levels in these mice. Together, these observations leave little doubt that the staining of transgenic muscle fibers reliably reflects the presence of CAT. On the other hand, some nuclei were occasionally stained in both transgenic muscle and wild-type muscle, indicating that this staining is nonspecific. We do not know why nuclear staining is seen inconsistently (e.g., compare Fig. 2, *a* and *b*), but we had no difficulty in distinguishing it from specific cytoplasmic staining, and we do not consider it further here.

Examination of CAT-stained sections of muscle revealed two noteworthy features. First, specific staining was confined to muscle fibers; intramuscular blood vessels, connective tissue, and nerves were not detectably stained (Fig. 2, *c* and *d*). Thus, the tissue specificity of transgene expression documented previously (Rosenthal et al., 1989) reflects a cell-type specificity of expression seen histologically. Sec-

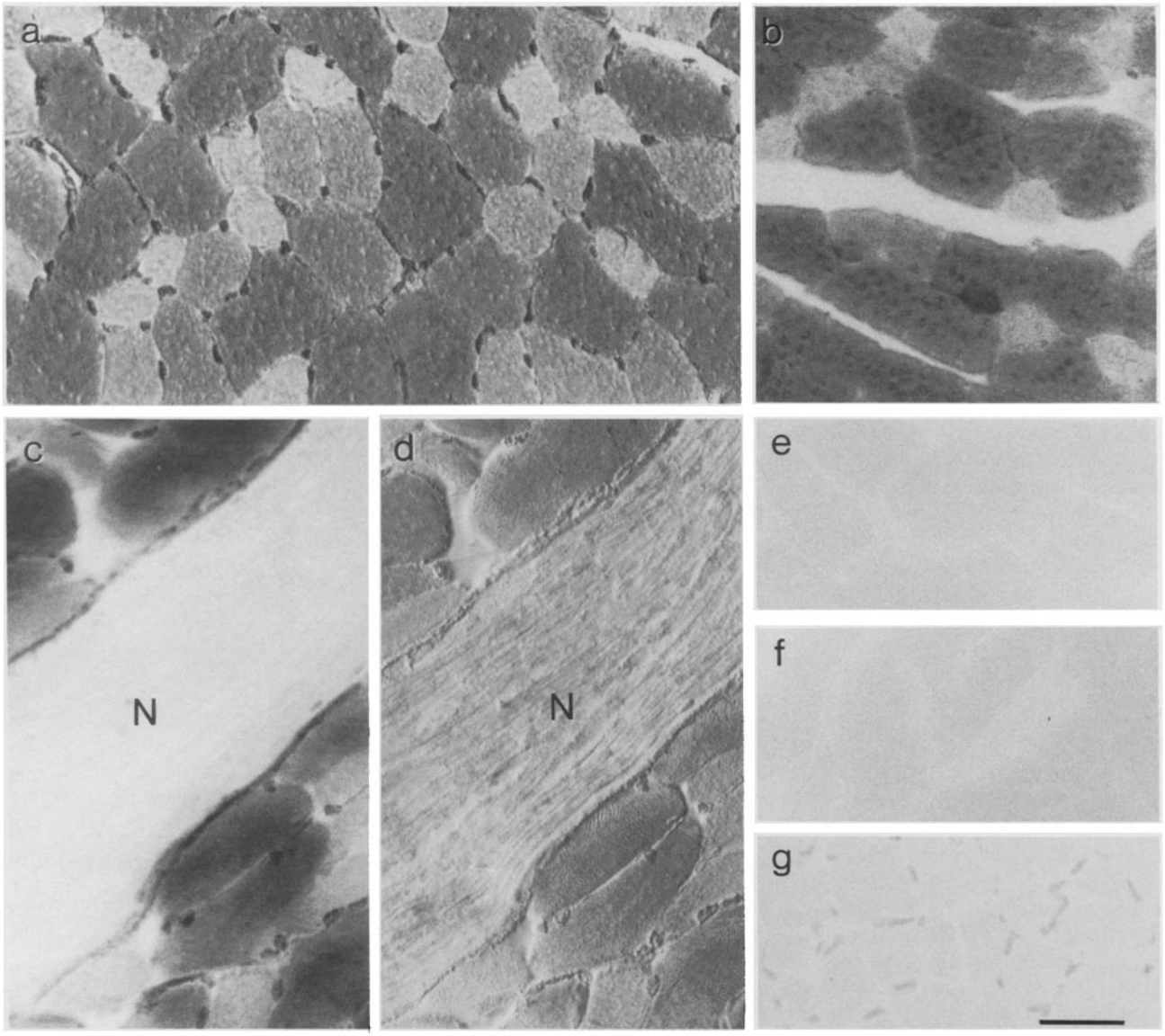

Figure 2. Distribution of CAT in skeletal muscles of MLC1-CAT transgenic mice. Most muscle fibers are stained in tibialis anterior (*a* and *c*) and gastrocnemius (*b*), but the intensity of staining varies considerably among fibers. Nuclei are stained in some muscles (compare *a* with *b*) but this staining appears to be nonspecific. Nerves (*N*) are unstained (*c*; *d* is same field photographed with Normarski optics to show position of nerve). No staining of muscle fibers was observed in transgenic muscle (gastrocnemius) incubated in a mixture lacking chloramphenicol (*e*) or AcCoA (*f*), or in wild-type muscle (tibialis anterior) incubated with the complete staining solution (*g*) Bar, 50 μm.

ond, there was considerable variation in the intensity of CAT staining among muscle fibers. Each muscle contained a mixture of intensely stained, moderately stained and virtually unstained muscle fibers (Figs. 2, *a* and *b* and 3), and the proportions of stained and unstained fibers varied from muscle to muscle (Fig. 4, *a* and *c*). For any individual fiber, the level of staining varied little from section to section (Fig. 3), and for any muscle, the proportions of stained and unstained fibers varied little from animal to animal (see below), indicating that the variations do not result simply from capriciousness of the histochemical method. Thus, different muscle fibers appear to express different levels of CAT.

