Integration Site-dependent Expression of a Transgene Reveals Specialized Features of Cells Associated with Neuromuscular Junctions

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Abstract. After skeletal muscle is denervated, fibroblasts near neuromuscular junctions proliferate more than fibroblasts distant from synaptic sites, and they accumulate adhesive molecules such as tenascin (Gatchalian, C. L., M. Schachner, and J. R. Sanes. 1989. *J. Cell Biol.* 108:1873–1890). This response could reflect signals that arise perisynaptically after denervation, preexisting differences between perisynaptic and extrasynaptic fibroblasts, or both. Here, we describe a line of transgenic mice in which patterns of transgene expression provide direct evidence for differences between perisynaptic and extrasynaptic fibroblasts in normal muscle.

Transgenic mice were generated using regulatory elements from a major histocompatibility complex (MHC) class I gene linked to the *Escherichia coli* β-galactosidase (*lacZ*) gene. Expression of lacZ was detected histochemically. In each of eight lines, lacZ was detected in different subsets of cells, none of which included lymphocytes. In contrast, endogeous MHC is expressed in most tissues and at high levels in lymphocytes. Thus, the MHC gene sequences appeared inactive in the transgene, and lacZ expression was apparently controlled by genomic regulatory elements that were specific for the insertion site.

In one line, cells close to the neuromuscular junction were lacZ positive in embryonic and young postnatal mice. Electron microscopy identified these cells as fibroblasts and Schwann cells associated with motor nerve terminals, as well as endoneurial fibroblasts, perineurial cells, and Schwann cells in the distal branches of motor nerves. No intramuscular cells >200 μm from synaptic sites were lacZ positive. These result indicate that there are molecular differences between perisynaptic and extrasynaptic fibroblasts even in normal muscle and that diverse perisynaptic cell types share a specific pattern of gene expression.

FIBROBLASTS are predominant cells of the connective tissue in most organs of the vertebrate body (Fawcett, 1986). Although fibroblasts are known to be heterogenous in composition and function (for example see Parker, 1932; Branwood, 1963; Schneider et al., 1977), only a few available markers label subsets of fibroblasts (for example see Garrett and Conrad, 1979). It has therefore been difficult to define the full range of fibroblast types or to learn how diversity within this broad category is generated.

Recently, we documented an apparent heterogeneity of fibroblast-like cells in rodent muscle. After denervation, several adhesive molecules (tenascin, N-CAM, fibronectin, and a heparan sulfate proteoglycan) accumulate in interstitial spaces surrounding the denervated synaptic sites (Covault and Sanes, 1985; Sanes et al., 1986*b*). A major cellular source of these deposits is a population of perisynaptic fibroblasts: denervation stimulates these cells to proliferate (Connor and McMahan, 1987; Gatchalian et al., 1989) and to synthesize increased amounts of the four molecules listed above (Gatchalian et al., 1989). These responses are selective in that the proliferative and biosynthetic responses to denervation are greater in perisynaptic fibroblasts than in fibroblasts distant from synaptic sites. Because the vast majority of regenerating motor axons form new synapses at original synaptic sites, and because these sites occupy a very small fraction (<0.1%) of the muscle fiber surface, we suggested that perisynaptic fibroblasts, and/or the adhesive molecules they produce, could play a role in guiding axons to synaptic sites (Gatchalian et al., 1989; Sanes, 1989).

A question that arises from these observations is whether the selective responsiveness of perisynaptic fibroblasts to denervation reflects preexisting differences between perisynaptic and extrasynaptic fibroblasts, or the existence of signals elicited by denervation that act locally on an initially uniform cell population. Distinguishing between these alternatives may be prerequisite to elucidating the nature of the nerve-dependent signal that influences fibroblast proliferation and biosynthetic activity. However, the paucity of available subtype-specific fibroblast markers made it difficult to determine whether perisynaptic and extrasynaptic fibroblasts differ in normally innervated muscle; indeed, we favored the interpretation that they do not (Gatchalian et al., 1989).

In the course of characterizing transgenic mice that express the *Escherichia coli* β-galactosidase (*lacZ*) gene, we

have obtained results that challenge this interpretation. Our initial aim was to produce mice that express lacZ in many tissues. We used regulatory elements from a major histocompatibility complex (MHC)[1] class I H-2K^b gene, which is broadly expressed (Klein, 1986; Morello et al., 1986), and chose *lacZ* as a reporter because its product can be detected histochemically in individual cells (Sanes et al., 1986*a*). Surprisingly, only a limited number of cell types were lacZ positive (we call the gene *lacZ* and the protein lacZ) in each line of mice, and patterns of expression varied dramatically from line to line. These results suggested that chromosomal elements near its site of insertion regulated the *lacZ* gene, rather than (or in addition to) the regulatory elements intrinsic to the transgene; that is, the transgene acted as an "enhancer trap" (O'Kane and Gehring, 1987; Gossler et al., 1989; further references in Discussion). Of particular interest, in light of our previous studies, was the observation that perisynaptic fibroblasts were lacZ positive during early postnatal life in mice of one line whereas extrasynaptic fibroblasts were lacZ negative. In addition, Schwann cells and perineurial cells near synaptic sites were lacZ positive in these mice. Here, we document this pattern of expression, describe how it changes during development and after denervation, consider its implications for the origin of perisynaptic fibroblasts, and discuss the general use of such transgenic mice for marking discrete cell populations.

Material and Methods

Production of Transgenic Mice

Mice were rendered transgenic by microinjection of DNA into the pronuclei of fertilized ova, using standard methods (Hogan et al., 1986). The DNA construct injected is shown in Fig. 1. It consisted of a 2-kb HindIII/NruI fragment of a murine MHC class I (H-2K^b) gene (Kimura et al., 1986), the *E. coli* β-galactosidase (lacZ) gene from pMMuLV-SV-LacZ (Sanes et al., 1986), and a 3′ splicing and polyadenylation signal from pSV2-CAT (Gorman et al., 1982). For injection, a 6.6-kb fragment was excised from the pUC18/Bluescript plasmid with SalI and NotI and isolated by electrophoresis in agarose. Mice were obtained from Jackson Laboratory (Bar Harbor, ME). Injections were made into fertilized ova obtained from C57Bl/6 females that had been mated with C57Bl/6 males (line 15) or (CBA.J × B10.M) F1 females that had been mated to B10.M males (all other lines). Mice bearing the transgene were identified by Southern blot analysis or polymerase chain reaction, using DNA from the tail and probes from the *lacZ* gene (Fine, 1989). Nine transgenic "founders" were mated with B10.M mice and their offspring studied. Southern analysis revealed a single integration site in each line. Initially, transgenic offspring were identified by the polymerase chain reaction. However, it soon became apparent that mice of line 42, on which we focus here, expressed high levels of lacZ in the skin at all postnatal ages. We therefore took 2-mm-diam punch biopsies from the dorsal neck skin of neonates, and stained them for lacZ as described below to distinguish transgenic from wild-type animals in this line.

Histology

Mice were anesthetized with ether and perfused through the heart with 2% paraformaldehyde and 0.4% glutaraldehyde in PBS at room temperature. Tissues were then dissected, postfixed for 10 min in the same solution, washed twice with PBS, and stained for 6–15 h at 37°C. The staining solution contained 2 mM 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-gal; United States Biochemical, Cleveland, OH) or 5-bromo-3-indolyl-β-D-galactoside (Bluo-gal; BRL Life Technologies, Gaithersburg, MD), 20 mM potassium ferrocyanide, 20 mM potassium ferricyanide, and 2 mM MgCl in PBS (modified from Sanes et al., 1986*a*).

After staining, tissues were washed twice with PBS and examined with a dissecting microscope. Areas containing lacZ-positive cells were then processed for light and electron microscopy. For routine histology, tissues were frozen in liquid nitrogen and sectioned in a cryostat; sections were stained with haemotoxylin and eosin. To examine single muscle fibers, muscles were stained for cholinesterase by the method of Karnovsky (1964) to reveal endplates, then cleared in glycerol. Single fibers were then teased from fiber bundles and mounted in glycerol. For electron microscopy, tissues were incubated overnight in 2% paraformaldehyde plus 2% glutaraldehyde in PBS, refixed for 1 h in 1% OsO4, and embedded in Araldite. Semithin sections were cut with a glass knife and stained with paraphenylenediamine or toluidine blue. Thin sections were cut with a diamond knife and examined without further staining.

