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Investigation of an *hsp68-lacZ* Transgene Inserted at the *dystonia musculorum* Locus and Expressed in the Neural Tube Floor Plate.

by

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A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of Doctor of Philosophy

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Abstract

Experiments involving the random insertion of foreign DNA constructs into the genome have frequently had unanticipated but informative consequences. In the Tg4 line of transgenic mice, an *hsp68-lacZ* transgene caused an insertional mutation of the *dystonia musculorum* (*dt*) locus. In the same line, the transgene gained an ectopic expression pattern in the neural tube floor plate. The co-occurence of these events suggested that the *dt* phenotype of postnatal sensory neuropathy might arise from a primary defect expressed in floor plate cells. To test this hypothesis, the cellular target of the *dt* mutation was determined by analyzing *dt*/*dt*↔+/+ chimeras, in which wild type neurons and their axons were identified by their expression of a human neurofilament trangene. In these chimeras, axonal pathology was confined to the axons of mutant neurons, thus defining an intrinsic neuronal *dt* defect, and ruling out an extrinsic influence of floor plate cells. The expression of β-galactosidase in the Tg4 line was further used to examine, using histochemical staining, the morphology of floor plate cells, which play a role in the guidance of commissural axons. Floor plate cells were found to possess a number of morphological specializations which appeared to establish a high degree of membrane apposition with commissural axons. Unexpectedly, β-galactosidase activity was also detected adjacent to stained floor plate cells in profiles that could not be distinguished from midline axon segments, suggesting that a process of macromolecular transfer may normally operate between floor plate cells and commissural axons.

Résumé

L'insertion au hasard de séquences d'ADN exogène dans le génome entraîne fréquemment des conséquences inattendues mais aussi très informatives. Ainsi dans la lignée de souris transgénique Tg4, une mutation a été causée par l'insertion d'un transgène *hsp68-lacZ* au niveau du locus *dystonia musculorum* (*dt*). Dans la même lignée, le transgène est exprimé de façon ectopique au niveau de la plaque neurale. La concomitance de ces duex phénomènes suggère que le phénotype *dt* de neuropathie sensorielle post-natale puisse résulter d'un défaut primaire des cellules affectés par la mutation *dt*. Afin de vérifier cette hypothèse, nous avons identifié les cellules affectée par la mutation *dt* en analysant des chimères *dt/dt*↔+/+ dont les neurones et leurs axones pouvaient être visualisés grâce à l'expression chez cuex-ci d'un transgène de neurofilament humain. Dans les chimères, l'état pathologique des axones était confiné aux seuls neurones mutants. Ce résultat a démontré que le défaut *dt* était intrinséque aux neurones et a permis d'exclure l'hypothèse de l'influence extrinséque des cellules de la plaque neurale. L'expression de la β-galactosidase dans la lignée transgénique Tg4 a de plus servi à étudier, à l'aide de méthodes de coloration histochimique, la morphologie des cellules de la plaque neurale qui jouent un rôle dans le quidage des axones commissuraux. Ces études ont révélé que les cellules de la plaque neurale possède un certain nombre de spécialisations morphologiques qui semblent former un degré élevé d'apposition membranaire avec les axones commissuraux. Étonnamment, l'activité de la β-galactosidase a aussi été détectée dans les segments axonaux médians, adjacents aux cellules colorées de la plaque neurale, ce qui suggère qu'un transfert de macromolécules puisse s'opére entre les cellules de la plaque neurale et les axones commissuraux.

Preface

The Guidelines Concerning Thesis Preparation issued by the Faculty of Graduate Studies and Research at McGill University reads as follows:

> "The candidate has the option, subject to the approval of their Department, of including as part of the thesis, copies of the text of a paper(s) submitted for publication, or the clearly-duplicated text of a published paper(s), provided that these copies are bound as an integral part of the thesis.
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> In the case of manuscripts co-authored by the candidate and others, the candidate is required to make an explicit statement in the thesis of who contributed to such work and to what extent; supervisors must attest to the accuracy of such claims at the Ph.D. Oral Defense. Since the task of the Examiners is made more difficult in these cases, it is in the candidate's interest to make perfectly clear the responsibilities of the different authors of co-authored papers."

I have chosen to write my thesis according to the above-quoted option with one paper published and one paper submitted for publication. The thesis is organized in five chapters. Chapter I is a general introduction and literature review. Chapters II-III contain the two manuscripts, each with its own abstract, introduction, methods, results, discussion and references. Chapter IV is a general discussion of all results and Chapter V contains the claims to original research.

Publications Arising from the Work of this Thesis

1. Campbell, R. M. and Peterson, A. C. (1992). An intrinsic neuronal defect operates in *dystonia musculorum*: a study of *dt/dt*↔+/+ chimeras. *Neuron* 9, 693-703.

2. Campbell, R. M. and Peterson, A. C. (1992). Development of the floor plate in transgenic mice: β-galactosidase histochemistry reveals cell morphology and evidence for macromolecular transfer to commissural axons. *Development* (submitted).

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Table of Contents

List of Figures and Tables

Chapter II

Chapter III

List of Abbreviations

BSA	bovine serum albumin
CMFDG	chloromethylfluorescein di-β-D-galactopyranoside
CNS	central nervous system
DAB	diaminobenzidine
DNA	deoxyribonucleic acid
DRG	dorsal root ganglion
E	embryonic day
EGF	epidermal growth factor
Fig.	figure
FDG	fluorescein di-β-D-galactopyranoside
GABA	gamma amino butyric acid
HRP	horseradish peroxidase
hsp	heat shock protein
HSV-TK	herpes simplex virus thymidine kinase
IDPN	β-β'-iminodipropionitrile
kD	kilodalton
kg	kilogram
Lys	Lysine
M	molar
mg	milligram
ml	milliliter
μM	micrometer (micron)
mRNA	messenger RNA
N-CAM	nerve cell adhesion molecule
NF-H	neurofilament-heavy protein
NF-L	neurofilament-light protein
NF-M	neurofilament-medium protein
NGF	nerve growth factor
NHS	normal horse serum
PBS	phosphate-buffered saline
PNS	peripheral nervous system
Pro	Proline
RNA	ribonucleic acid
Ser	Serine

Chapter 1 General Introduction

Preface

Trangenes have proven to be effective tools for investigating the regulation of tissue-specific gene expression, and for causing genes to be expressed in ectopic sites (Palmiter and Brinster, 1986). However, because DNA constructs integrate randomly within the genome, such experiments have in addition had unanticipated consequences. First, in a number of cases the genomic insertion of transgenes has resulted in mutation of endogenous genes. Such insertion mutations have revealed the existence of previously unknown genes (Overbeek *et al.*, 1986; McNeish *et al.*, 1988; Singh *et al.*, 1991) and in some cases allowed their cloning and subsequent characterization (Woychik *et al.*, 1985, 1990; Zeller *et al.*, 1989; Maas *et al.*, 1990). Second, foreign DNA constructs have acquired novel tissue specific expression patterns when they have come under the influence of genomic enhancer elements neighbouring their site of insertion (Hamada, 1986a, 1986b). The "enhancer trapping" strategy, and in particular its derivative, "gene trapping" have been used successfully to identify novel genes (O'Kane and Gehring, 1987; Allen *et al.*, 1988; Ghysen and O'Kane, 1989; Gossler *et al.*, 1989; Skarnes *et al.*, 1992).

In an experiment to identify the location of the regulatory elements that direct the normal developmental expression profile of the mouse *hsp68* gene, seven lines of transgenic mice bearing an *hsp68* promoter-*E. coli lacZ* construct were derived (Kothary *et al.*, 1988: Appendix I). In one line, Tg4, this transgene's insertion was accompanied by both the insertional mutation of the *dystonia musculorum* (*dt*) gene and the conferral on the transgene of an ectopic expression pattern in the neural tube floor plate. The co-occurrence of these events raised the possibility that the regulatory elements of the *dt* gene were directly responsible for the transgene's expression pattern, and therefore that the *dt* gene might normally be expressed in, and the *dt* mutant defect localized to, the neural tube floor plate.