In considering factors that might account for the variation in CAT staining among muscle fibers, we were immediately struck by the similarity of our histochemical results to the patterns of fiber type diversity that have been revealed by numerous histochemical and immunohistochemical methods (Pette and Staron, 1990). In addition, the variations among muscles suggested two alternative explanations for the positional gradient of transgene expression documented previously (Donoghue et al., 1991): different levels of CAT per muscle could arise from positional-dependent variations in the proportions of CAT-rich and CAT-poor fibers or from position-dependent gradations in the average level of CAT per fiber. Therefore, we used the histochemical stain for CAT to test the idea that levels of transgene expression are correlated with fiber type, to elucidate the cellular basis of the positional gradient, and to ask whether fiber type– and position-dependent expression are regulated separately.

Fiber Type–dependent Transgene Expression

Although all skeletal muscle fibers share numerous features, it has long been recognized that they are not all identical.

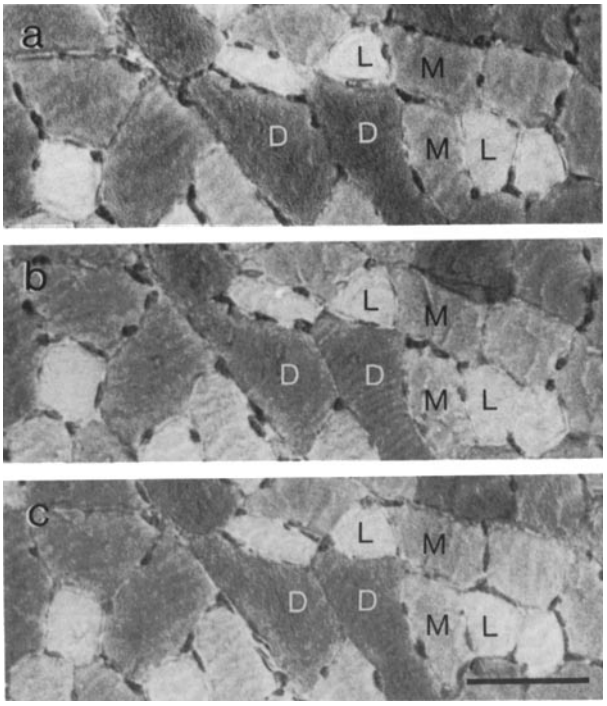

Figure 3. Three serial sections of a tibialis anterior muscle from an MLC1-CAT mouse, stained for CAT. The same fibers are stained lightly (*L*), moderately (*M*), or darkly (*D*) in each section, demonstrating that the variations reflect differences in CAT level rather than capriciousness of the stain. Bar, 100 μm.

Thus, even within a single muscle, fibers are heterogenous in their electrical, metabolic, mechanical, and histological properties. Several of these properties have been used to categorize mammalian muscle fibers as slow-twitch (type I) or fast-twitch (type II). Each individual muscle contains fibers of several types, and the proportions of fiber types vary from muscle to muscle (Schmalbruch, 1985; Pette and Staron, 1990). Perhaps the most reliable method for categorizing muscle fibers is by determining the myosin heavy chain (MHC) genes that they express. Four MHCs, called I, IIA, IIB, and IIX can be distinguished in adult mammalian trunk and limb muscles (Sciaffino et al., 1989). Both immunohistochemical and electrophoretic analyses have revealed that most fibers express only a single one of these MHCs, and can therefore be classified as type I, IIA, IIB, or IIX fibers; a minority of fibers contain detectable levels of two MHCs (Termin et al., 1989; Gorza, 1990). We therefore combined immunohistochemical staining for MHC isoforms with histochemical staining for CAT to ask whether CAT levels correlated with fiber type.

We used three muscles for this portion of the study: soleus, tibialis anterior, and the internal intercostal of the eighth thoracic (T8) interspace. All three muscles are from the caudal half of the animal and express relatively high levels of CAT (Donoghue et al., 1991), but they are diverse in structure and function. Muscles were frozen, and sets of six serial sections were prepared from each. Five sections from each set were stained with a panel of five monoclonal antibodies to MHC isoforms (Table I), and the sixth was stained for CAT. The sections were then photographed, and the micrographs were assembled into photomontages (see Fig. 4 for examples). By counting the number of immunoreactive fibers on the five antibody-stained montages, we derived the fiber type composition of each muscle. We then followed individual muscle fibers from montage to montage to determine the MHC isoform(s) expressed by CAT-rich and CAT-poor fibers.