In the course of this study, we compared two histochemical substrates for lacZ, X-gal, and Bluo-gal. These are closely related indoxyl derivatives of galactose, differing only in whether the indolyl ring is (X-gal) or is not (Bluo-gal) substituted by chloride at the 4-position (Holt and Sadler, 1958). The reaction product derived from X-gal was bright blue, whereas that from Bluo-gal was a bluish gray that was sometimes difficult to distinguish from the surrounding tissue. For this reason, we usually used X-gal as a substrate for detecting lacZ-positive cells in whole mounts and thick sections. However, the X-gal reaction product diffuses some distance before it precipitates, leading to occasional staining of cells or extracellular spaces that neighbor lacZ-positive cells (Sanes et al., 1986*a*). This diffusion is reduced but not eliminated by increasing the concentrations of ferrocyanide and ferricyanide in the reaction mixture from the 5 mM used originally (Sanes et al., 1986*a*) to 20 mM (Gray et al., 1988). On the other hand, substitution of Bluo-gal for X-gal decreased diffusion of reaction product to negligible levels, and thus stained lacZ-positive cells crisply. Bluo-gal was therefore preferable to X-gal when stained tissue was to be examined at high magnification. Furthermore, the precipitates formed from the two substrates differed at the ultrastructural level: the Bluo-gal reaction product formed a fine, very electron-dense precipitate that bound tightly to internal membranes (e.g., Fig. 4), whereas the precipitate formed by X-gal was less dense, more difficult to distinguish from endogenous structures, and sometimes displaced to extracellular sites (Fig. 6 *b*). Bluo-gal was therefore preferable to X-gal for electron microscopy.

Results

We produced nine lines of mice that contained a transgene in which the *lacZ* gene was linked to regulatory elements from the MHC class I H-K^b gene. Based on the broad distribution of endogenous MHC class I transcripts (Klein, 1986; Morello et al., 1986; Fahrner et al., 1987), and on the nearly ubiquitous expression of another transgene containing the same MHC sequences that we used (Morello et al., 1986), we expected that lacZ would be expressed in most tissues of MHC-*lacZ* mice. However, this result was not obtained. Instead, histochemically detectable levels of lacZ were present in only a small subset of cells in each line. The cell types stained varied little among animals within each line, but varied dramatically from line to line (Table I). For example, the kidney tubules were intensely stained only in mice of line 15 (Fig. 2, *a* and *b*), whereas lungs were stained only in line 25 (Fig. 2 *c*) and arterial walls were stained only in mice of line 30 (Fig. 2 *d*). Although lacZ-positive cells were readily detected in eight of the nine lines examined, none of these expressed detectable lacZ in circulating lymphocytes or in lymphoid tissues, which normally express high levels of endogenous MHC gene products (Morello et al., 1986; Fahrner et al., 1987). These patterns suggested that transcription of lacZ was being regulated by chromosomal elements near the transgene's site of integration, rather than by (or in addition to) sequences of the MHC gene (see Discussion). Further descriptions of all lines are presented in Fine (1989). Here, we focus on a single line, number 42, in which the pattern of lacZ expression proved to be

1. *Abbreviations used in this paper*: E, embryonic day; MHC, major histocompatibility complex; P, postnatal day.

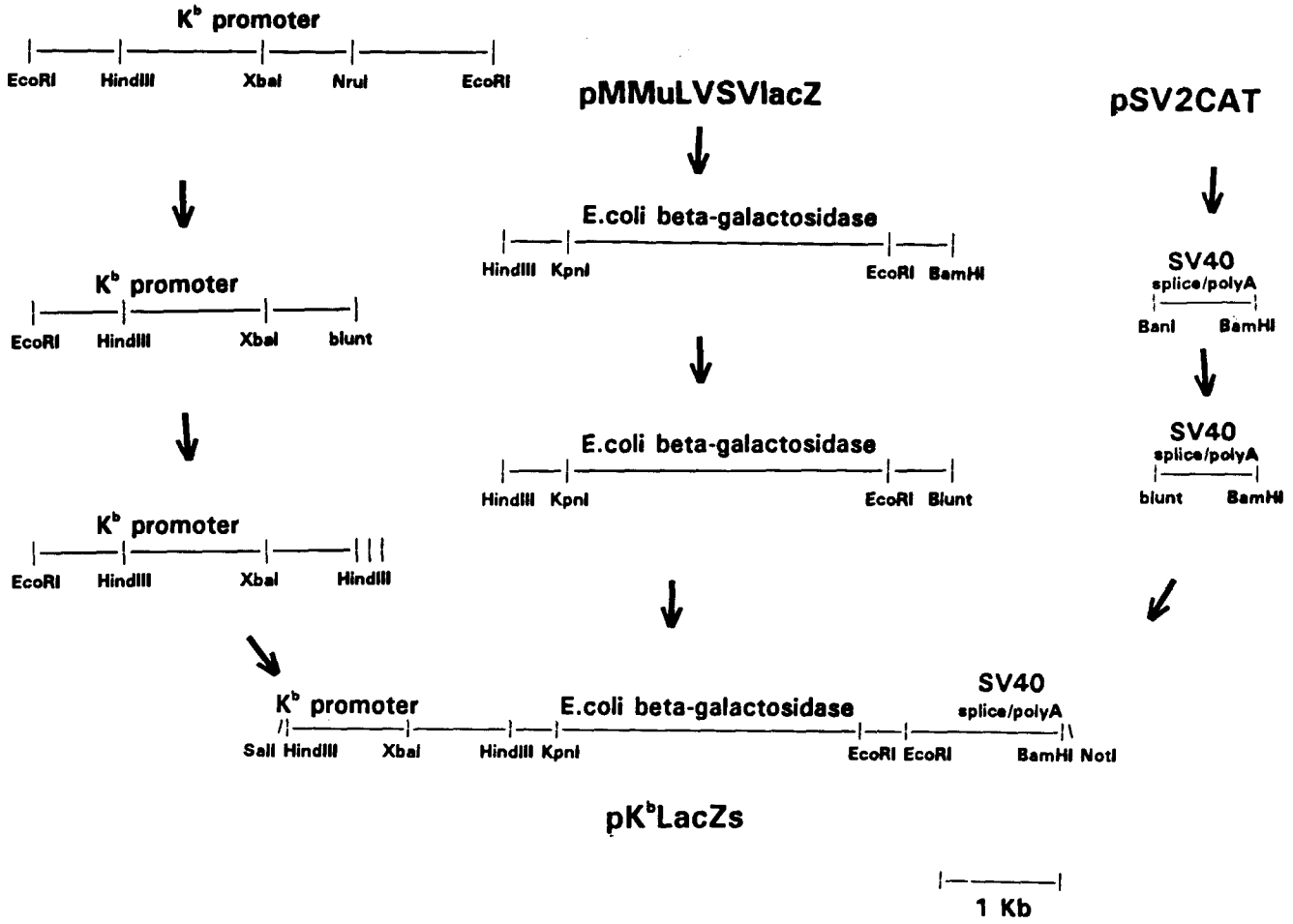

Figure 1. Assembly and structure of the transgene containing MHC class I (H_k) regulatory elements, the *E. coli* β-galactosidase gene (*lacZ*), and a splicing and polyadenylation signals from SV-40. Restriction sites used in the construction are indicated.

relevant to our previous studies on cellular heterogeneity in this tissue (Sanes et al., 1986*b*; Gatchalian et al., 1989).

lacZ-expressing Cells at Neuromuscular Junctions

Our attention was attracted to mice of line 42 by a narrow band of lacZ-positive material in the region of intercostal (Fig. 3 *a*) and diaphragm (Fig. 3 *c*) muscles of 3–10-d-old (P3-P10) transgenic animals. The position of the stained bands corresponded to that of the "endplate zones," within which intramuscular nerves run and neuromuscular junctions are concentrated (see, for example, Fig. 1 in Merlie and Sanes, 1985). We also found concentrations of lacZ reaction product in the synaptic regions of numerous other striated muscles in the trunk and limbs. In each muscle, lacZ-positive cells formed small clusters in or near terminal branches of motor nerves (Fig. 3 *c*). All transgene-positive mice of line 42 stained between P3 and P10 exhibited this pattern ($n \cong 15$). No lacZ-positive cells in the muscles were found outside of the endplate zones. Furthermore, no cells were detectably lacZ positive in the skeletal muscles of wild-type mice at any age (Fig. 3 *b*) or of other lines of transgenic mice produced with the same *lacZ* construct (Table I). Thus, the transgene in line 42 is selectively expressed in areas near neuromuscular junctions.

To confirm the association of lacZ-positive cells with synapses, we counterstained muscles for cholinesterase to mark synaptic sites. In such preparations, it was apparent that >95% of the lacZ-positive cells were within 100–200 μm of an endplate. This association was particularly clear in single fibers teased from muscles doubly stained for lacZ and cholinesterase: lacZ-positive cells frequently directly abutted cholinesterase-positive synaptic sites (Fig. 3 *d*).