2. Insertional mutation of the *dt* locus by an *hsp68-lacZ* transgene.

2.1 The Tg4 transgenic mouse line. Heat shock proteins function in the repair of damage to cellular protein as a result of heat or other stress, which induce their expression (Lindquist, 1986). The related heat shock cognate genes are expressed constitutively, and assist in cellular processes mediating protein folding and protein-protein interaction. In the adult mouse, hsp68 is the major inducible heat shock protein, while the cognate proteins are hsc70 and hsc74 (Pelham, 1986). Unexpectedly, analysis of *hsp68* mRNA expression during development showed this gene to be expressed in some tissues of unstressed embryos (Kothary *et al.*, 1987). *hsp68* mRNA was detected in the placenta beginning on embryonic day (E) 8.5, in the yolk sac beginning on E11.5, and within embryonic tissues from E15.5 onwards. This finding, along with other reports of *hsp68* expression in early embryos (Blensaude *et al.*, 1983), suggested that this protein might play a role during development.

To more precisely define both the promoter regions directing this constitutive expression and the developmental pattern of *hsp68* expression, trangenic mice were produced using a construct comprising the *hsp68* promoter fused to *lac*Z as a reporter gene (Kothary *et al.*, 1989). The promoter fragment that was used consisted of an 800 base pair region that included the *hsp68* TATA box, CAAT box and at least five copies of the heat shock consensus elements, which are palindromic sequences that have been shown to mediate the heat-inducibility of other heat shock genes (Lindquist, 1986).

In seven lines of mice made with the same construct, expression of β-galactosidase could be elicited in cultured tail fibroblasts by subjecting them to heat or sodium arsenite stress, demonstrating that the construct was present and able to be transcribed in all lines. However, in none of these lines was constitutive expression detected in the tissues that had been shown previously to

express *hsp68* mRNA. It was concluded that the promoter region employed in the construct, although capable of conferring inducible expression, did not include the sequences necessary to respond to the *trans*-acting factors that normally regulate constitutive developmental expression of the *hsp68* gene (Kothary *et al.*, 1989).

In one of these lines, the Tg4 line, two unexpected events occurred. First, mice homozygous for the transgene displayed a phenotype of progressive ataxia, which became evident in the first postnatal week. The most likely explanation for this phenotype was that the *hsp68-lacZ* transgene had caused a recessive insertion mutation. The phenotype of affected mice matched that of a previously identified autosomal recessive mutation, *dystonia musculorum* (*dt*), which causes axonal degeneration of peripheral and central sensory neurons (Duchen, 1976). It was subsequently established that the insertion mutation was allelic with *dt* by crossing transgene hemizygotes with *dt* heterozygotes: offspring with the common affected phenotype were produced in the appropriate ratio. The transgene's insertion site was mapped using in situ hybridization methods to a position on chromosome 1 that corresponded to that of the *dt* locus (Kothary *et al.*, 1988).

2.2 Transgenic insertional mutations. The insertional mutation of the *dt* locus was consistent with a number of previous reports of similar insertional mutations, comparison with which suggested a means of characterizing the *dt* gene. Transgenic insertion mutations, which occur in an estimated 15-20% of integration events (Covarrubias *et al.*, 1985), have revealed a number of genes, and in some cases facilitated their molecular characterization. Many insertion mutations cause prenatal lethality (Wagner *et al.*, 1983; Mark *et al.*, 1985; Covarrubias *et al.*, 1985, 1986, 1987; Overbeek *et al.*, 1986; Mahon *et al.*, 1988); another group result in non-lethal developmental abnormalities, particularly in

limb morphogenesis (Overbeek *et al.* 1986; McNeish *et al.*, 1988; Woychik *et al.*, 1985). The *legless* mutation causes truncation of hindlimbs, lack of digits on the forelimbs, defects in anterior brain structures, and craniofacial malformations (McNeish *et al.*, 1988); the presence of a transgene of known sequence allowed this mutation to be chromosomally mapped and may allow the normal gene to be cloned (Singh *et al.*, 1991). Another mutation, *limb deformity*, causes fusion of the distal bones and digits of all four limbs (Woychik *et al.*, 1985). Although allelic to previously identified spontaneous mutations, the presence of a known sequence tag at the gene locus has allowed the gene to be cloned, and the identity and distribution of its mRNA transcripts to be characterized (Zeller *et al.*, 1989; Maas *et al.*, 1990, Woychik *et al.*, 1990). This has suggested a normal role for the gene product in the specification of anteroposterior polarity in the developing limb bud, which is consistent with the mutant phenotype (Zeller *et al.*, 1989). The success of these analyses suggested that, in the case of the *dt* insertional mutation, cloning of the *dt* gene could lead to identification of its product and hence to an understanding of the pathogenesis of the disease (Kothary *et al.*, 1988).

2.3 Transgene expression in the Tg4 line. A more direct approach to the investigation of *dt* pathogenesis was provided by the coincident observation that the transgene was expressed constitutively in the Tg4 line. Unexpectedly, β-galactosidase activity was detected not in a pattern corresponding to that of *hsp68* expression, but rather in a linear pattern underlying the embryo's dorsal midline, in a structure which was later identified as the neural tube floor plate (Kothary *et al.*, 1988). Since the transgene had been shown to be present and inducible by heat shock in all other lines, the pattern of constitutive expression in this line appeared to be a consequence of its particular site of insertion. In other cases, foreign DNA constructs have similarly acquired ectopic expression

patterns as a result of coming under the influence of tissue specific enhancers (Hamada, 1986a, 1986b; O'Kane and Gehring, 1987; Allen *et al.*, 1988; Ghysen and O'Kane, 1989; Gossler *et al.*, 1989). As the insertion of the Tg4 transgene was known to have disrupted the *dt* locus, this raised the possibility that the regulatory elements responsible belonged to the *dt* gene, and therefore that the *dt* gene was normally expressed within the floor plate during development. This prompted a consideration of whether the identified features of *dt* pathology and pathogenesis could be produced by a defect in any of the floor plate's known functions.

3. *dystonia musculorum* : pathology and pathogenesis. The *dt* mutation, first reported by Duchen and others (Duchen *et al.*, 1963, 1964) arose spontaneously in a randomly bred colony of mice. Breeding tests showed *dt* to be transmitted as an autosomal recessive trait, and the *dt* locus was mapped to chromosome 1 (Green, 1981). The mutation subsequently arose spontaneously in several different mouse colonies (Schlager and Dickies, 1967; Duchen, 1976; Messer and Strominger, 1980; Sotelo and Guenet, 1988). The frequency with which the mutation has arisen following administration of mutagenic agents has led to the hypothesis that it is a large gene (Sotelo and Guenet, 1988).

3.1 Clinical features of *dt* Mice afflicted with *dt* exhibit a progressive movement disorder culminating in their premature death. The first symptoms appear between six and ten days of age, at which time mice that are lifted by the tail flex their hindlimbs tightly to the trunk instead of extending them away from the body, as is the normal reflex in mice. In addition, affected mice at this age spontaneously flex their forelimbs to the thorax while pronating the paw (Duchen *et al.*, 1964). Over the next several weeks limbs are held in abnormal positions as if in spasm, or make flailing movements during attempts at

locomotion. Twisting of the trunk is also seen. Symptoms progress to the point where affected mice lie on their bellies with fore- and hind-limbs extended from the body and move only by swimming movements. Affected mice are runted in comparison to their normal litter mates and most die by the time of weaning, although the lifespan varies depending upon the particular *dt* allele carried, as well as upon strain background, and some affected mice survive for up to eight months (Duchen *et al.*, 1964; Duchen, 1976).

3.2 Histopathological features of *dt*. The most prominent feature of *dt* is axonal degeneration of the peripheral and central neurons that carry sensory information. When the mice are born, the number and appearance of axons in dorsal roots and peripheral nerves is normal (Duchen *et al.*, 1964). However, at four to seven days of age, axons in these regions develop fusiform swellings, up to 40 μm in diameter (Janota, 1972; Duchen *et al.*, 1964). Electron microscopy shows swellings in the very youngest animals to be filled with vesicular structures, but within a week they contain tangles of neurofilaments and to a lesser extent degenerate organelles (Janota, 1972; Moss, 1981a). Over the next several weeks myelinated axons begin to be lost, and Schwann cells containing myelin breakdown products are observed (Janota, 1972). By the third or fourth week dorsal roots contain few myelinated fibers, are hypercellular and contain increased collagen. By this time degeneration begins to be seen in unmyelinated fibers (Janota, 1972; Duchen, 1976). Despite the dramatic loss of axons, the perikarya of DRG neurons retain a normal appearance until late in the disease, when they exhibit chromatolysis and eccentric nuclei (Duchen *et al.*, 1964; Duchen, 1976).