As summarized in Table I, all three muscles contained a mixture of fiber types, but their proportions were different in each muscle. Interestingly, the antibody to type IIA MHC consistently stained fibers more intensely in tibialis anterior and intercostal T8 than in soleus (compare Fig. 5, *d* and *j*). We have no explanation for this difference but note that others have previously reported differences between type II fibers in soleus and those in other hindlimb muscles (Ausoni et al., 1990; Bateson and Parry, 1983). Comparison of serial sections showed that the majority of muscle fibers in all three muscles expressed only a single MHC, but that a small number contained two MHC types: examples of I/IIA and IIA/IIX fibers were encountered in soleus, IIA/IIX and IIB/IIX in tibialis anterior, and IIA/IIX in intercostal. We did not determine the proportion of fibers that exhibited this hybrid character, but believe that they comprise <10% of the total in each muscle studied (see also Gorza, 1990). In general, the fiber type compositions of tibialis anterior and intercostal T8 agree with previous studies on rat and mouse (Ariano et al., 1973; Armstrong and Phelps, 1984; Cunningham et al., 1991; Parry and Wilkinson, 1990; Gorza, 1990). For soleus, our results confirm the finding that the fiber type composition of this muscle differs considerably between mouse and rat, with few type II fibers present in the latter (Lewis et al., 1982; Ausoni et al., 1990).

With these assurances that MHC immunohistochemistry permits reliable categorization of mouse muscle fibers, we analyzed several fields from each set of photomontages (Fig. 4), to ask whether levels of transgene expression correlated with fiber type. Fig. 5 shows examples from soleus and tibialis anterior, and Table II summarizes the results from all three muscles. In each muscle, nearly all slow-twitch fibers (type I) were CAT-negative, whereas most fast-twitch (type II) fibers were CAT positive (Fig. 5, *a–c* and *g–i*). Although we have not quantitated the difference in CAT level between type I and II fibers, the calibration method described below (see Fig. 9) permits a rough estimate to be made: the darkest staining type II fibers contain >10-fold higher CAT levels than do type I fibers. In this respect, the regulation of transgene expression mirrors that of the endogenous MLC1f/3f gene (see Discussion).

In addition to the difference between type I and II fibers, CAT levels varied considerably among type II fibers, and these differences were correlated with subtype (Fig. 5, *d–f* and *j–l* and Table II). Thus, in tibialis anterior and intercostal T8, the average level of transgene expression was greatest in type IIB, slightly less in type IIX, and less still in type IIA fibers. For soleus, a muscle with no type IIB fibers, the most intensely staining fibers were type IIX, whereas some IIA fibers were barely stained. In all muscles, hybrid fibers had intermediate levels of expression; for example IIA/IIX fibers generally stained more intensely than IIA fibers in soleus, and IIB/IIX fibers stained less intensely than IIB fibers in tibialis anterior. Thus, expression of CAT in MLC1-CAT transgenic mice varies systematically with fiber type.