Table I. Occurrence of lacZ-positive Cells in MHC-lacZ Transgenic Mice

	Line								
Site	11	13	15	19	25	30	31	38	42
Brain	+	−	+	+	+	+	+	+	+
DRG	+	−	+	−	−	−	−	+	+
Thymus	+	−	−	−	+	−	−	+	+
Skin	−	−	−	−	+	−	+	+	+
Stomach	+	−	−	−	+	−	−	−	−
Lung	−	−	−	−	+	−	−	−	−
Kidney	−	−	+	−	−	−	−	−	−
Arteries	−	−	−	−	−	−	+	−	−
Muscle	−	−	−	−	−	−	−	−	+
Lymphocytes	−	−	−	−	−	−	−	−	−

+ indicates that some cells in the tissue indicated express lacZ. Note that particular cells stained within a tissue, e.g., brain, vary from line to line. For details, see Fine (1989).

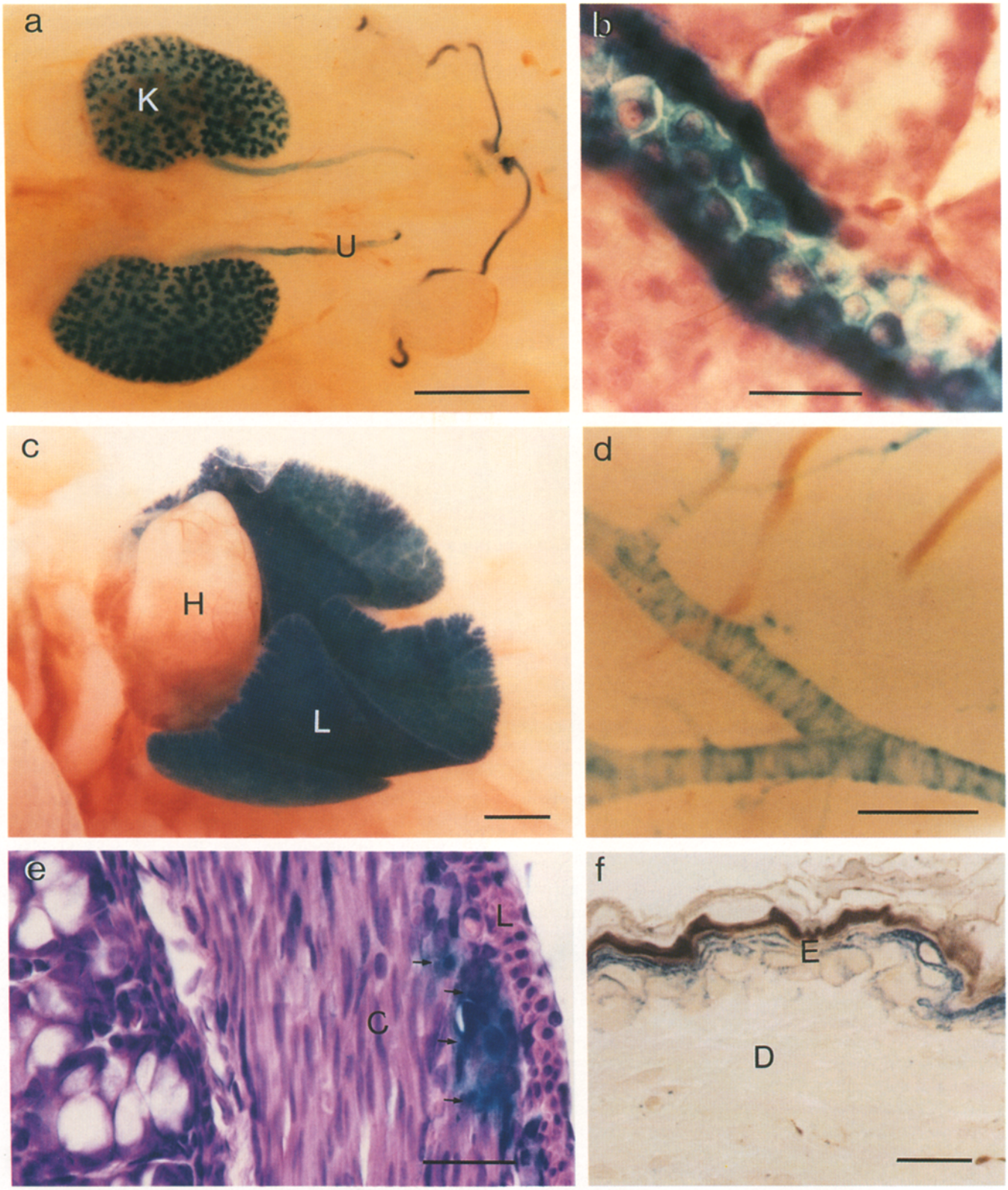

Figure 2. Patterns of lacZ expression differ among lines of MHC-*lacZ* transgenic mice. (*a* and *b*) Line 15. (*a*) Kidneys (*K*) and ureters (*U*) are lacZ positive. E15. (*b*) Section through adult kidney shows that lacZ-positive cells in kidney are those of the collecting ducts. (*c*) Line 25, E17. Lungs (*L*) are lacZ positive, while nearby organs such as the heart (*H*) are unstained. (*d*) Line 31, adult. Arteries in brain contain lacZ-positive cells. (*e* and *f*) Line 42. (*e*) lacZ-positive neurons (*arrows*) in the myenteric (Auerbach's) plexus of the intestine, between the circular (*C*) and longitudinal (*L*) layers of smooth muscle. P20. (*f*) Cells in the strata granulosum and spinosum of the epidermis (*E*) are lacZ positive but underlying dermis (*D*) is unstained. P6. *a* and *c* are photographs of partially dissected embryos; *b* is a cryostat section counterstained with haemotoxylin and eosin; *d* is a whole mount of a hand-cut slice of brain; *e* is a paraffin section, counterstained with haemotoxylin and eosin; and *f* is a semi-thin section of resin-embedded skin, counterstained with paraphenylenediamine. See Table I for a list of tissues stained in nine transgenic lines. Bars: (*a* and *c*) 1 mm; (*b*, *e* and *f*) 25 μm; (*d*) 500 μm.