In the spinal cord, degeneration is seen in the central projections of DRG neurons, both within dorsal columns, which are smaller than normal, and in the spinal cord grey matter (Duchen *et al.*, 1964; Sotelo and Guenet, 1988).

Degeneration is seen primarily in large calibre myelinated afferents, but also occurs within a subset of small calibre myelinated and unmyelinated fibers which possess acid phosphatase activity and terminate in the superficial laminae of the dorsal horn (Sotelo and Guenet, 1988). Swollen and degenerating fibers are also seen within ventral and ventrolateral white matter, indicating that second order sensory neurons are also involved (Sotelo and Guenet, 1988; Duchen, 1976). Using electron microscopy and silver impregnation stains to detect degenerating fibers, Sotelo and Guenet (1988) found the most severely affected of these neurons to be those in the nucleus of Clarke, which give rise to spinocerebellar fibers. Degenerating axons from these neurons were traced the length of the spinocerebellar tract to their termination as mossy fibers in the cerebellum.

Degeneration is also seen in axons belonging to the sensory ganglia of the mixed sensory nerves, V, VII, IX, and X. The peripheral sensory receptors associated with these and the DRG-derived fibers appear to be normally innervated early in the disease, but become denervated in animals more than three weeks old. In particular, Pacinian corpuscles and muscle spindles lose their innervation, become atrophied, and appear to be lost (Duchen *et al.*, 1964; Duchen, 1976)

In contrast to the degeneration seen in the axons associated with dorsal root ganglia, ventral roots show no evidence of degeneration apart from infrequent axonal swellings (Janota, 1972). However, in affected mice over one month of age, atrophy of bundles of muscle fibers as well as signs of partial motor denervation are seen: whereas the terminal branch of motor axons normally innervates a single muscle fiber, in affected mice these elaborate collateral sprouts to innervate several fibers. This has been interpreted as an

attempt by surviving axons to re-innervate denervated fibers (Duchen *et al.*, 1964).

3.3 Theories of *dt* pathogenesis. Based on the observations that axonal swellings contained accumulations of neurofilaments and other organelles, Janota (1972) and Hanker and Peach (1976) proposed that an early event in *dt* pathogenesis was a disturbance of axonal flow. The sparing of DRG neuronal perikarya until late in the disease was interpreted as further evidence for a primary axonal lesion. Janota (1972) also noted that, although abnormalities in myelination exist in *dt*, they occurred late in the disease and appeared to represent a response to a primary degeneration of axons and not the primary lesion. In particular, he found that many swellings in young mice occurred in groups of axons that had not yet been myelinated, and that swellings in older mice were covered by a layer of myelin that appeared to be stretched but otherwise normal.

In contrast, Moss proposed that a primary defect in myelination underlay the axonal lesions in *dt* (Moss, 1980, 1981a, 1981b). In teased sciatic nerve segments from affected *dt* mice, he found several abnormalities in the pattern of myelination, including a lower-than-normal number of Schmidt-Lanterman clefts, a diminished average internodal length, and elongated nodal gaps. Moss interpreted this to be indication of a primary defect causing segmental demyelination of axons (Moss, 1980). He reported that in sciatic nerves widespread segmental demyelination was an early feature of *dt*, and that demyelinated areas of large axons were not confined to regions containing swellings. These abnormalities were reported to be more common, in sciatic nerves, than were axonal swellings or degeneration, and, since in phrenic nerves the myelination abnormalities appeared to precede axonal lesions, he proposed that they were the primary event in the disease (Moss, 1981a).

To test this hypothesis, Moss performed sciatic nerve transplant experiments between normal mice and affected *dt/dt* mice. When nerve segments from *dt/dt* affected mice were transplanted into the sciatic nerves of normal mice, they developed axonal swellings as well as indications of the previously observed segmental demyelination. Since the normal host axons regenerating through these grafts would be expected to be myelinated by *dt/dt* Schwann cells, these results were interpreted to mean that both the axonal swellings and the myelination abnormalities were secondary to a primary *dt/dt* defect manifested within Schwann cells. In contrast, segments of normal nerves transplanted into *dt/dt* mice did not develop abnormalities, although the condition of affected host mice and their limited survival time made interpretation of these experiments difficult.

In contrast, Sotelo and Guenet (1988) hypothesized, on the basis of the distribution of affected neurons, that *dt* caused a primary defect within peripheral sensory receptors. This was in turn proposed to cause retrograde degeneration of peripheral sensory neurons and, via a transneuronal mechanism, degeneration of second order neurons in the spinal cord. To support this hypothesis the authors cited their observation that the second order neurons within the spinal cord that showed the most severe degeneration, those at the origin of the spinocerebellar tract, were those that received the highest concentration of degenerating primary afferents. However, as they noted, an inconsistency with this theory was that the cuneate and gracile nuclei, which received input from the severely affected dorsal column axons, showed no evidence of degeneration (Sotelo and Guenet, 1988). In addition, the localized axonal nature of the peripheral lesions, in which the innervation of peripheral sensory receptors as well as the apparent sparing of DRG perikarya were

maintained until well after significant pathology was seen in the CNS (Janota, 1972), would appear also to argue against this sequence of events.

3.4 Other sites of pathology in *dt*. Other sites of involvement have been sought to explain the motor symptoms of *dt*, in particular the ataxia, spasm, and involuntary movements. Messer and Strominger (1980) found that in affected *dt/dt* mice changes developed over the first month of life in the magnocellular or coarse neurons of the red nucleus, which give rise to the rubrospinal tract. These neurons had abnormal contour, eccentric nuclei, and central chromatolysis. These abnormalities often occured only on one side of an affected mouse's brain, and were variable in magnitude although this did not correlate with the severity of the symptoms. They were also variable between the Albany allele of *dt* (dt^{Alb}) and the allele that arose in the Jackson Laboratories (dt^{J}), which showed similar clinical symptoms. However, in contrast to this variability in histological appearance, a test for the functional capacity of these rubral neurons, their ability to synthesize acetylcholinesterase, showed these neurons to be equally impaired in mice affected by either allele (Stanley *et al.*, 1983).

Abnormalities in neurotransmitter levels have also been observed. Messer and Gordon (1979) found a reduction in the GABA biosynthetic capacity of *dt* mutant striatum and substantia nigra. Riker *et al.* (1981) found an increased level of noradrenaline metabolic activity in the cerebellum of mutant mice, but normal amounts of noradrenaline in the cerebellum and no morphological or biochemical changes in Purkinje cells in mutant mice. They hypothesized that the elevated level of noradrenaline metabolism was a response to an increase in tonus of noradrenergic inputs from the locus coeruleus to the cerebellum, caused by a decrease in the spinal ascending input. This theory is consistent with the subsequently observed degeneration within the spinocerebellar tract (Sotelo and Guenet, 1988).

Ebendal and Lundin (1984) tested the hypothesis that a reduced level of NGF was responsible for the axonopathy of *dt*. Although a low level of NGF was found in *dt/dt* sciatic nerves, the level increased when these nerve segments were explanted into tissue culture. The authors concluded that the general condition of the mice and disturbances in development caused the *in vivo* deficiency, rather than that being the primary cause of the disorder.

4. The neural tube floor plate. The floor plate is a column of cells, triangular in cross section, that connects the right and left sides of the neural tube at the ventral midline. It was recognized as a distinct structure by His, who divided the neural tube into four longitudinal zones: the roof plate, floor plate, and lateral plates—these in turn divided into the alar and basal plates, corresponding to the presumptive sensory and motor divisions of the spinal cord, respectively (Kingsbury, 1920). Whereas the lateral plates were observed to be the zones in which neurons differentiate from dividing precursor cells, the roof and floor plates were noted to lack neuroblasts but to be composed instead of "indifferent (ependymal) cells alone" (Kingsbury, 1920). By these morphological criteria, Kingsbury determined that the ventral midline floor plate extended throughout the spinal neural tube and rhombencephalon but terminated abruptly at the fovea isthmi, a pit in the ventricular floor marking the midbrain's caudal limit. Rostral to this point, dividing and differentiating neurons, arranged into ventricular and marginal layers, as in the lateral plates, were present in the ventral midline region (Kingsbury, 1920, 1930).