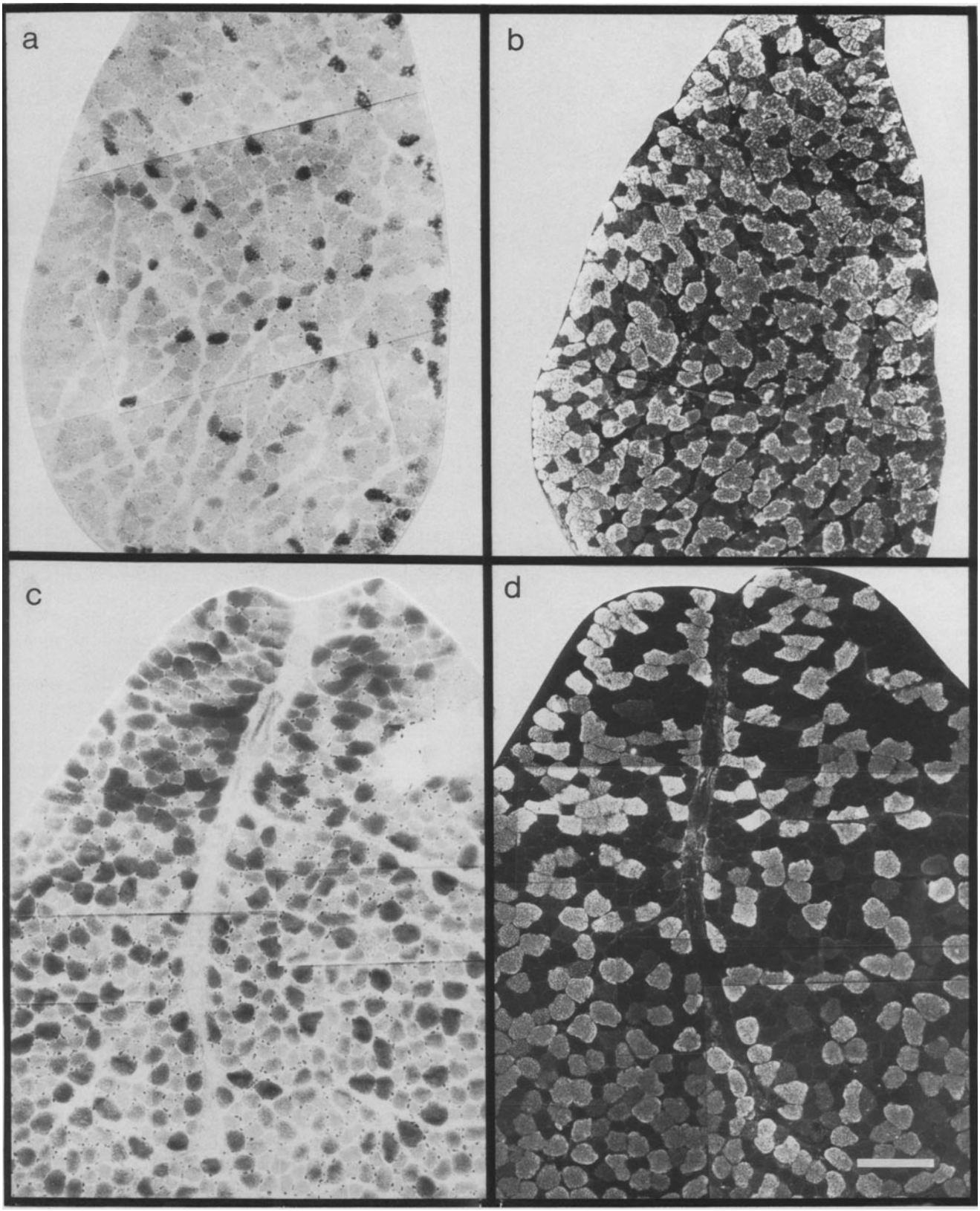

Figure 4. Photomontages of soleus (*a* and *b*) and tibialis anterior (*c* and *d*) muscles stained histochemically for CAT (*a* and *c*) or immunohistochemically with antibodies to MHCs (*b* and *d*). Analyses of fiber type composition and of fiber type-dependent CAT expression used montages such as these. Section in *b* was stained with anti-type I MHC, and section in *d* was stained with anti-type IIB MHC. Nearly all of the soleus but only about one-fifth of the tibialis anterior is shown. Bar, 200 μm.

Table I. Fiber Type Compositions of Soleus, Tibialis Anterior, and Internal Intercostal T8 Muscles Determined with MHC-specific Antibodies

Antibody	Fiber type stained				Muscle (percent of fibers)		
	I	IIA	IIB	IIX	Tibialis anterior	Soleus	Internal intercostal T8
BA-D4	+	−	−	−	1, 0	57, 55	5, 1
MY-32	−	+	+	+	ND	47, 45	ND
SC-71	−	+	−	−	13, 26	38, 38	33, 39
BF-F3	−	−	+	−	67, 47	0, 0	42, 49
BF-35	+	+	+	−	19, 27	5, 7	20, 10

The name and specificity of each antibody is listed on the left. The percent of fibers in each muscle that were negative for BF-35 or positive for the other antibodies is listed on the right. Values are from two muscles, each analyzed from montages such as those shown in Fig. 4. *ND*, Not determined, because >95% of the fibers were positive.

Position-dependent Expression of the Transgene

Our interest in the MLC1-CAT transgene stems largely from the observation that its expression is positionally graded: muscles that are derived from successively more caudal somites express successively higher levels of CAT activity both in vivo (Donoghue et al., 1991) and in vitro (Donoghue, M. J., J. P. Merlie, and J. R. Sanes. 1991. *Soc. Neurosci. Abst.* 17:10). The assays that revealed this pattern provided measures of the total amount of CAT per muscle. They were not, however, able to distinguish among several ways in which differences in CAT levels among muscle fibers might lead to the generation of the observed differences among muscles. At one extreme (Model 1, Fig. 6), a positional gradient of transgene expression could arise from positionally graded variations in the proportions of CAT-rich and CAT-poor fibers. Given the results on fiber type–dependent transgene expression presented above, a specific hypothesis would be that fiber type composition is positionally graded; for example, that the CAT-poor and CAT-rich fibers correspond to type I and type II fibers, respectively. At the other extreme (Model 2, Fig. 6), the concentration of CAT per muscle fiber might be more or less continuously graded with body position, such that the absolute level of CAT per cell would be independently influenced by position and fiber type. Because these alternatives suggest very different cellular and molecular mechanisms for the generation and maintenance of the gradient, we used the histochemical stain for CAT to distinguish between them.

To test directly the interaction of fiber type and position as determinants of transgene expression, we compared CAT levels in fibers that were matched for fiber type but differed in position. We used intercostal muscles for this series of experiments because they are a clearly segmented set of muscles, each of which arises from a single somite and is innervated by a single thoracic nerve (see Cunningham et al., 1991, for references). Fig. 7 shows CAT staining of identified IIA and IIB fibers in sections of T2, T6, and T10 intercostals. Within each muscle, type IIB fibers are more intensely stained than IIA fibers. However, the average level of CAT staining varied from muscle to muscle for each fiber type, being barely detectable in T2 intercostal, intermediate in T6 intercostal, and highest in T10 intercostal. Similar results were obtained when type IIX fibers were compared, whereas, type I fibers were CAT-negative at all levels (not shown). Thus, the fiber type dependence of transgene expression does not explain its positional gradient.

Knowing that fiber type– and position-dependent transgene expression vary separately we tested the models sketched in Fig. 6 by quantitating CAT levels in larger populations of fibers. Intercostal muscles T2, T4, T6, T8, and T10 were sectioned at constant thickness, mounted on a single slide, and stained for CAT. The relative optical density of the reaction product within 60 fibers per muscle was then measured as described in Materials and Methods, using a computer-based image analysis system. Values obtained from a single animal are presented in the left half of Fig. 8, and results from three animals are compiled in the right half of Fig. 8. The relative optical density of stained muscle fibers varies several-fold among fibers within each muscle. This variation presumably reflects the fiber type dependence of expression documented above, although no clear subdivision into categories was revealed by densitometry. Despite the intramuscular variation, however, it is apparent that the overall population (both its mean and its extremes) is increasingly intensely stained in increasingly caudally derived muscles. Thus, the densitometric analysis supports Model 2 in Fig. 6—that levels of CAT expression within individual muscle fibers are influenced by the fibers' position along the rostrocaudal axis.