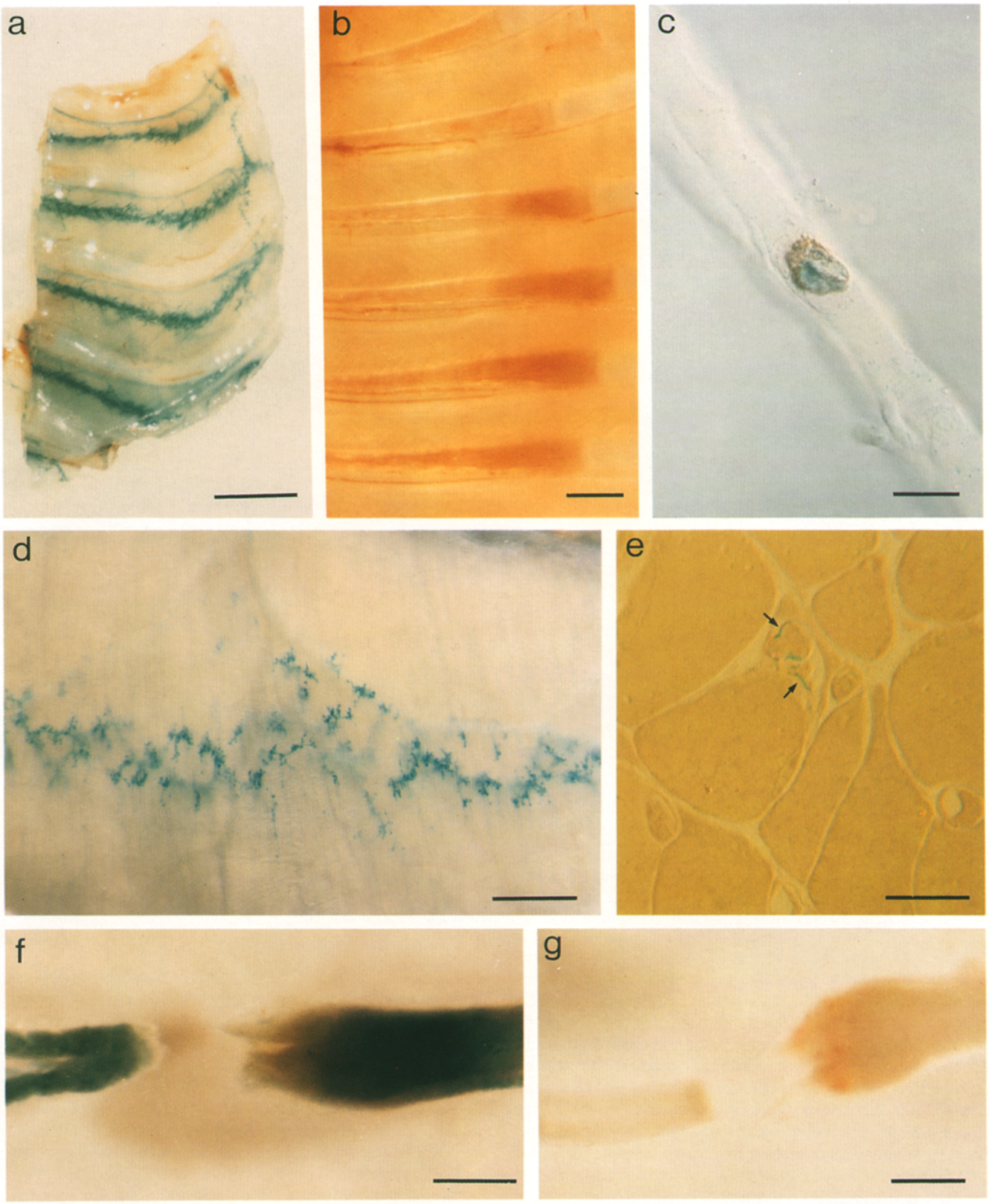

Figure 3. lacZ-positive cells in muscles and peripheral nerves of line 42 mice. (*a*) Ribcage of a transgenic mouse (P3) stained with X-gal. Reaction product is concentrated in bands that correspond to endplate zones. (*b*) Ribcage of a 7-d-old wild-type mouse of the same strain as in *a*. No lacZ expression is detectable in skeletal muscles. (*c*) In the diaphragm of a 5-d-old transgenic mouse, the close association of the X-gal staining with the endplate zone is evident. Muscle fibers run vertically. (*d*) A teased muscle fiber of a 12-d-old transgenic mouse doubly stained for lacZ and acetylcholinesterase. A lacZ-positive cell is located atop the endplate. (*e*) Intercostal muscle of a 6-d-old transgenic mouse stained with Bluo-gal, embedded in araldite, cross-sectioned at a thickness of 2 μm, and counterstained with paraphenylendiamine. Two lacZ-positive cells (*arrow*) are located close to a muscle fiber at what appears to be a synaptic site. There is no blue label in muscle fibers. (*f*) Proximal (*right*) and distal (*left*) stump of a sciatic nerve of a 35-d-old transgenic mouse 5 d after transection. The stumps are intensely stained with X-gal. (*g*) Proximal (*right*) and distal (*left*) stump of a sciatic nerve of a wild-type mouse of the same age and strain as in *f* 5 d after transection. Both stumps are lacZ negative after the same staining as in *f*. Bars: (*a*, *b*, *d*, *f*, and *g*) 500 μm; (*c*) 50 μm; (*e*) 20 μm.

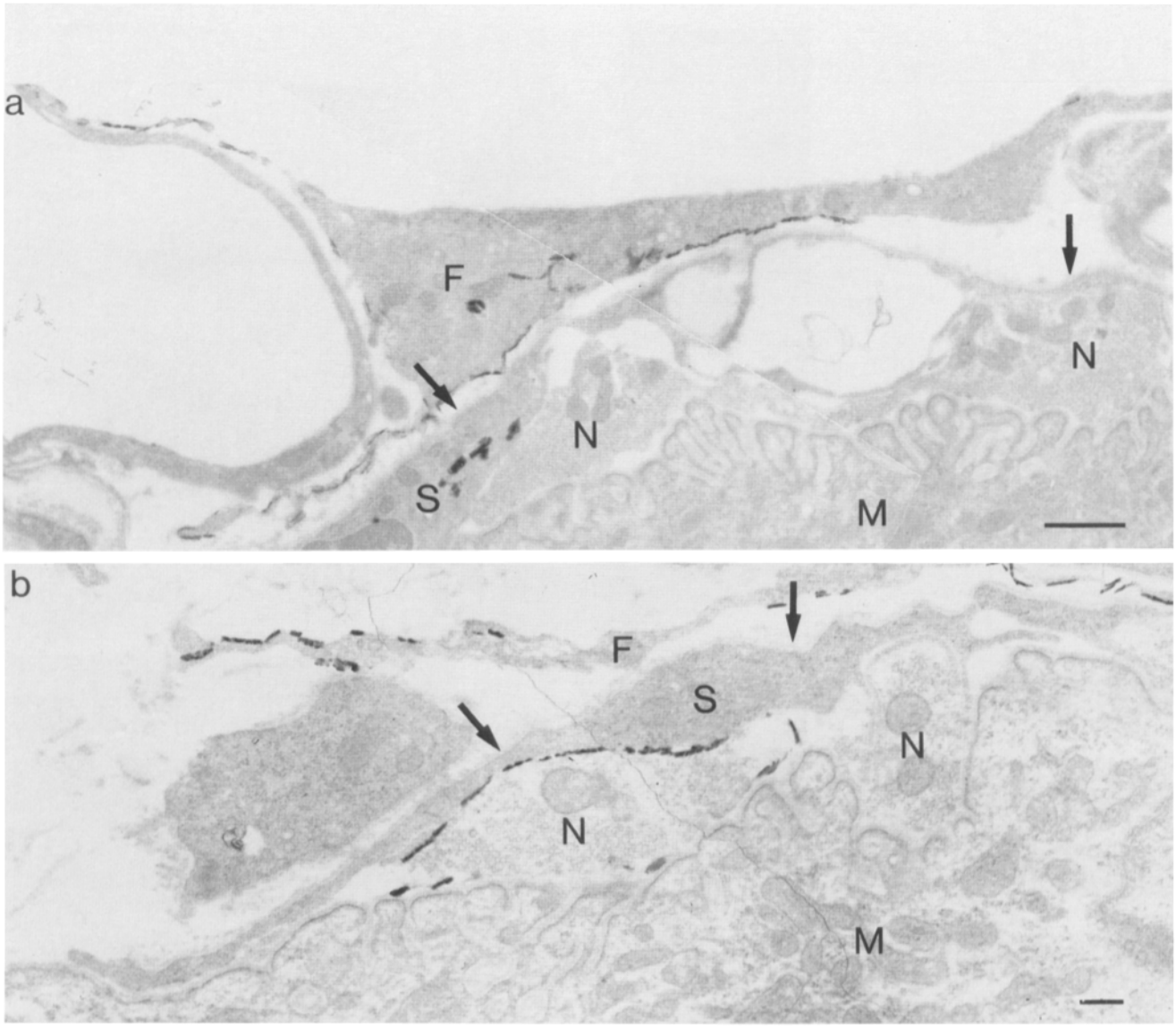

Figure 4. Electron micrographs of neuromuscular junctions in the intercostal muscles of 12- (*a*) and 11- (*b*) d-old transgenic mice. Nerve terminals (*N*) are opposed to synaptic folds in the muscle fiber (*M*) membrane. Terminals are ensheathed by Schwann cells (*S*) which are themselves coated with basal lamina (*arrows*). The Schwann cells are overlaid by fibroblasts (*F*) that lack basal lamina. Both fibroblasts and Schwann cells are labeled with the dark reaction product of the Bluo-gal stain. Bars, 1 μm.

To identify the lacZ-positive cells, synapse-rich regions were embedded in plastic and sectioned for light and electron microscopy. Light microscopy indicated that reaction product was concentrated in mononucleated cells between muscle fibers, and was not detectable within the muscle fibers themselves (Fig. 3 *e*). Electron microscopy of adjacent sections revealed that the lacZ-positive cells occurred in two distinct compartments: interstitial spaces adjacent to synapses, and small terminal nerve branches. In interstitial spaces (Fig. 4), the majority of labeled cells were identified as fibroblasts by the criteria detailed in Gatchalian et al., (1989): these cells bore numerous long processes, had abundant rough endoplasmic reticulum, and lacked a basal lamina. Often, a lacZ-positive fibroblast extended processes over the entire endplate (Schwann cells, nerve terminals, and synaptic portions of muscle fibers). In addition, Schwann cells that cover motor nerve terminals were frequently lacZ positive. Within distal nerve branches (Fig. 5), both endoneurial fibroblasts and Schwann cells were frequently labeled. Some of the perineurial cells that ensheathe the nerve branches were also stained (Fig. 5 *c*); these cells are derived from fibroblasts (Bunge et al., 1989). Other cell types identified in thin sections of muscle were rarely if ever lacZ positive. These included capillary endothelial, lymphoid, and mast cells in interstitial spaces; muscle fibers; fibroblasts distant from synaptic sites; and fibroblasts, Schwann cells, and perineurial cells in proximal portions of the nerve. Thus, two major intramuscular cell types, fibroblasts and Schwann cells, expressed lacZ; this expression was seen in both interstitial and intraneural compartments, but was confined to areas near synaptic sites (Fig. 6).