The function of the floor plate remained undefined until the latter half of the 1980's, when a series of studies revealed this structure to exert a variety of influences on neural tube development. These include participating in the inductive events that determine the dorsal or ventral identity of lateral plate

neurons, as well as exerting both a long range and local influence on the guidance of the axons that form the spinal cord's ventral commissure (Jessell *et al.*, 1989).

4.1 Role of the floor plate in polarization of the neural tube. The floor plate participates in a chain of inductive events that affects the differentiation of cells throughout the neural tube. Before the neural tube is formed by folding of the neural plate during neurulation, the floor plate constitutes a morphologically indistinguishable midline portion of the neural plate overlying the notochord, termed the "notoplate" (Jessell *et al.*, 1989). When individual progenitor cells in pre-gastrulation embryos are labelled in fate-mapping experiments, those injections that label the notochord tend also to label the floor plate, but not more lateral regions of the neural tube (Gimlich, 1986; Dale and Slack, 1987; Kimmel and Warga, 1988). This suggests that the floor plate shares an embryological origin closer to that of the notochord than the adjoining neural tube cells. Analysis of Xenopus embryos at the time of gastrulation shows that the cells destined to become the floor plate lie adjacent to, but segregated from prospective notochordal cells in the dorsal lip of the blastopore (Keller, 1985; Keller *et al.*, 1985). Whereas the cells forming the notochord involute and extend dorsally during gastrulation, floor plate cells extend dorsally without involuting to intercalate among prospective neuroepithelial cells (Keller, 1985). Similarly, in the chick embryo, floor plate cells appear to have an origin distinct from that of other neuroepithelial cells: grafts of quail epiblast tissue placed immediately anterior to Hensen's node in chick embryos contribute to the floor plate along its entire length, whereas those placed lateral to Hensen's node contribute to the lateral wall of the neural tube (Schoenwolf *et al.*, 1989).

Although prospective floor plate cells appear to be set apart before gastrulation, the differentiation and maintenance of the floor plate once the

neural tube has formed depends on a continuing inductive influence by the notochord. Where the notochord is absent due to experimental manipulation (Kitchin, 1949; Youn and Malacinski, 1981; Clarke *et al.*, 1991) or mutation (Dunn *et al.*, 1940; Theiler, 1959; Bovolenta and Dodd, 1991), the floor plate fails to develop. Conversely, in cases where a developmental malformation (*duplicitas anterior*) causes duplication of the notochord in rostral regions of the neural tube, duplicate floor plates overlie each notochord, or, where they approach each other, a single, broad floor plate develops (Watterson *et al.*, 1955; Limborgh, 1956). Similarly, transplant of a notochord fragment to an ectopic location adjacent to the neural tube leads to the formation of an ectopic floor plate (van Straaten *et al.*, 1985; 1988). Such ectopic floor plates express characteristic cell surface epitopes (Yamada *et al.*, 1991) and possess floor plate-specific biological activity (Placzek *et al.*, 1990).

Grafting of the floor plate alone to the lateral region of the neural tube can produce an additional, ectopic floor plate and motor neurons (Yamada *et al.*, 1991). This is consistent with findings obtained from study of the zebrafish *cyclops* (*cyc*) mutant, in which the floor plate fails to develop although the notochord is present. When wild type cells are transplanted into mutant embryos, mutant neuroepithelial cells fail to respond to wild type notochord cells but participate in forming a floor plate when intermixed with wild type floor plate cells. This suggests that induction of the floor plate is a two-step process, in which the floor plate, once induced by the notochord, produces a homeogenetic signal to maintain itself as a cohesive differentiated structure (Hatta *et al.*, 1991). According to this hypothesis, the *cyc* neuroepithelial cells would be insensitive to the inductive signal from the notochord, but sensitive to that from other floor plate cells.

While the differentiation of the floor plate is dependent on an inductive influence from the notochord, in turn, the notochord and floor plate together appear to exert an inductive influence on the differentiation of neurons in more lateral regions of the neural tube (Glover, 1991; Hirano *et al.*, 1991; Lumsden, 1991). When the notochord is absent, not only does the floor plate fail to develop but motor neurons fail to differentiate, with the result that, as determined by the expression of neuronal epitopes, the entire neural tube acquires a dorsal identity (Placzek *et al.*, 1990c; Clarke *et al.*, 1991; Yamada *et al.*, 1991). Grafting of the notochord to ectopic regions of the neural tube suggests that it is able to induce the differentiation of the floor plate and motor neurons independently. When placed less than 30 microns from the neural tube, both a floor plate and motor neurons are induced, whereas at a distance of from 30 to 80 microns (or closer if grafted at later stages), only motor neurons are induced (Glover, 1991; Lumsden, 1991). As in the normal embryo, the ectopic floor plate and motor neurons are separated by an intervening region, termed region X by Yamada *et al.*(1991). This does not appear to be a null region, as in the rhombencephalon it contains a specific population of serotonin-expressing neurons, which are also induced when the notochord is transplanted to ectopic rhombencephalic locations (Yamada *et al.*, 1991).

4.2 Chemotropic influence of the floor plate Commissural axons can be distinguished by their expression of the axon-specific glycoprotein TAG-1 (Dodd *et al.*, 1988). Arising from neurons in the dorsal neural tube, they initially project in the ventral direction. As they reach the developing motor neuron pool midway down the neural tube they alter their trajectory to project ventromedially toward the floor plate. During this ipsilateral projection, commissural axons do not fasciculate, but neither do they appear to follow any other physical guidance cues, such as aligned extracellular space or cellular

processes (Holley and Silver, 1987). In the absence of a physical substrate for guidance, Tessier-Lavigne *et al.* (1988) investigated the possibility that this trajectory change represented a long-range attraction to a floor plate derived chemotropic factor. Explants of the dorsal neural tube, containing commissural neurons, were co-incubated in a three-dimensional collagen gel matrix with either floor plate explants or control explants of the ventral neural tube lacking the floor plate. The presence of floor plate tissue not only increased the number of axons that projected from the dorsal neural tube explant into the collagen gel, but these axons also projected preferentially toward the explanted floor plate. When the floor plate explant was placed in such a way that it was not aligned with the direction in which commissural axons would have projected *in vivo*, these axons re-oriented their trajectory within the explant to grow directly toward the explanted floor plate. The effect was specific for commissural axons, identified by their expression of TAG-1. It did not affect the survival of commissural neurons or the rate at which they extended neurites within the explant, which were identical in the presence or absence of the floor plate explants, but rather the extension of these neurites into the collagen gel and the direction these assumed. A similar effect on the ability of commissural axons to extend into the collagen gel was observed when dorsal neural tube explants were cultured in media conditioned by floor plate explants, suggesting that a diffusible factor was responsible, although, as would be expected in the absence of a gradient of this factor, neurite extension showed no preferential direction (Placzek *et al.*, 1990a).

The effect of the floor plate explant in reorienting axons operated within the neuroepithelium over a distance of approximately 250 microns, indicating that it would be theoretically capable of acting over the 100-150 micron distances at which commissural axons first re-orient to grow toward the floor plate *in vivo*

(Placzek *et al.*, 1990a). In the chick embryo, commissural axons were observed to adjust their trajectory to project toward transplanted floor plate tissue that was placed adjacent to the lateral neural tube (Placzek *et al.*, 1990b). A similar re-orientation of commissural axons was seen when a segment of neural tube was rotated dorso-ventrally by 180°, such that the floor plate lay in the position normally occupied by the roof plate (Yaginuma and Oppenheim, 1991). In this case, commissural axons from adjacent, unrotated regions of the neural tube adopted abnormal trajectories which caused them to project toward the ectopically positioned floor plate. Together, these experiments suggested that the hypothesized chemotropic activity was not an artefact of the culture environment but was cabable of acting during the normal projection of commissural axons *in vivo* (Tessier-Lavigne and Placzek, 1991).