Finally, we asked how the intensity of the histochemical stain for CAT related to measures of CAT activity obtained from conventional radiometric assays of tissue homogenates. To this end, the mean intensities of fibers from T2, T4, T6, T8, and T10 intercostals were plotted against the specific activities determined in our previous study (Donoghue et al., 1991). This plot revealed that optical density determined histochemically was proportional to the log of the specific activity determined in solution (Fig. 9). A logarithmic relationship is the expected one, because gray level is a logarithmic measure of light intensity; in other words, the specific activity of the enzyme is directly proportional to the density of the stain (see Inoué, 1986 for Discussion). In essence, this correspondence establishes a calibration curve for the histochemical stain, that can then be used to quantitate variations among muscle fibers within a muscle. In intercostal T6, for example, the lightest and darkest fibers differ by >10-fold in the levels of CAT they accumulate.

Discussion

Although all mammalian skeletal muscle fibers share numerous structural, molecular, and developmental attributes, they

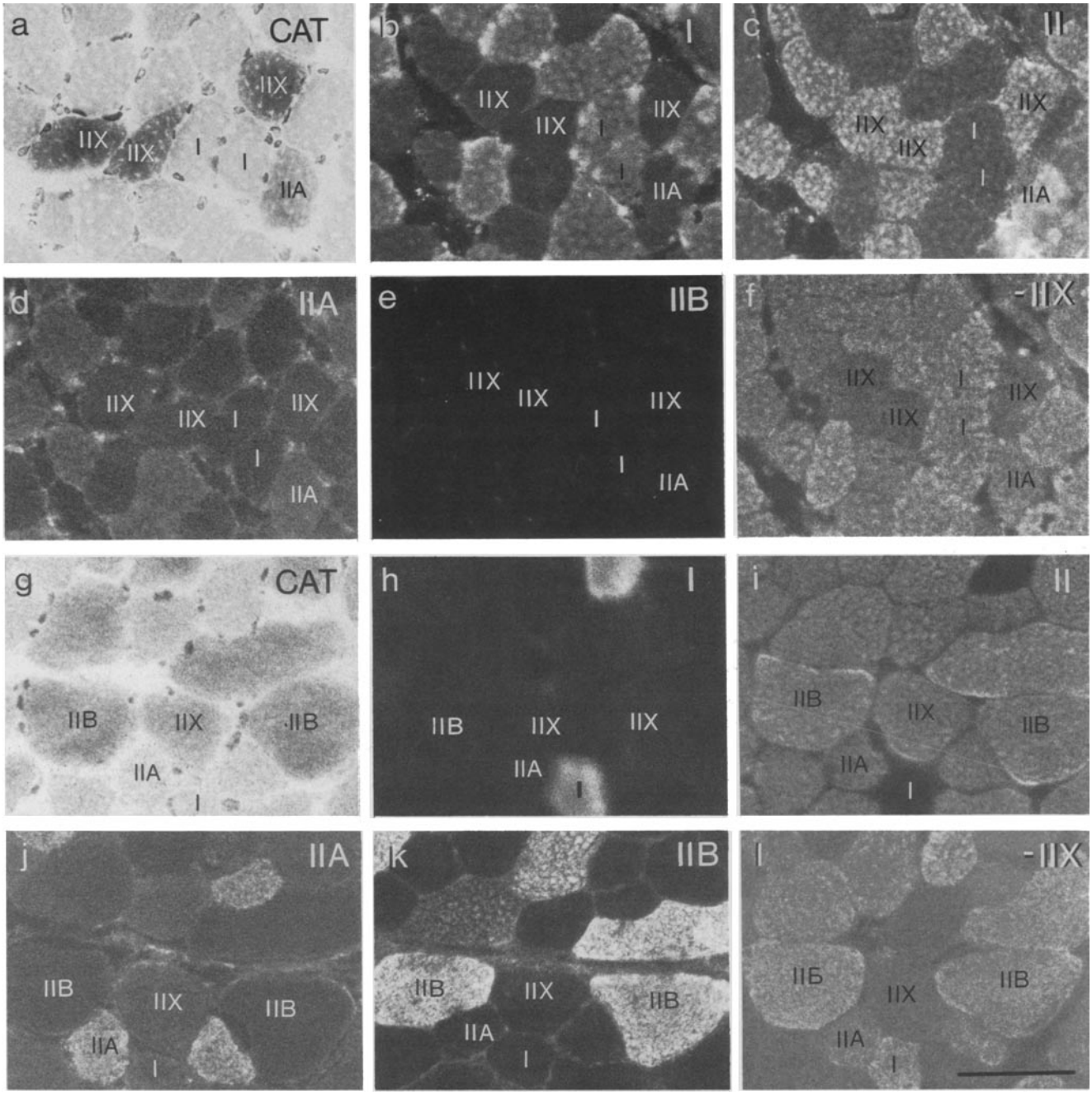

Figure 5. CAT levels in individual muscle fibers correlate with fiber types. Serial sections of soleus (*a–f*) or tibialis anterior (*g–l*) from MLC1CAT mice were stained histochemically for CAT (*a* and *g*) or immunohistochemically with a panel of MHC-specific monoclonal antibodies (*b–f* and *h–i*). Fibers were typed by the criteria compiled in Table 1, and the relationships between fiber type and CAT level are summarized in Table II. Micrographs in *a* and *g* were taken at different exposures and should not be compared with each other. *-IIX* indicates that this antibody stains all fibers except IIX. Bar, 100 μm.

are also heterogeneous to a considerable extent. The two classes of heterogeneity that concern us here are: (*a*) the division of fibers into discrete types that differ in contractile and metabolic properties; and (*b*) the positional differences among muscles originally revealed by their graded preferences for innervation from rostrocaudally matched motor axons. These forms of heterogeneity are of interest both to developmental biologists, as an experimentally tractable system to study determinants of cell type diversification (see, for example, Stockdale and Miller, 1987), and to neurobiologists, as determinants of synaptic specificity (see Thompson et al., 1990, on fiber types, and see below on positional differences). The studies of transgene expression in MLC1-CAT mice reported here provide new information on both forms of heterogeneity, and suggest new strategies for their further study. In addition, the histochemical stain for CAT that we developed in the course of this work may be generally useful in studies of gene expression.