Developmental Regulation of lacZ Expression

To learn whether intramuscular patterns of lacZ expression

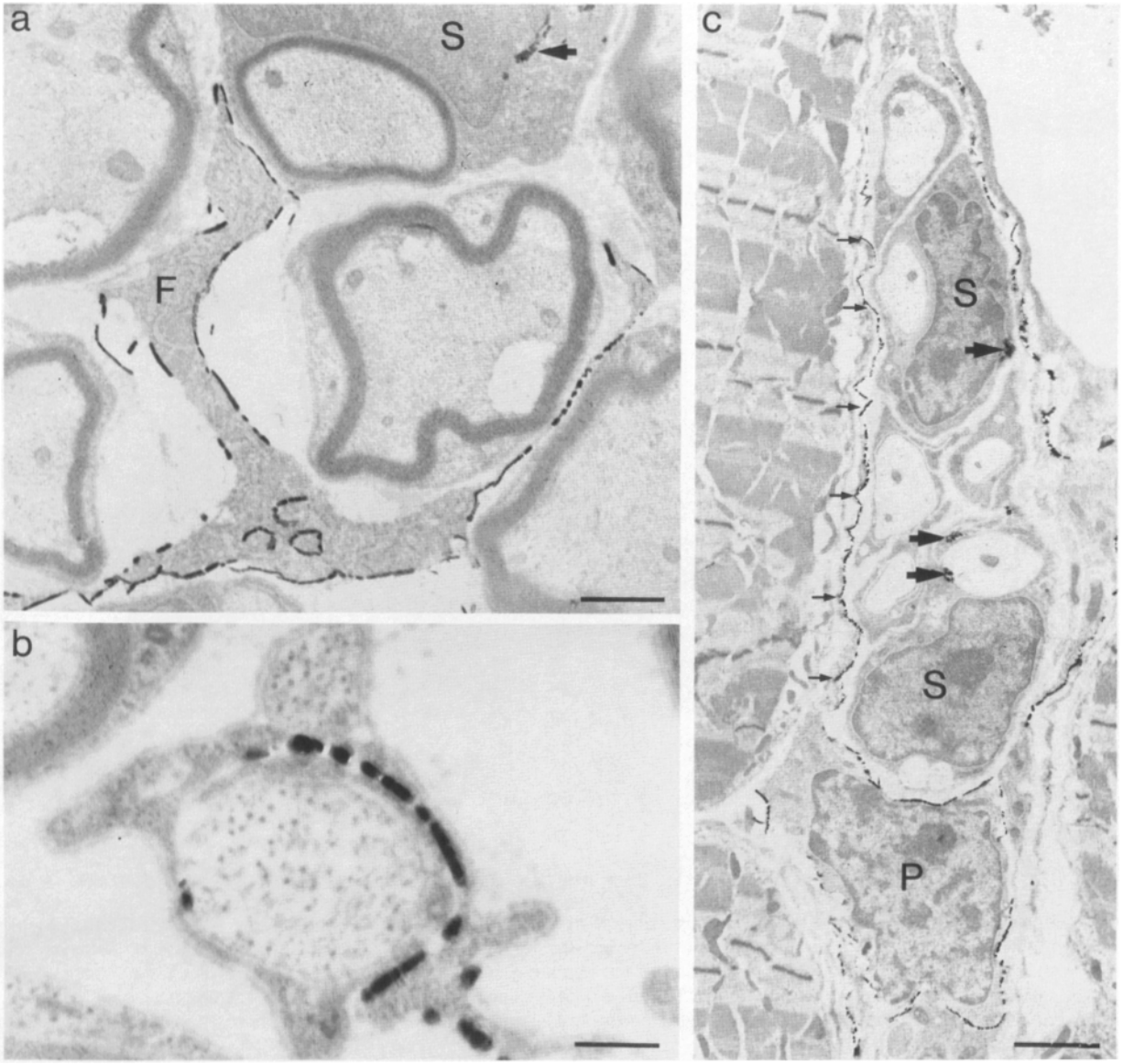

Figure 5. Electron micrographs of intramuscular nerve fascicles in line 42 mice. (*a*) Labeled endoneurial fibroblast (*F*) and Schwann cell (*S*) in a P11 mouse. (*b*) Unmyelinated axon ensheathed by a lacZ-positive Schwann cell in a P14 mouse. (*c*) Perineurial (*P*) and Schwann cells are lacZ positive in a P9 mouse. Deposits of reaction product in Schwann cells are marked by large arrows in *a* and *c*; a labeled perineurial process is indicated by small arrows in *c*. Bars (*a*) 1 μm; (*b*) 0.4 μm; (*c*) 2 μm.

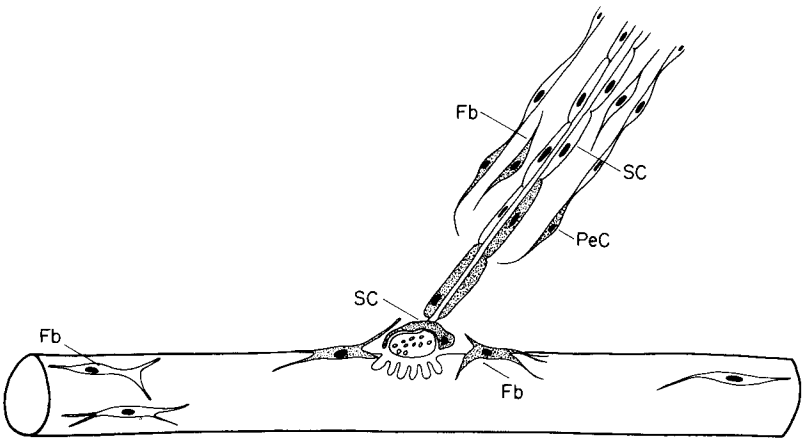

Figure 6. Schematic summary of lacZ expression at neuromuscular junctions of line 42 mice. Perisynaptic fibroblasts (*Fb*) and Schwann cells (*SC*) as well as Schwann cells, perineurial cells (*PeC*), and endoneurial fibroblasts (*Fb*) in the distal part of the motor nerve are lacZ positive (*stippled areas*). Extrasynaptic fibroblasts (*Fb*) and cells in the proximal parts of the motor nerves are lacZ negative (*open areas*).