4.3 Local role of floor plate in commissural axon guidance Where the floor plate is absent, as a result of either a mutation or experimental perturbation, the trajectories of axons in the region of the ventral commissure are disrupted, suggesting an additional role for the floor plate in local axon guidance in this region. The *Danforth's short tail* (*Sd*) mutation results in the failure of development of the notochord, and thus the floor plate, in caudal regions of the neural tube. In these regions of *Sd* mutant embryos, commissural axons reach the ventral midline by travelling an abnormal circumferential route, but, once there, either fail to cross the midline, exiting the neural tube, or cross the midline but fail to turn rostrally. Similarly, in zebrafish embryos lacking the floor plate as a consequence of the *cyclops* mutation, some axons follow their normal commissural ascending pathway but others either turn rostrally before reaching the floor plate, or turn rostrally at a shallower angle than normal (Bernhardt *et al.*, 1992). Finally, in Xenopus embryos lacking the floor plate following U.V. exposure, commissural axons cross the midline obliquely, rather than

perpendicularly, to the neural tube's longitudinal axis. In addition, some axons turn caudally rather than rostrally after crossing the midline (Clarke *et al.* 1991). Evidence from all three species therefore implicates the floor plate in providing a local guidance influence to commissural axons at the ventral midline.

5. Possible participation of the floor plate in *dt* pathogenesis. A comparison of the influences of the floor plate on neural tube development with the histopathological features of *dt* suggests two ways in which a mutant defect expressed in this structure could lead to the pathological features of the disease. In each case, the defect would influence the second order sensory neurons in the developing neural tube. First, a defect in the inductive influence of the floor plate on neural tube cell differentiation could lead to abnormal differentiation of these neurons. Since both sensory and motor divisions of the spinal cord appear to be intact in neonatal *dt* mice, this defect would have to be more subtle than merely the absence of a single factor solely responsible for the effect. Second, failure of floor plate cells to exert long or short range guidance signals for commissural axons could lead to their misrouting. In each case, failure of these second order neurons to establish connections with their proper targets could lead to their degeneration, and in turn lead to the degeneration of peripheral sensory neurons projecting on them.

If this hypothesis were true, axonal degeneration in *dt* would not result from an intrinsic action of the *dt* mutant defect within affected neurons, but would instead arise secondarily in response to expression of the mutant defect by another cell type. A direct means of evaluating this hypothesis was available: the analysis of aggregation chimeras composed of both *dt*/*dt* and wild-type cells.

6. Use of chimeric mice to localize mutant defects affecting the nervous system

The nervous system is characterized by an exceptionally high degree of interdependence among its component cell types, both during its development and its functioning in the mature animal. As evidenced by the variety of pathogenetic hypotheses proposed for *dt*, this compiicates the analysis of mutants affecting the nervous system: mutation of a gene expressed in one cell type may lead to a more histologically apparent lesion in one or more of the other cell types with which it interacts. Since the extensive catalog of nervous system mutants in the mouse provides a rich opportunity for the investigation of both the developing and mature nervous system, numerous methods have been applied to the analysis of these mutants. One of the most powerful is the analysis of chimeras constructed by aggregating mutant and normal embryos (Tarkowski, 1961; Mintz, 1962; Mintz, 1965). In such chimeras, the consequences of a cell's having mutant vs normal genotype can be directly compared. If only mutant cells display pathological features, this indicates that the mutation acts intrinsically within that cell type. Conversely, the pathological involvement of both mutant and wild type cells would indicate an extrinsic influence of the mutant on that cell type, acting via a primary defect within another cell with which it interacts.

The cerebellum is affected by many of the identified mouse mutants, and has thus been the focus of numerous chimera analyses. One of the first mutants examined was *Purkinje cell degeneration* (*pcd*), an autosomal recessive mutation causing the postnatal loss of almost all Purkinje cells (Mullen *et al.*, 1976). Mullen (1977) constructed *pcd*/*pcd*↔+/+ chimeras, in which mutant cells could be identified histochemically by the allele of the gene coding for β-glucuronidase (*Gus*) they carried. In these chimeras, only wild type Purkinje cells survived,

indicating that *pcd* mutation acts intrinsically within Purkinje cells to cause their death.

Mice affected by the autosomal recessive mutation *staggerer* (*sg*) develop ataxia and tremor by the second postnatal week. In the cerebellum, which is smaller than normal, Purkinje cells lie in ectopic locations; their dendritic arbours are dysplastic, with few spines, and Purkinje cell-parallel fiber synapses are absent. The granule cells, from which parallel fibers arise, eventually degenerate (Caviness and Rakic, 1978; Herrup and Mullen, 1979). The synaptic relationship of the two affected cell types led to hypotheses that a primary degeneration of Purkinje cells led to secondary degeneration of granule cells (Sotelo and Changeux, 1974; Landis and Sidman, 1978), or, alternatively that the defect is pleiotropic and affects both cell types via a more basic defect (Yoon, 1976). In +/+↔*sg/sg* chimeras, cerebella were of intermediate size between mutant and normal, indicating that the defect was expressed in chimeras. In these cerebella, all Purkinje cells of normal size and in the normal location were of wild type genotype, while small, abnormally placed Purkinje cells, which were also present, were all of *sg/sg* genotype. This correlation of genotype with phenotype indicated that the mutation acted intrinsically within Purkinje cells to cause the phenotype. The inability of the β-glucuronidase marker to reliably distinuish granule cell genotype did not permit evaluation of the intrinsic or extrinsic nature of the mutant defect's action in that cell type (Herrup and Mullen, 1979).

The cerebellum of mice homozygous for the *weaver* (*wv*) mutation is reduced in size, as a result of the absence of most granule cells. In addition, there is a reduction in the number of both Purkinje cells and Bergmann glial cells, which have abnormal morphology and are found in ectopic locations (Goldowitz and Mullen, 1982b). In *wv*/+ heterozygotes, the phenotype is attenuated, with some granule cells surviving but in inappropriately superficial locations, with

corresponding ectopia of Purkinje and Bergmann glial cells (Caviness and Rakic, 1978; Goldowitz, 1989a). Developing granule cells migrate from the external to the internal granular layer on the radial processes of Bergmann glia (Rakic, 1971), leading to several theories of *weaver* pathogenesis, including primary defects in Bergmann glial cells which impede granule cell migration (Rakic and Sidman, 1973a, 1973b, 1973c), and defects in the granule cells themselves that impair their ability to migrate (Sotelo and Changeux, 1974). In *wv*/+↔+/+ chimeras (Goldowitz and Mullen, 1982b), only *wv*/+ but not wild type granule cells were found in ectopic positions; in *wv*/*wv*↔+/+ chimeras (Goldowitz, 1989a), some granule cells successfully migrated to the internal granular layer, and all were of wild-type genotype. This indicated that both granule cell ectopia and granule cell death are due to an intrinsically acting *wv* defect. By similar comparison of genotype and phenotype in these chimeras, Purkinje cell death was found to result from the intrinsic action of the *weaver* mutation (Smeyne and Goldowitz, 1990), while Purkinje cell ectopia was secondary to an extrinsic defect, as this property was exhibited by both mutant and wild type cells (Goldowitz and Mullen, 1982b; Smeyne and Goldowitz, 1990).

The use of chimeras has not been limited to the central nervous system. Peterson and Bray (1984) showed that abnormalities in myelin thickness in the peripheral nerves of shiverer mice resulted from an intrinsic defect within Schwann cells, while Peterson (1985) showed that ensheathment abnormalities by Schwann cells in dystrophic mice conversely required expression of the mutant defect within a non-Schwann cell type.

6.1 Neuronal genotype markers The analysis of chimeras depends on a comparison of cellular phenotype and genotype. To allow the genotype of origin of particular cells to be distinguished, chimeras have generally been constructed such that one component embryo carries a genetically determined characteristic

that can be recognized at the cellular level in the histological preparations that permit a cell's mutant or normal phenotype to be recognized. To allow a simultaneous and accurate comparison of genotype and phenotype to be made, the ideal marker would be recognizable in all of the cells derived from one component but not present in any cells of the other component. It would also be recognizable whether the cell possesses mutant or normal phenotype and its presence should not influence expression of the mutant phenotype.