Table II. Levels of Transgene Expression Vary with Fiber Type

Fiber type	Muscle		
	Tibialis anterior	Soleus	Intercostals
I	−	−	−
IIA	−/+	−/+	−/++
IIB	++	0	++
IIX	+/++	++	+/++

Results were obtained from analysis of CAT- and anti-MHC-stained serial sections, such as those shown in Figs. 5 and 7. CAT staining varied among fibers of each type; symbols indicate range of intensities for the majority (>80% of fibers. −, no staining above background; +, clear staining; ++, intense staining; 0, there are no IIB fibers in soleus.

Fiber Type–dependent Transgene Expression

The properties that distinguish muscle fiber types have been studied in great detail (Pette and Staron, 1990). Many proteins have been identified that are differentially distributed by fiber type, and genes have been isolated for several of these (Kedes and Stockdale, 1989). In addition, several factors have been implicated in the generation and maintenance of fiber type diversity, including patterns of nerve-evoked activity, levels of circulating hormones, and hereditary commitments of myoblasts (Stockdale and Miller, 1987). However, no genomic regulatory elements have hitherto been shown to direct fiber type–specific gene expression, and it has therefore been difficult to initiate molecular analysis of the mechanisms by which lineage and environment act to specify a muscle fiber's type.

One factor that has slowed progress toward this goal is the lack of suitable tissue culture systems in which to study fiber type diversification: with few exceptions (Miller and Stockdale, 1986), myoblasts cultured from both fast and slow muscles form myotubes of only a single type of culture (e.g., Rubenstein and Holtzer, 1979; Düsterhoft et al., 1990), and muscle cell lines have not been well characterized as to fiber type. Therefore, it is attractive to use transgenic mice for at least the initial identification of fiber type–specific regulatory elements. In fact, transgenic mice have been prepared that contain promoters and enhancers from several muscle-specific genes (Shani, 1985, 1986; Merlie and Kornhauser, 1989; Johnson et al, 1989; Petropoulus et al., 1989) but in only one case (Hallauer et al., 1988) was transgene expres-

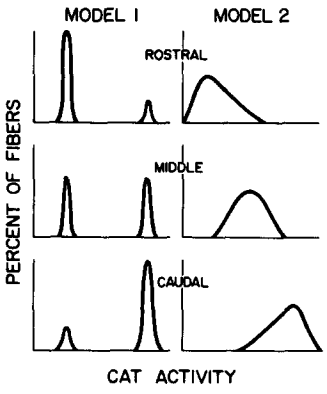

Figure 6. Two models to account for the rostrocaudal gradient of transgene expression observed in muscles from MLC1CAT mice. In *MODEL 1*, the proportions of CAT-poor and CAT-rich fibers vary with position. In *MODEL 2*, levels of CAT in individual fibers vary with position.

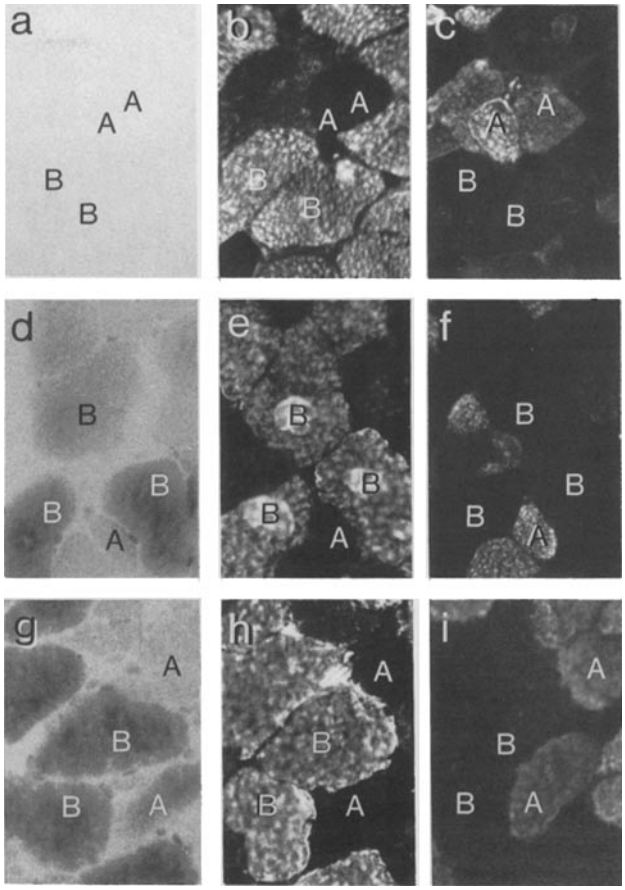

Figure 7. Position-dependent expression of CAT in identified type IIA and IIB muscle fibers. Serial sections of intercostal T2 (*a–c*), T6 (*d–f*), and T10 (*f–i*) muscles were stained for CAT, (*a*, *d*, and *g*) type IIB MHC (*b*, *e*, and *h*), or type IIA MHC (*c*, *f*, and *i*). For both IIA and IIB fibers, levels of CAT expression varied in the order T2 < T6 < T10.