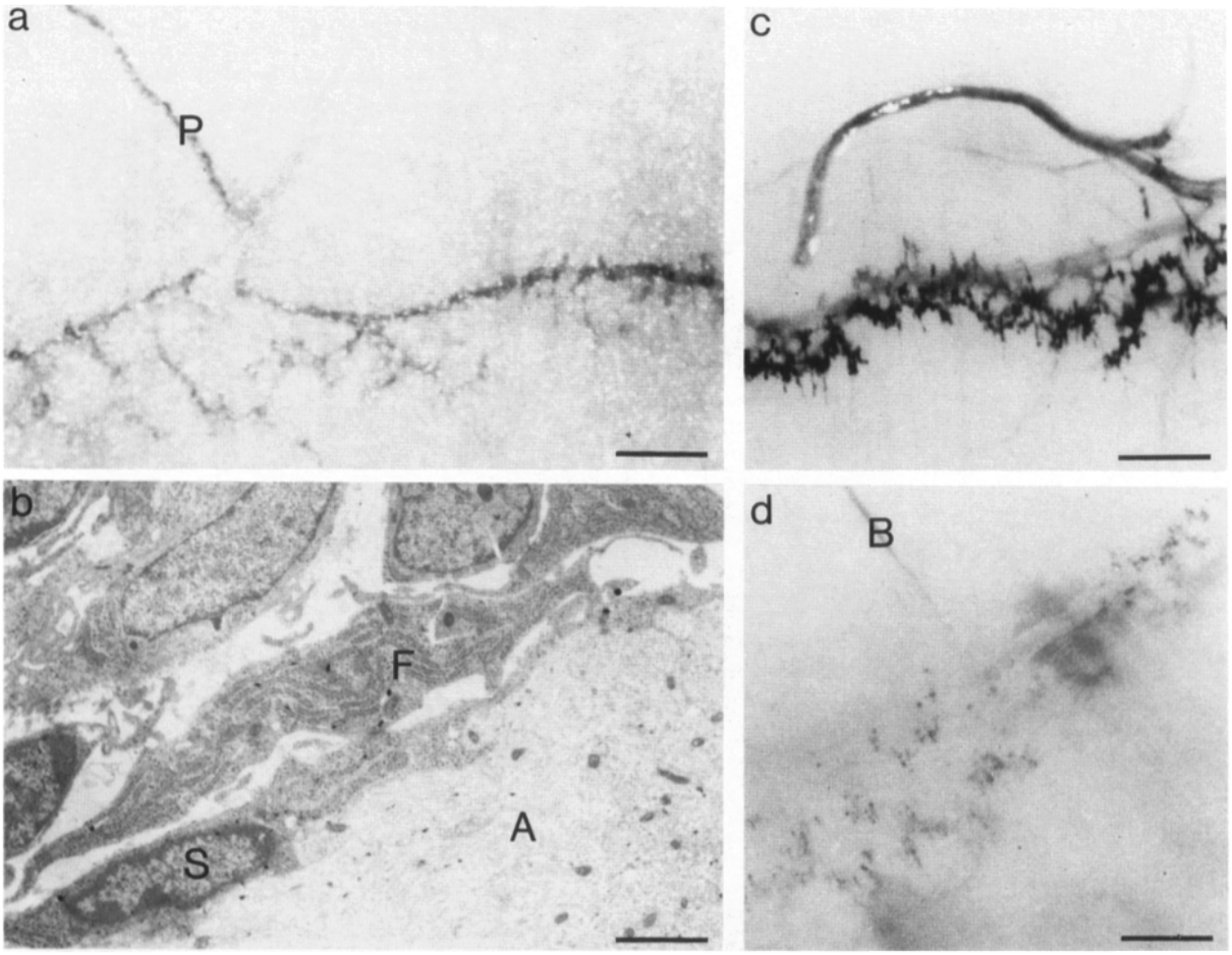

Figure 7. Diaphragms of transgenic mice (line 42) at different stages of development. (*a*) At E15, X-gal staining is confined to the phrenic nerve (*P*) and its branches. (*b*) In an electron micrograph of the same diaphragm, a nerve associated fibroblast (*F*) and a Schwann cell (*S*) are labeled with X-gal. *A*, axon. (*c*) By P3, the distal part of the phrenic nerve including its fine terminal branches at synaptic sites is labeled with X-gal. (*d*) At P9, lacZ expression is limited to cells adjacent to neuromuscular junctions, in the endplate zone. The dark structures in the background are blood vessels (*B*) filled with erythrocytes, not nerves. *a*, *c*, and *d* are photographs of whole mounts. Bars: (*a*) 100 μm; (*b*) 2 μm; (*c* and *d*) 300 μm.

varied with age, we examined muscles from embryos and postnatal mice by light and electron microscopy. At all times, lacZ-positive intramuscular cells were confined to synaptic areas. However, the number and type of lacZ-positive cells in these areas changed with development.

At embryonic day (E)15, the earliest time examined, lacZ-positive cells were already present in intercostal muscles and diaphragm. Nerve terminals were infrequent and rudimentary at this stage, and few Schwann cells or fibroblasts were associated with synapses per se. However, stained cells were present in the intercostal and phrenic nerves and their intramuscular branches (Fig. 7 *a*). Electron microscopy showed that nerve-associated Schwann cells and fibroblasts were both lacZ positive (Fig. 7 *b*).

During the subsequent 2–3 wk, the number of perisynaptic lacZ-positive cells increased. By the first postnatal week, as described above, lacZ-positive fibroblasts and Schwann cells were associated with most synaptic sites (Figs. 3 *c*, 4, and 7 *c*). During the same period, staining of the nerve decreased, first in extramuscular and then in intramuscular (distal) portions. By postnatal days (P)10–12, staining of Schwann cells and perineurial cells in nerves was confined to the distalmost branches (Fig. 7 *d*). Thus, expression of lacZ simultaneously decreased in the nerve and increased in perisynaptic interstitial spaces leading to an apparent gradual redistribution of lacZ-positive cells from within nerves to abutting nerve terminals. We do not know whether the redistribution reflects the movement of individual cells, or a proximo-distal wave of transgene expression.

Finally, between ~P12 and P20, the intensity and apparent number of lacZ-positive perisynaptic cells declined. By 3 wk after birth, lacZ expression was not detectable in any intramuscular cells. Muscles of older mice remained lacZ negative.

lacZ Expression After Denervation

The postnatal decline in lacZ expression by perisynaptic cells is roughly coincident with several steps in the maturation of the postsynaptic apparatus. For example, acetylcholine receptors and N-CAM are initially present throughout the muscle fiber membrane, but are lost from extrasynaptic areas during the first postnatal weeks. Some such processes are

Table II. Effect of Nerve Transection on lacZ Expression in Nerve and Muscle

					lacZ-positive cells		
No.	Muscle	Age at denervation	Age at sacrifice	Time denervated	Perisynaptic	Nerve: proximal stump	Nerve: distal stump
				d			
1	Diaphragm	P7	P9	2	++*		
2	Leg	P7	P13	6	++*	++	++
3	Leg	P7	P13	6	++*	++	++
4	Diaphragm	P8	P10	2	++*		
5	Intercostal	P12	P20	8	−		
6	Intercostal	P13	P26	13	−		
7	Intercostal	P17	P21	4	−		
8	Diaphragm	P19	P25	6	−		+
9	Diaphragm	P19	P25	6	−		++
10	Leg	P21	P26	4	−	+	+
11	Leg	P23	P26	3	−	−	−
12	Leg	P30	P35	5	−	++	++
13	Leg	P30	P35	5	−	++	++
14	Intercostal	P31	P39	8	−		
15	Diaphragm	P31	P35	4	−		++
16	Diaphragm	P35	P43	8	−		++
17	Diaphragm	P36	P38	2	−		−
18	Diaphragm	P36	P40	4	−		++
19	Diaphragm	P36	P46	10	−		−
20	Leg	P38	P43	5	−	++	++
21	Leg	P38	P43	5	−	++	++

* Equivalent to innervated muscles in same animal. Nerve stumps were examined in only a subset of mice. Blank spaces, not determined.

clearly nerve dependent, in that they are prevented by neonatal denervation and/or reversed by denervation of adult muscle (for example see Covault and Sanes, 1985; Schuetze and Role, 1987). In addition, denervation provokes proliferation of the perisynaptic (Connor and McMahan, 1987; Gatchalian et al., 1989) and endoneurial cells (for example see Bradley and Asbury, 1970; Salonen et al., 1988) in which lacZ is expressed. In these contexts, it was interesting to ask whether denervation affected the expression of lacZ by cells in nerve or muscle. Accordingly, we denervated the intercostal, diaphragm, or lower leg muscles of line 42 mice, by cutting the intercostal, phrenic, or sciatic nerve, respectively, then stained both nerve and muscle for lacZ at various times thereafter. Nerves were cut before (P7), during (P12–20), or after (P30–38) the period when interstitial lacZ expression declines, and animals were studied 2–13 d after denervation.

Results of these experiments are summarized in Table II. In short, axotomy did not detectably speed, slow, prevent, or reverse the postnatal loss of lacZ expression by cells in terminal nerve branches or in perisynaptic interstitial areas. On the other hand, nerve damage did evoke a transient appearance of lacZ in both the proximal and distal nerve stump. Between 4 and 8 d after nerve section, the distal end of the proximal stump and the entire distal stump, including its major branches, contained lacZ-positive cells (Fig. 3 *f*). Electron microscopy demonstrated that both Schwann cells (Fig. 8) and endoneurial fibroblasts reexpressed lacZ. In one transgenic animal, expression had declined markedly by 10 d after surgery even though regeneration of axons had been prevented by physical separation of the proximal and distal stumps. In no case were cut nerves of wild-type mice detectably stained (Fig. 3 *g*), indicating that the staining of transgenic nerves was due to lacZ. Thus, nerve damage can upregulate expression of lacZ in nerves of line 42 mice, but does not do so in perisynaptic areas of muscle.

Extramuscular Expression of lacZ

Although expression of lacZ in the muscles of line 42 mice is confined to synaptic areas, there are, as noted above, some extramuscular sites of expression in these mice. Because intramuscular lacZ-positive cells include both fibroblasts and Schwann cells, we asked whether other populations of fibroblasts and/or other neural crest derivatives expressed the transgene. To this end, we screened the major organs of line 42 mice for lacZ expression at selected stages of embryonic and postnatal development. Results of this survey are summarized in Table III, and our main conclusions are as follows. (*a*) Few if any extramuscular fibroblasts expressed detectable levels of lacZ. Heart, lung, and spleen, all of which are rich in fibroblasts, were entirely lacZ negative. Likewise, the fibroblast-laden tendons that attach muscles to bones or ligaments were unstained. In pancreas and liver, a few cells in interstitial compartments were stained with X-gal. However, this pattern of staining was seen in wild type as well as transgenic mice, and thus reflects background staining rather than lacZ expression. Skin was intensely stained at all stages examined, but staining was confined to the epidermal layer; dermal fibroblasts were unstained (Fig. 2 *f*). Thus, in muscle, it appears to be the lacZ-positive perisynaptic fibroblasts rather than the lacZ-negative extrasynaptic fibroblasts that constitute a special population. (*b*) Some neural crest derivatives express lacZ, but many do

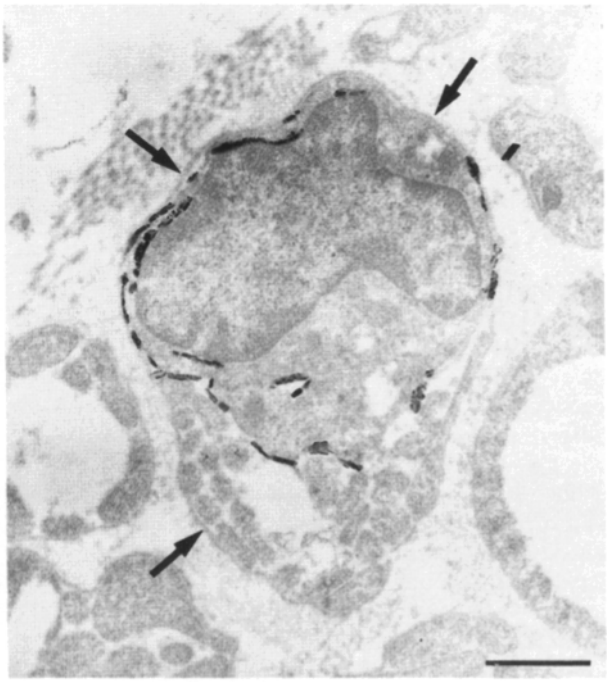

Figure 8. lacZ-positive Schwann cell in the distal stump of a sciatic nerve of a transgenic mouse 6 d after transection. A basal lamina (*arrows*) ensheaths the cell and identifies it as a Schwann cell. Bar, 1 μm.