The genotype markers that have been employed previously fulfill these criteria to varying degrees. One frequently used marker is the β-glucuronidase enzyme, encoded by the *Gus* gene. Different alleles of the *Gus* gene result in either high or low levels of β-glucuronidase acitivity, which can be distinguished by histochemical staining *in situ*. Since different mouse strains are homozygous for one allele or the other, constructing chimeras with wild type mice of a strain carrying the opposite *Gus* allele to that carried by the mutant strain under study allows the genotype of individual cells to be distinguished (Herrup and Mullen, 1981; Goldowitz and Mullen, 1982b; Smeyne and Goldowitz, 1990). However, the gene is not expressed in all cell types: therefore, while it can be used for the analysis of Purkinje cells, it can not distinguish the genotype of granule cells (Goldowitz and Mullen, 1982b).

Another marker is the *ichthyosis* (*ic*) mutant. A proportion of cells carrying this gene can be distinguished by the centralized clumping of heterochromatin in their nuclei (Goldowitz and Mullen, 1982a; 1982b; Goldowitz, 1989a). However, this system has several limitations. It requires a cell-by-cell analysis of the area of centrally clumped heterochromatin vs nuclear size. Furthermore, the ranges of this calculated value in *ic* vs non *ic* mice overlap, so that populations must be analyzed to determine whether one genotype is overrepresented in the class of cells having mutant or normal phenotype. Analysis of chimeras is thus tedious,

as the genotype of an individual cell can not be known with certainty on the basis of its nuclear characteristics (Goldowitz and Mullen, 1982b).

A more flexible marking system takes advantage of the different satellite DNA sequences present in the *Mus musculus* vs *Mus caroli* mouse species. These can be detected by hybridization of labelled DNA probes to tissue sections using *in situ* methods (Rossant *et al.*, 1983; Goldowitz, 1989a, 1989b).

6.2 Development of a transgene-based axonal marker Because all of the above markers label only cell bodies, they can not be used for the chimera analysis of diseases such as *dt*, in which the pathological phenotype can be reliably detected only in axons. As a solution to this problem, a line of mice bearing a transgene encoding the human neurofilament light (hNF-L) protein was derived (Julien *et al.*, 1987). Neurofilaments, the class of intermediate filaments that participate in the formation of the neuronal cytoskeleton, are heteropolymers composed of three subunit proteins, with apparent molecular weights of 68 kiloDaltons (kD) (NF-L), 145 kD (NF-M), and 200 kD (NF-H) (Hoffman and Lasek, 1975; Liem *et al.*, 1978). Each of the subunit proteins has a central 30 kD alpha helical rod domain, which is common to all intermediate filaments. In addition, each contains a 10 kD amino terminal 'head' domain and a carboxy terminal 'tail' domain of variable size, from 20 to 150 kD, which is responsible for the size variability of the subunit proteins as a whole (Geisler *et al.*, 1984, 1985; Julien *et al.*, 1985, 1986; Lewis and Cowan, 1985). The rod domains of all the neurofilament proteins are able to co-assemble to form 10nm-diameter filaments via the formation of coiled coil structures (Geisler and Weber, 1981; Moon *et al.*, 1981; Zachroff *et al.*, 1982; Gardner, *et al.*, 1984; Minami *et al.*, 1984). *In vivo*, the core of mature neurofilaments consists principally of the assembled rod domains of NF-L, NF-M, and NF-H. The currently accepted model for neurofilament structure is one in which the tail domains of NF-M and NF-H

project radially from this core as sidearms, which interact with the the sidearms of neighbouring neurofilaments to form cross bridges, and may also mediate interaction of neurofilaments with other organelles (Willard and Simon, 1981; Liem and Hutchinson, 1982; Sharp *et al.*, 1982; Hirokawa *et al.*, 1984; Hisanaga and Hirokawa, 1988). Neurofilaments are assembled in the neuronal cell body (Morris and Lasek, 1982) before entering the axon, where they form the major component of slow axoplasmic transport (Hoffman and Lasek, 1975).

The *hNF-L* transgene was found to be expressed widely in the nervous system, and the hNF-L protein appeared to be incorporated into the neurofilament cytoskeleton both in cell bodies and their axonal or dendritic processes (Julien *et al.*, 1987). Most importantly, the neurons and axons of the trangenic line can be selectively stained with an antibody that recognizes human, but not mouse, NF-L. This allows the specific detection of transgenic neurons and their processes in tissue sections (Julien *et al.*, 1987; Vidal-Sanz *et al.*, 1991). These characteristics met the requirements for the chimera based analysis of *dt*, since they allowed the comparison of neuronal genotype and axonal phenotype in histological sections. Therefore, the availability of this marker made feasible the analysis of *dt*/*dt*↔+/+ chimeras described here.

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Chapter II — An Intrinsic Neuronal Defect Operates in *dystonia musculorum*: A Study of *dt*/*dt*↔+/+ Chimeras.

Preface

Although the *dt* mutant was identified in 1963, numerous histopathological and transplant studies led to no consensus as to the pathogenesis of the disease, including the cell type or types primarily affected by the mutant defect. The co-occurrence, in the Tg4 line, of *dt* insertional mutation and ectopic transgene expression in the neural tube floor plate raised the possibility that *dt* pathogenesis proceeded from the expression of a primary mutant defect in floor plate cells (Kothary *et al.*, 1988: Appendix I). A means of testing this hypothesis, the analysis of mutant↔wild type chimeras, had been previously used to identify the primary cellular targets of a number of other neurological mutants. To apply this method to *dt*, whose pathological features are most easily recognized in axons, required a means of distinguishing the axons of wild type neurons from those of mutant neurons. For this purpose, a mouse line expressing an *hNF-L* transgene was included as the wild type component of chimeras. Although an antibody, DP-5, that specifically recognizes the hNF-L protein had been identified at the time the study was undertaken, the staining it provided using the existing immunocytochemical protocol was neither intense nor consistent enough to allow the genotype of individual axons in sections of chimera tissue to be distinguished with the certainty required for chimera-based analysis. Therefore, to improve hNF-L immunolabelling, a number of fixatives were evaluated; of these, periodate-lysine-paraformaldehyde was found to provide the most intense staining. However, as staining remained inconsistent, further modifications to the protocol were required. It was determined that axons could be most reliably and intensely stained by incubating floating tissue sections in immunohistochemical reagents, rather then applying these reagents to sections affixed to slides. To this end, a method for embedding and sectioning tissue that made feasible the processing of large numbers of floating cryostat sections while preserving the integrity of stained tissue was developed: the resulting protocol is described in the Methods section of this chapter.

Abstract

In the mouse mutant *dystonia musculorum* (*dt*), peripheral and central sensory axons develop focal swellings and degenerate. To identify the primary cellular target of the mutation, we have analyzed the spinal cords of *dt/dt*↔+/+ aggregation chimeras. In these chimeras, characteristic swellings appeared only on the axons of mutant genotype neurons; the axons of wild-type neurons, identified by their expression of a transgene-encoded human neurofilament protein, were normal. This direct correlation of genotype and phenotype indicates that the *dt* mutation acts via a mechanism intrinsic to affected neurons. In addition, we show here that the *dt* mutation leads to a disorder of neurofilament processing in which phosphorylated neurofilament epitopes accumulate inappropriately in neuronal perikarya.

Introduction

Mice affected by the autosomal recessive mutation *dystonia musculorum* (*dt*) undergo a dramatic deterioration in motor function. Abnormalities in limb positioning and coordination typically appear in the second week of *ex utero* life; efforts to walk soon become severely compromised by twisting of the trunk and prolonged extension of the limbs into strikingly abnormal positions. Although the rate at which the disease progresses differs among several *dt* alleles, most affected mice die by four weeks of age (Duchen *et al.*, 1964; Duchen, 1976).

The most prominent histopathological lesion in *dt* is the degeneration of axons in the sensory system, characterized in its initial stages by the appearance of fusiform swellings on affected fibers. In the peripheral nervous system (PNS), axons of dorsal root ganglia (DRG) and some cranial nerve sensory ganglia are involved, and in later stages of the disease peripheral nerves become markedly depleted of sensory fibers (Duchen *et al.*, 1964). In the spinal cord, swellings and degeneration are also seen in the axons of second order sensory neurons (Sotelo and Guenet, 1988). In contrast to the pronounced fiber loss observed in dorsal roots, the number of axons in ventral roots remains within normal limits and, apart from rare swellings, these fibers show little evidence of degeneration (Janota, 1972). Nonetheless, signs of partial motor denervation have been observed in older surviving *dt/dt* mice (*Duchen et al.*, 1964).