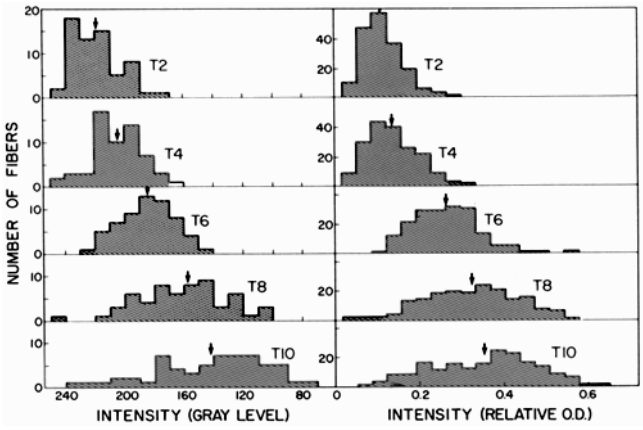

Figure 8. Position-dependent expression of CAT in intercostal muscles. T2, T4, T6, T8, and T10 intercostals were mounted on a single slide and stained for CAT; optical densities of 60 fibers per section were measured as described in Materials and Methods. Panels on the left show values from a single animal, expressed as average gray level for each cross-sectioned fiber on a scale where 0 = black and 256 = white. For the right panels, values from each of three animals were normalized to the most intensely stained fiber and then combined. Arrows indicate mean values for each segmental level.

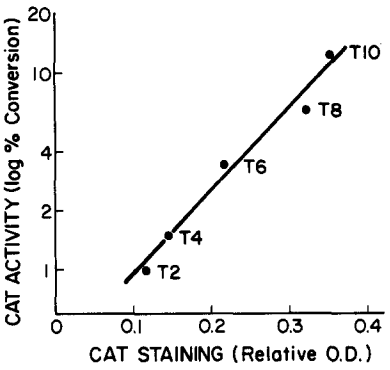

Figure 9. Relationship of CAT activity determined histochemically (*x*-axis) and biochemically (*y*-axis) in intercostal muscles. Values for staining intensity are taken from the right panels of Fig. 8; values for the radiometric assay are from Donoghue et al. (1991). Each set of values is the average from three separate MLC1-CAT mice of line 52. Intensity of staining is proportional to the log of the enzyme activity per unit protein.

sion assayed in muscles that differ in fiber type composition. In that study, a quail troponin I gene was shown to be expressed at far higher levels in gastrocnemius than in soleus muscle in each of three lines tested. Hallauer et al. (1988) interpreted this result as indicating that the troponin I transgene contained sequences that directed expression in fast-twitch (type II) but not slow-twitch (type I) fibers. However, in light of data demonstrating that mouse soleus contains roughly equal proportions of type I and type II fibers (Table I; Lewis et al., 1982) cellular studies of the type reported here might be expected to show that the troponin I transgene is expressed in only a subset of type II fibers.

In considering the correlation of CAT levels with fiber type in MLC1-CAT mice, a crucial issue is whether the fiber type dependence of transgene expression faithfully mirrors the expression of the endogenous gene. In principle, this correspondence could be tested by staining serial sections histochemically for CAT and immunohistochemically for MLC1f. Unfortunately, no type-specific antibodies to mammalian MLC were available to us, and a widely used monoclonal antibody specific for chick MLC1f/3f (Crow et al., 1983) proved to recognize both fast and slow MLCs in rodents (our unpublished results). On the other hand, numerous electrophoretic analyses of microdissected single fibers have shown convincingly that MLC1f and MLC3f are expressed in most type II fibers but are absent from most type I fibers; among the muscles for which this result has been obtained are rat soleus and extensor digitorum longus (Mizusawa et al., 1982), rabbit soleus, adductor magnus, tibialis anterior and psoas (Pette and Schnez, 1977; Staron and Pette, 1987*a*,*b*; Mabuchi et al., 1982), bovine masseter, rectus abdominous and sternomandibularis (Young and Davey, 1981), and human rectus femoris and triceps (Billeter et al., 1981). This pattern of preferential (in human) or exclusive (in dog, cow, and rat) expression of MLC1f and 3f in type II fibers (see Pette and Vrbová, 1985; Pette and Staron, 1990, for further references) corresponds to the pattern of transgene expression that we observed here. We therefore suggest that the MLC1f/3f sequences included in the transgene contain elements that direct fiber type-selective expression, perhaps by responding to a type II-restricted transcriptional regulator.

While the most striking correlation of CAT level with fiber type is that expression is type II-specific, we also observed differences in CAT level among type II fibers, in the order IIB > IIX > IIA. This pattern, and particularly the difference between type IIA and IIB fibers was evident and consistent in each of several muscles studied. It is intriguing that the complete order of CAT expression, IIB > IIB/X > IIX > IIX/A > IIA > I, matches the sequence of fiber type transitions inferred from studies of MHC expression in normal and electrically stimulated muscles (Gorza, 1990). However, we know of no data on whether or not endogenous MLC1f expression differs among type II fibers. Some MLC1f is clearly present within both IIA and IIB fibers but there are quantitative differences in MLC1f levels among type II fibers, and these might correlate with subtype (Pette and Staron, 1990). We therefore do not know whether the differences in CAT activity among type II fibers mirror expression of the endogenous gene or represent an anomalous specificity of the transgene.