Table III. Extramuscular Distribution of lacZ-positive Cells in Line 42 mice

	Age				
	E15	PO	P10	P20	P30
Brain					
Olfactory cortex	+	+	+		
Cerebral cortex					
Basal ganglia	+	+	+	+	+
Retina		+	−	−	+
Ganglia					
Sensory	+	+	+	+	+
Sympathetic			+		
Enteric			+		
Adrenal gland	−	−			+
Skin	+	+	+	+	+
Thymus	+	+	+	+	−
Heart	−	−	−	−	−
Lung	−	−	−	−	−
Liver	−	−	−	−	−
Kidney	−	−	−	−	−
Spleen	−	−	−	−	−
Aorta	−	−	−	−	−
Stomach	−	−	−	−	−

Blank spaces, not determined.

not. In particular, neurons in sympathetic, sensory, and enteric ganglia expressed the transgene at all stages examined (e.g., Fig. 2 *e*). On the other hand, chromaffin cells in the adrenal medulla, and crest-derived connective tissue in the heart (LeDouarin, 1982) as well as Schwann cells in extramuscular nerves were lacZ negative. Thus, lacZ does not serve as a general marker of the neural crest lineage in these mice. (*c*) Specific subsets of neurons expressed lacZ in developmentally regulated patterns. In addition to the crest-derived ganglion cells noted above, some classes of neurons in retina, cerebral cortex, and cerebellar cortex were lacZ positive (not shown). No obvious morphological or functional features were common to the lacZ-positive neuronal subsets.

Effect of the Mutation Created by Transgene Insertion

Integration of a transgene into the mouse genome disrupts chromosomal DNA. Because the MHC-regulatory elements in the transgene do not account for the observed pattern of lacZ expression, it seems plausible that transcription of the transgene is influenced by regulatory elements of a nearby endogenous gene (see Discussion). If the insertional mutation disrupts the endogenous gene, then mice homozygous for the transgene might be phenotypically abnormal. The following results suggest that the insertional mutation in line 42 in a recessive lethal.

Among offspring of two transgenic heterozygotes, classical genetic theory predicts that 25% of all pups and 33% of the transgenic pups should be homozygous for the transgene, and thus for the insertional mutation. No offspring of matings between a transgenic and a wild-type mouse will be homozygous for the transgene. During the course of our studies, 330 line 42 mice from 49 litters were screened for lacZ expression by X-gal staining of skin punch biopsies at P6–9. For 29 litters, both parents were transgenic heterozygotes; 20 litters were born to one transgenic and 1 wild-type parent. In litters with two transgenic parents, 34% of the transgenic pups (=24% of all pups) were found dead or were killed because of extreme weakness, at about the time of weaning (P16–23). In contrast, only 4% of pups died before P30 in litters with only one transgenic parent; this figure does not differ significantly from the value for infant mortality of wild-type mice in our colony. Transgenic or wild-type pups of either group that survived until P30 were healthy and showed no signs of abnormal development. The correspondence between observed and predicted values for mortality (Table IV) suggests that the transgene has disrupted an endogenous gene whose expression is inessential for embryonic development but essential for survival past weaning.

We also noticed that the intensity of lacZ staining of skin biopsies was variable among transgenic offspring of two transgenic parents. On the assumption that homozygotes would express more lacZ than heterozygotes, we asked whether mice with more intensely stained skin (putative homozygotes) were more likely to die than mice with less intensely stained skin. All biopsies were from the same dorsal area of the neck, and were classified by intensity of staining (−, +, or ++) before any phenotypic abnormalities were apparent. Out of 50 transgenic pups in the + category, only 4 (8%) died or were killed because of extreme weakness before P30. In the ++ class, however, 43 of 87 pups (49%) were dead before P30. The relationship between staining intensity and mortality was highly significant ($P < 0.001$ by the chi-square test). Although it is apparent that the skin test distinguishes heterozygotes from homozygotes imperfectly, these results support the hypothesis that mice homozygous for the transgene are also homozygous for a recessive lethal mutation.

Initial pathological studies have not provided an explanation of how the insertional mutation exerted its effects. lacZ-positive perisynaptic cells did not differ detectably in number or morphology between heterozygotes and presumed homozygotes. The transgenic pups that died were emaciated and 30–50% smaller than their litter mates. Autopsy revealed pale livers and empty stomachs in the dead presumed homozygotes, whereas the livers of wild-type litter mates were red and their stomachs were filled with milk. Histology of major organs of two dead transgenic pups showed pneumonia in one mouse and meningitis in the other. These findings suggest that death resulted from infections that were secondary to malnutrition.

Table IV. Mortality of Line 42 Mice with One or Two Transgenic Parents

Parents	Pups	Total	Dead before P30	Predicted value for recessive lethal mutation
tg × tg	tg	137	47 (34%)	33%
	wt	64	2 (3%)	0
	total	201	49 (24%)	25%
tg × wt	tg	56	4 (7%)	0
	wt	73	1 (1%)	0
	total	129	5 (4%)	0

tg, transgenic mice of line 42; *wt*, wild type.

Discussion

Dependence of Transgene Expression on Chromosomal Position

Exogenous genes used to produce transgenic animals are frequently constructed by linking regulatory elements from one gene to protein-coding sequences from another gene, the "reporter." Generally, the transgene is transcribed in patterns dictated by its regulatory sequences, and the reporter gene "reports" on the properties (e.g., organ specificity, developmental regulation, or hormone responsiveness) of these sequences (Palmiter and Brinster, 1986; Jaenisch, 1988). In contrast, O'Kane and Gehring (1987) devised a strategy for *Drosophila* in which a transgene reports on the existence and nature of endogenous chromosomal regulatory elements. In this method, fly embryos were injected with a transgene in which the reporter gene *lacZ* was fused to a weak promoter that was unable to drive detectable levels of transcription on its own. Many transgenic *Drosophila* generated from such constructs expressed lacZ in small subsets of cells; the patterns of expression were constant from animal to animal within a line, but varied greatly from line to line. The original interpretation (O'Kane and Gehring, 1987), which has since been substantiated in several cases (for example see Wilson et al., 1989; Bier et al., 1989; Mlodzik et al., 1990) was that transcription of *lacZ* in these flies was directed by enhancer-like elements that were associated with endogenous genes near the transgene's chromosomal site of integration. Thus, a transgene can serve as an "enhancer trap" to report on the expression pattern of an endogenous gene and to facilitate its cloning.

It appears that the MHC-*lacZ* transgene in our mice is behaving more like an enhancer trap than like a conventional transgene. Evidence favoring this interpretation is as follows. (*a*) Small subsets of cells were lacZ positive in eight transgenic lines, whereas endogenous MHC class I genes are expressed in a wide variety of cell types. (*b*) Patterns of expression varied dramatically from line to line, although all lines bore the identical transgene (albeit with varying numbers of copies). (*c*) Patterns of expression were relatively constant from animal to animal within each line, arguing against extragenic or unlinked genetic determinants of expression. (*d*) Little expression was observed in lymphocytes or lymphoid tissues, which express high levels of endogenous MHC class I gene products. (*e*) Considerable expression is observed in brain, which expresses the lowest levels of endogenous MHC class I antigens. (*f*) Similar overall levels of expression are observed in embryos and adults, whereas endogenous MHC expression increases many fold late in development. (*g*) Interferon has no apparent effect on lacZ expression (Fine, 1989), although it is a potent inducer of endogenous MHC class I genes. (See Ozato et al., 1984; Klein, 1986; Morello et al., 1986; Fahrner et al., 1987 for studies of endogenous MHC expression).

Taken together, these results suggest that sequences necessary for the initiation of transcription (i.e., a basal promoter) are active in the transgene, but that sequences necessary for developmentally regulated, tissue-specific, and interferon-inducible expression are inactive or absent. Instead, specific patterns of transgene expression are apparently determined by sequences in the mouse genome near the site of transgene integration. An alternative we cannot exclude is that the patterns of expression observed result from mutations or rearrangements within the transgene itself, which create novel specificities in each integrant. This is unlikely, however, in light of the high frequency with which novel patterns of expression occurred (eight of nine lines). It is striking that the same MHC genomic elements we used here were effective in promoting tissue-appropriate and developmentally regulated expression of another reporter, human growth hormone, in transgenic mice generated by Morello et al. (1986). We have no explanation for the different behavior of the same regulatory sequences in these two contexts.