Since *dt* is likely to be a model for a heritable human neurodegenerative disease such as Friedreich's ataxia (Sotelo and Guenet, 1988), the mechanism by which the *dt* mutation leads to this phenotype is of considerable interest. However, little is understood of *dt* pathogenesis: the gene has not yet been cloned and neither its product nor the cell types in which it might normally be expressed are known. The finding that the axonal swellings in *dt/dt* mice contain aggregrations of neurofilaments and other axonal components led Janota (1972)

to propose that *dt* represented a primary neuronal disorder involving a specific disturbance of axonal transport . However, since axonal swellings can arise in response to a variety of extraneuronal influences, including trauma, disorders of myelination, and metabolic disorders (Jellinger, 1973), defects originating within some non-neuronal cell types have also been considered. Moss (1980, 1981a) observed abnormal patterns of myelination in the peripheral nerves of *dt/dt* mice, which in some cases appeared to precede axonal degeneration. On the basis of nerve grafting experiments, he argued that *dt* represents a primary disorder of Schwann cells which affects axons only secondarily (Moss 1981b). In contrast, Sotelo and Guenet (1988) proposed that the primary defect could lie within the peripheral sensory receptors, whose failure to provide normal trophic support would lead secondarily to dying-back of peripheral sensory axons and subsequently to trans-neuronal degeneration of second order sensory neurons in the CNS.

When similar uncertainty has arisen concerning the primary cellular target of other mutations that affect the mouse nervous system, the analysis of aggregation chimeras composed of mutant and wild type cells has proven effective (Herrup and Mullen, 1979; Goldowitz and Mullen, 1982; Peterson and Bray, 1984; Peterson, 1985; Goldowitz, 1989a). Here we have employed a chimera preparation to determine whether the *dt* mutation acts via an intrinsic neuronal defect or, alternatively, causes a primary defect within some other cell type that then acts on otherwise normal axons. Chimeras were constructed by aggregating preimplantation *dt/dt* and +/+ embryos, thereby obliging cells of both genotypes to coexist throughout development. If *dt* were to cause a defect intrinsic to affected neurons, only axons arising from the mutant neurons in these chimeras should be affected. In contrast, if the primary defect were manifested within some other cell type, such as myelinating cells or sensory receptors,

mutant cells in those populations should act equally on all axons regardless of the genotype of the neuron from which they arise. Depending on the nature of such an extrinsic defect, axons of mutant neurons might be rescued, or axonal swellings and degeneration might be imposed on wild-type neurons; in either case, no correlation should be observed between the genotype of neurons and the phenotype of their axons.

Materials and Methods

Mice. The *dt* mutant mice used throughout this study were derived from a transgenic line in which a mouse *hsp68- E. coli lac* Z construct caused an insertional mutation of the *dt* locus (Kothary *et al.* 1988). This mutation is allelic with spontaneously-arising alleles of *dt*, but hemizygous carriers can be conveniently identified by DNA hybridization techniques using transgene-specific probes (described in Sapienza *et al.*, 1987). Since *dt/dt* homozygotes do not survive to reproduce, all homozygous mice, either for chimera production or postnatal analysis, were obtained amongst the progeny of hemizygous matings. For chimera construction, all embryos produced from such matings were used. Homozygotes analyzed as two-week old pups were identified by clinical phenotype.

Wild-type embryos for chimera construction were derived from mice homozygous for the *hNF-L* line 29 transgene locus (Julien *et al.*, 1987). Control mice homozygous for *dt* and also hemizygous for the *hNF-L* transgene locus were obtained from matings between *dt/+* females and *dt/+; hNF-L/-* males. Offspring with the *dt/dt* affected phenotype were processed for immunohistochemical analysis at two weeks of age. Half were expected to also carry the *hNF-L* transgene–these were identified by immunolabelling with the DP-5 antibody.

Construction of chimeras. E2.5 embryos from each transgenic line were obtained by timed matings (the day of the vaginal plug designated E0.5). Chimeras were produced by aggregating pairs of eight-cell embryos *in vitro* and culturing these aggregates for 24 hours in B.M.O.C. medium (Gibco BRL) at 37°C. Embryos that successfully aggregated and developed into blastocysts over this period were transplanted into the uterus of a pseudopregnant female to complete development (Mullen and Whitten, 1971). Because only cells in the inner cell

mass are allocated to the formation of the embryo, such chimeric blastocysts give rise to mice with varying proportions of the two component genotypes, including, in some cases, the absence of one or the other component.

The embryos aggregated to generate the desired chimeras were derived from a mixed genetic background segregating for coat color; thus, while a mixed coat color indicated a chimeric genotype, a uniform coat color did not rule it out. Of the 60 mice born from embryo aggregations, only 1/4 of the chimeras were expected to contain cells of *dt/dt* genotype, since this component was derived from matings between *dt/+* heterozygotes. Therefore, to ensure that *dt/dt*↔+/+ chimeras would be included in the sample analyzed, all mice with chimeric coat color, with or without a clinical phenotype, as well as all phenotypically affected mice of uniform coat color were sacrificed at 14-16 days of age and processed for immunocytochemistry.

To identify those chimeras containing *dt/dt* cells, cryostat sections of spinal cord from each animal were first examined unlabelled with differential interference contrast optics; then, if swellings were observed, sections were processed for immunolabelling with RT-97 and re-examined. To estimate the proportion of wild-type axons in a chimera, alternate serial sections were immunolabelled with RT-97 and DP-5. On polaroid photomicrographs at 40X, counts of stained axons were made in the region of the ventral funiculus immediately bordering the ventral median fissure. This region was chosen because axons here are derived from spinal cord neurons, and therefore should be representative of the population examined for axonal swellings. Also, these axons could be reliably counted, since they appeared in cross section as round profiles. From right and left sides of three sections labelled with each antibody, stained profiles within an area corresponding to a square 125μm on a side were counted. The results for each antibody were averaged; division of the number of

DP-5-immunolabelled axons by the number labelled by RT-97 provided the estimated proportion of wild-type axons in the chimera.

Perfusion. 14-16 day-old mice were anaesthetized with Avertin (8 mg/kg) and perfused transcardially using a 25-guage butterfly needle attached to a peristaltic pump operating at 5 ml/minute. 10 ml of phosphate buffered saline (0.15M NaCl, 0.1M phosphate, pH 7.3) was delivered first to clear the vasculature, followed by 100ml periodate-lysine-paraformaldehyde (2% paraformaldehyde, 0.1M lysine and 10mM $NaIO_4$ in 0.1M phosphate buffer, pH 7.2) (McLean and Nakane, 1974). The spinal cord was removed and incubated at 4°C in the same fixative for 5 hours, then transferred to 30% sucrose in 0.1M phosphate buffer pH 7.3 for a further 2 to 3 days. Tissue was embedded in 15% gelatin and the blocks were trimmed, frozen in isopentane at -40°C, and stored under isopentane at -80°C until sectioning.

Antibodies. The mouse monoclonal antibody RT-97, directed against the mouse neurofilament-heavy protein (NF-H), was kindly supplied by Dr. John Wood (London, England). RMO 55, directed toward a phosphorylated epitope on rat NF-M and RMO 24, recognizing a phosphorylated epitope on rat NF-H, were generously provided by Dr. Virginia Lee (Philadelphia). DP-5, which recognizes human, but not mouse, NF-L was purchased from Dr. Martha Soriano (Paris, France).