Position-dependent Transgene Expression

Electrophysiological experiments have shown that mammalian motor axons selectively innervate and preferentially reinnervate muscles from corresponding segmental positions (Wigston and Sanes, 1982, 1985; Laskowski and Sanes, 1987, 1988; Laskowski and High, 1989). These results imply the existence of molecular differences among muscles that are rostrocaudally graded and that bias the efficacy of synapse formation. However, it was not possible to infer from the physiological studies whether "positional information" resides within the muscle fibers themselves or whether synaptic specificity might arise from extramuscular cues that axons encounter (see, for examples, Landmesser, 1984; Suzue et al., 1990). The discovery that CAT expression is rostrocaudally graded in adult MLC1-CAT mice (Donoghue et al., 1991) provided the first evidence that muscle fibers themselves bear a "positional memory." More recently, we have found that this positional memory is hereditary and stable in vitro: myoblasts from individual muscles divide in culture and form myotubes that express levels of CAT characteristic of their muscles of origin (Donoghue, M. J., J. P. Merlie, and J. R. Sanes. 1991. *Soc. Neurosci. Abst.* 17:10). Results presented here extend this work in two ways.

First, the positional gradient detected in muscle homogenates might have arisen from positionally varying proportions of discrete classes of cells and/or from position-dependent gradations in the average concentration of CAT per fiber (*Model 1* and *Model 2* in Fig. 6). Clearly, these two "microscopic" patterns of transgene expression imply different classes of mechanisms for the generation and maintenance of the "macroscopic" gradient. In the first model, fibers might be specified as "CAT-rich" or "CAT-poor" in the embryo, and no molecular memory of body position would need to persist into adulthood in any individual cell. In the second model, in contrast, continued maintenance of graded levels of transgene expression suggests that some aspect of the transcriptional apparatus varies with position in adults. Our results favor the second model, and thereby motivate a search

for regulators that are graded in abundance or activity along the rostrocaudal axis.

Second, the positional gradient might have been greatly influenced by, if not due to, differences in fiber type composition among muscles. Instead, our results suggest that fiber type and position are largely independent determinants of transgene expression. Thus, levels of CAT are positionally graded within fibers of a given type, and levels of CAT vary with fiber type within muscles derived from a single somite (the intercostals). Further evidence that fiber type and positional determinants do not interact appreciably is that the order of expression by fiber type (IIB > IIX > IIA > I) is the same in muscles from each of several rostrocaudal levels and that the positional gradient of expression appears to be of similar magnitude at least in the type II subtypes (A, B, and X). In practical terms, the clarity with which the positional gradient is seen in whole muscles reflects the fact that most muscles are similar in fiber type composition (e.g., few muscles other than soleus have >20% type I fibers; Schmalbruch, 1985). In molecular terms, our results suggest that fiber type- and position-responsive elements in the MLC1-CAT transgene are distinct, and thereby motivate studies aimed at physically separating them.

A Histochemical Stain for CAT

Many studies of gene expression make use of constructs in which regulatory sequences from one gene are fused to protein-coding sequences from a second gene, the "reporter." Assays of the reporter-encoded mRNA or protein then provide indirect but useful measures of the recombinant gene's rate of transcription. As noted in the Introduction, studies in which transgene expression must be localized at the cellular level have most often used *lacZ* as the reporter, largely because of the simple histochemical stain available to detect β-galactosidase activity. On the other hand, CAT is a preferable reporter in some cases, and the histochemical stain for CAT may be generally useful as is shown in the following examples. (*a*) Vertebrate cells have endogenous β-galactosidase but no endogenous chloramphenicol acetylating activity; "background" levels in CAT assays are therefore low, and do not obscure detection of low levels of authentic CAT. (*b*) Expression patterns of recombinant genes sometimes depend on sequences in the reporter gene as well as those in the regulatory elements (Russo et al., 1988; Merlie J. P., and J. R. Sanes. 1990. *Neurosci. Abst.* 16:1003), so that complete analysis may require comparison of results obtained with two or more reporters. (Note that we have shown that the positional gradient of expression in MLC1-CAT mice is not reporter-dependent; Donoghue et al., 1991.) (*c*) CAT is currently the most widely used reporter in studies of eukaryotic gene expression (Alam and Cook, 1990). Thus, there is a large body of data for comparison, and a large number of CAT-containing vectors already in use. (*d*) In some cases (e.g., lineage tracing; see Galileo et al., 1990) it is helpful to visualize two reporters simultaneously. The histochemical stain for lacZ is compatible with Karnovsky's stain for acetylcholinesterase (J. R. Sanes and J. P. Merlie, unpublished observations), which produces the same product as the stain for CAT (see Results). It should therefore be possible to doubly stain tissues for CAT and lacZ.

Whether the histochemical stain for CAT will be useful for these purposes in its present form is unclear. MLC1-CAT mice express high levels of CAT in caudal muscle, relative to levels of CAT produced from many other expression vectors. Levels of CAT in rostral muscles of MLC1-CAT mice are detectable by the radiometric assay (Donoghue et al., 1991), but not with the current staining protocol (data not shown). On the other hand, we have not yet systematically optimized the histochemical stain for CAT, and suspect that its sensitivity can be improved without compromising its specificity. Work toward this goal is in progress.

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