Although unexpected, our results are consistent with two previous sets of observations on transgenic mice. First, although many mouse lines harboring a transgene with a tissue-specific promoter have shown appropriately tissue-specific expression of the reporter, occasional lines have shown "ectopic" expression that did not reflect the characteristic pattern of the regulatory elements used. The ectopias have generally been attributed to influences of endogenous sequences near the insertion site (Lacy et al., 1983; Kothary et al., 1988), and this has been demonstrated directly in one case: Al-Shawi et al. (1990) recovered the transgene from a mouse that exhibited ectopic expression, and used it to generate a second round of transgenic animals, which proved to express the transgene in the "normal" pattern. Thus, in this case, the possibility that ectopic expression resulted from a mutation in the transgene was definitively excluded. Second, while our work was underway, three groups reported on the production of mice bearing transgenes in which weak promoters were linked to *lacZ* (Allen et al., 1988; Gossler et al., 1989; Bonnerot et al., 1990). In many such mice, lacZ was expressed in restricted subsets of cells that varied from animal to animal. All three groups argued, as do we, that expression was dependent on the transgene's site of integration in the genome.

Whatever their source, enhancer trap transgenic mice can be useful in several respects. (*a*) Expression of lacZ can allow distinctions to be made among cells that cannot be distinguished by conventional markers. (*b*) Conversely, expression of lacZ at distinct sites or different developmental stages may suggest relationships among cells previously thought to be unrelated. (*c*) Regulation of lacZ expression within a given cell type (e.g., during development or following some intervention) can serve as an indicator of changes in gene expression. (*f*) If the *lacZ* gene disrupts an endogenous gene, causing a detectable insertional mutation, it can serve as a "tag" to facilitate cloning of the gene. Our analysis of transgene expression in line 42 illustrates the first three of these uses, and sets the stage for exploiting the fourth.

Selective Expression of lacZ in Perisynaptic Cells

The skeletal neuromuscular junction is the best characterized of all synapses. The structure, function, and development of its pre- and postsynaptic components (the motor nerve terminal of the motoneuron and the endplate of the muscle fiber, respectively) have been studied in detail, and many of their major components have been identified (Salpeter, 1987). Less well studied, however, are several populations of mononucleated cells that are associated with neuromuscular junctions. These include Schwann cells that cap the nerve terminal, perisynaptic fibroblasts that are selec-

tively responsive to denervation, and cells of the distal nerve branches (fibroblasts, Schwann cells, and perineurial cells) that ensheath immediately preterminal portions of the motor axons. The patterns of transgene expression we observed in mice of line 42 reveal new features of these perisynaptic cells.

Our main conclusions are as follows. (*a*) lacZ is selectively expressed in perisynaptic fibroblasts in embryos and young postnatal mice. This result provides the first evidence for molecular differences between these cells and extrasynaptic fibroblasts in normal muscle. (*b*) lacZ is expressed in Schwann cells that cap motor nerve terminals and that occupy distal nerve branches, but not in those of proximal portions of peripheral nerves. Thus, there must be some molecular distinction among Schwann cells within nerves that correlates with their distance from the spinal cord and/or peripheral targets. (*c*) lacZ is expressed in three discrete perisynaptic cell types (Schwann cells, fibroblasts and perineurial cells) and in two different perisynaptic sites (intraneural and interstitial), whereas extrasynaptic fibroblasts and cells in proximal portions of the nerve are lacZ negative. Together, these results suggest that existence of a hitherto unsuspected molecular similarity among perisynaptic cells. (*e*) Expression of lacZ in perisynaptic cells declines as the animals mature. Thus, the transgene is responsive to a developmental change that occurs postnatally. (*f*) lacZ reappears in cells of peripheral nerves following axotomy. This reappearance coincides with a wave of cellular proliferation that is known to follow nerve damage (for example see Bradley and Asbury, 1970; Salonen et al., 1988), and thus may serve as a marker for an activated state of nerve-associated cells. Given that axotomy leads to reexpression of lacZ in both fibroblasts and Schwann cells within the distal nerve stump, one might have expected that perisynaptic fibroblasts would also become lacZ positive after denervation. However, this was not observed.

Of particular interest, in light of our previous work, is the distinction between perisynaptic and extrasynaptic fibroblasts that the transgene reveals. As described in the Introduction, it is known that perisynaptic fibroblasts are selectively affected by denervation: they proliferate more and secrete larger amounts of several adhesive molecules than extrasynaptic fibroblasts (Covault and Sanes, 1985; Connor and McMahan, 1987; Gatchalian et al., 1989). The perisynaptic localization of these responses might reflect the presence of a localized signal (e.g., one derived from degenerating axons) and/or special features of synapse-associated fibroblasts. Our new results do not directly distinguish between these possibilities, nor do they provide direct evidence for or against either of them. However, they do demonstrate that perisynaptic fibroblasts are biochemically distinguishable from extrasynaptic fibroblasts during the course of normal development and before denervation occurs. It is therefore plausible that these differences contribute to the denervation-dependent responses documented previously.

The principal question that arises from these results is: What accounts for the selective expression of lacZ in multiple, discrete populations of perisynaptic cells? One possibility is that perisynaptic cells and their extrasynaptic counterparts arise from separate precursors and become committed to different programs of gene expression early in development. Indeed, lineage relationships have previously been inferred from patterns of transgene expression in endocrine and neuronal cells, although no direct evidence accompanied these speculations (Alpert et al., 1988). In our case, an extreme proposal would be that perisynaptic fibroblasts and at least some Schwann cells arise from a common precursor, whereas extrasynaptic fibroblasts (and possibly other Schwann cells) are derived from separate sources. One argument against this idea is that Schwann cells are known to arise from the neural crest whereas most fibroblasts are mesodermal derivatives. However, at least cranial crest can generate some mesenchymal cells (LeDouarin, 1982) and no studies to date have asked whether any perisynaptic intramuscular cells are crest derived as well.

A second possibility is that the selective perisynaptic expression of lacZ reflects the presence of a localized factor to which several cell types are responsive. For example, developing axons, the postsynaptic apparatus, or even one of the perisynaptic cell populations might release a soluble molecule that alters the metabolism of fibroblasts, Schwann cells, and perineurial cells in a way that leads to lacZ expression. In this model, the gradual decrease in lacZ expression that occurs postnatally might reflect either the loss of the signal or a decline in the cells' responsiveness to it. Similarly, the reexpression of lacZ after axotomy could be an indicator of appearance of the signal (e.g., from degenerating axons) or acquisition of cellular responsiveness (e.g., as intraneural cells reenter the mitotic cycle).

Finally, it is possible that both lineage-derived predispositions and localized environmental signals control expression of lacZ. The presence of histochemically detectable lacZ in diverse, discrete subpopulations of cells in several different organs (Table III) argues that neither lineage nor position alone will be able to explain the patterns observed.

Distinction among these (and other) possibilities will probably require characterization of the genomic elements that control lacZ expression in line 42 mice. Based on other studies of enhancer trap transgenes (discussed above), we suspect that expression of the MHC-*lacZ* transgene is controlled by regulatory elements of an endogenous gene located at or near the transgene's site of insertion. The observation that integration of the transgene has produced a recessive lethal mutation is consistent with this hypothesis, although the phenotype of the mutation has so far given no clue to the function or pattern of expression of the endogenous gene. At best, the temporal and spatial patterns of lacZ expression might faithfully reflect those of the endogenous gene, as has been observed in several cases in *Drosophila* (for example see Wilson et al., 1989). There is as yet insufficient data from mammals to know whether a similarly satisfying result is likely, or whether transgene expression here is more likely to reflect novel specificities that result from, for example, combinations of genomic and transgenic elements (for example see Russo et al., 1988). However, recent successes in the cloning of genes disrupted by insertional mutation (for example see Woychik et al., 1990) encourage the hope that molecular analyses of line 42 could lead to identification of a gene that is important for the development and/or function of cells associated with synapses.

We are grateful to Jeannette Cunningham, Dorothy Dill, and Grace Gray and Rose Hays for assistance, and E. Weiss (Biogen, Cambridge, MA) for providing the MHC gene. S. M. Fine thanks Dennis Loh (Department of Medicine, Washington University, St. Louis, MO) for support and advice during the course of this work.

Production of transgenic mice was supported by a grant from National Institutes of Health (NIH) to C. David. Their analysis was supported by a grant from NIH to J. R. Sanes. J. Weis was supported by fellowships from the Max Kade Foundation and the Deutsche Forschungs-Gemeinschaft. S. M. Fine was supported by an NIH grant to the MSTP program at Washington University.

Received for publication 17 January 1991 and in revised form 8 March 1991.

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