Immunocytochemistry. 12 µm-thick sections were cut in a cryostat and transferred to formol sucrose (7.5% sucrose and 4% formaldehyde in 0.07M phosphate buffer, pH 7.4) for 30 minutes. Sections were lifted from the knife using a wire loop containing a drop of the fixative and deposited in the fixative such that they spread flat on its surface. All subsequent steps were carried out in 0.5 M Tris buffer, pH 7.6; antibodies were diluted in this buffer to which was added 1% normal horse serum (NHS). After a 30 minute rinse in buffer, non-

specific protein binding sites were blocked by incubating sections in 7% NHS for 30 minutes. The sections were then incubated overnight at room temperature in the primary antibody, which was diluted as follows: (DP-5)–0.2 mg/ml; (RT-97)–diluted 1/800 from a supplied 1/10 dilution; (RMO 55 or RMO 24)–1/50 dilution of tissue culture supernatant. After rinsing, sections were incubated in biotinylated horse anti-mouse IgG (Vector), diluted at 1/200, for 1 hour. Following further rinses, sections were incubated in avidin-biotinylated HRP complex (ABC) (Vector) for 1 hour. HRP activity was detected using 1mg/ml diaminobenzidine with 0.03% H_2O_2 in 0.1M Tris, pH 7.6, for 10 minutes. To identify and count swellings, sections were examined at 40X magnification.

Results

The fundamental prerequisite of this study was a means of determining whether individual axons, either with swellings or without, belonged to mutant or wild type neurons. For this purpose, a line of mice that express a transgene encoding the human 68 kD neurofilament-light protein (hNF-L) formed the wild-type component of the chimeras. Both the cell bodies and axons of neurons expressing this transgene can be specifically identified by immunocytochemical techniques, using an antibody, DP-5, that recognizes human, but not mouse, NFL (Julien *et al.*, 1987; Vidal-Sanz *et al.*, 1991). No similar means was available to specifically label the sub-population of axons arising from neurons of *dt/dt* genotype. We therefore analyzed chimeras by comparing the phenotype of wild-type axons labelled with DP-5 to the phenotype of the total axonal population, labelled with RT-97, an antibody recognizing the 200 kD mouse neurofilament protein, NF-H (Anderton *et al.*, 1982).

The *dt/dt* component of the chimeras was derived from a line of mice bearing an insertional mutation of the *dt* locus. This mutation has been shown to be allelic with other *dt* mutant alleles, and to cause an equally severe clinical and histopathological phenotype (Kothary *et al.*, 1988). However, before chimeras were analyzed, we ensured that characteristic *dt/dt* swellings occurred in these mice and that these could be detected by both the RT-97 and DP-5 antibodies.

Neurofilament immunolabelling of *dt/dt* mice with RT-97. Swellings with similar morphology have been observed in both the peripheral and central nervous system of *dt/dt* mice (Duchen *et al.*, 1964). However, in our immunocytochemical preparations, the presence of endogenous mouse IgG in dorsal roots and peripheral nerves led to background labelling which obscured these profiles. Since background staining did not occur in central nervous system (CNS) tissue, we confined our analysis to the swellings found in the

spinal cord. Chimeras were analyzed at two weeks of age, when axonal swellings in the spinal cord are a prominent feature of *dt/dt* mice (Duchen *et al.*, 1964).

The RT-97 antibody specifically recognizes an epitope containing a phosphorylated serine residue that is multiply present on the C-terminal region of NF-H in assembled neurofilaments (Coleman and Anderton, 1990). Since this region of the protein is normally phosphorylated only after entering the axon, RT-97 does not label perikarya or dendrites in normal spinal cord neurons (Figure 1), and as a result the morphology of immunolabelled axons within the gray matter can be seen clearly.

In sections of *dt/dt* spinal cord, RT-97-immunolabelled swellings were found in the gray matter, with a smaller number found in the lateral and ventral white matter and fewer still observed in the dorsal columns (Figure 1). As observed by Duchen *et al.* (1964) and Sotelo and Guenet (1988), swellings were most abundant in ventral regions of the gray matter, with few found in the dorsal portions of the dorsal horns. Although degenerating axons of DRG neurons have been demonstrated within these dorsal regions (Sotelo and Guenet, 1988), at the age of the mice examined in this study most swellings in dorsal roots occurred within or immediately adjacent to the dorsal root ganglia, with few found on the more distal axon segments near the spinal cord (data not shown). These observations, together with that fact swellings were rare in dorsal columns, suggest that most of the swollen axons observed within the spinal cord belonged to gray matter neurons rather than to DRG neurons.

Ultrastructural studies have previously shown that most swellings in *dt/dt* mice contain abundant neurofilaments, while a small proportion are primarily composed of vesicular organelles (Janota, 1972; Sotelo and Guenet, 1988). Consistent with this, we found that the majority of axonal swellings were

strongly immunolabelled by RT-97, while only a small proportion, visible with differential interference contrast optics, stained poorly or not at all. Smaller swellings had a diameter two or three times that of the axon, but much larger swellings, some exceeding 20μm in diameter, were also observed. Most swellings were fusiform, with smoothly tapering ends, while others were spherical or sausage shaped. Focal swellings often adjoined a fine, poorly-labelled axon segment on one side and a longer (up to several hundred μm), more isodiametrically-distended axon segment on the other (Figure 1).

Unexpectedly, a small proportion of neuronal perikarya and dendrites in these sections were immunolabelled by RT-97 (Figure 1). The same distribution of stained perikarya was seen using the antibodies RMO 24 (data not shown), which also recognizes a phosphorylated epitope on NF-H, and RMO 55, which recognizes a phosphorylated epitope on NF-M; like RT-97, both of these antibodies label only axons in normal mice (Lee *et al.*, 1987). Such ectopic perikaryal staining ranged from pale to very intense. The nuclei of heavily-labelled cells frequently occupied an eccentric position, in some cases forming a distinct bulge on the side of the cell. Like swellings, immunolabelled perikarya were found throughout the intermediate zone and ventral horn gray matter. Notably, many sections contained large labelled perikarya in the extreme ventrolateral gray matter, which, on the basis of their size and location, appeared to be motor neurons.

Immunoreactivity of perikarya with RT-97, RMO 24 and RMO 55 represents an additional pathological feature of the *dt* phenotype. However, for the purpose of the present investigation, we restricted our criteria for *dt* pathology to the presence of axonal swellings; these are a well documented characteristic of *dt* and this preliminary experiment demonstrated that RT-97

immunolabelling allowed them to be readily detected and reliably distinguished from the morphological features of normal axons.

Labelling of axons with anti-human NF-L antibody in *dt/dt* mice. The chimera strategy employed here was based on the assumption that any extrinsic influence on axonal morphology would be revealed in chimeras by the development of swellings on wild type axons. This required, first, that expression of the hNF-L protein would not modify the axonal phenotype of *dt*, and, second, that if swellings did occur on wild-type axons they would be labelled by the DP-5 antibody. To directly test whether these requirements would be met in the chimera preparation, crosses were made to produce control mice that were homozygous for the *dt* allele and hemizygous for the *hNF-L* transgene. In DP-5-labelled spinal cord sections from such mice, many intensely stained axonal swellings were observed (Figure 2). Although it would be expected that all swellings containing mouse NF-L would also contain hNF-L, the number of detectable DP-5-immunolabelled swellings was only 90% of the the number observed when an equivalent group of sections was immunolabelled with RT-97 (Table 1). However, this small difference did not appear to reflect the failure of DP-5 to label a subpopulation of swellings, but to occur instead because this antibody also labelled cell bodies, which obscured some of the smaller swellings. We concluded from these results that if swellings were to occur on hNF-L-expressing axons in chimeras, they would be readily detected.

Identification of chimera genotype. Because *dt/dt* homozygotes do not survive to reproduce, the embryos that provided this component of the chimeras were derived from matings between *dt/+* heterozygotes. As a result, only 1/4 of the products of embryo aggregation were expected to be of the informative *dt/dt*↔*hNF-L/hNF-L* genotype. Therefore, an additional prerequisite of this investigation was a means of identifying this specific sub-population.

In a pilot study, a small proportion of the chimeras derived from the above combination displayed a mild form of the movement disorder seen in *dt/dt* mice, while the remainder were clinically unaffected. Like young *dt/dt* mice, affected chimeras flexed fore- and hindlimbs toward the trunk when suspended by the tail (Duchen, 1976). The pronounced gait disturbance seen in older *dt* homozygotes did not occur in chimeras, most of which attained a normal life span. Most importantly, the spinal cords of such affected chimeras contained axonal swellings identical in appearance to those in *dt/dt* spinal cords. This feature provided an unequivocal marker of the presence of *dt/dt* cells.

Thirty presumptive chimeras were analyzed at two weeks of age for the presence of axonal swellings. Eight were positive; of these, seven also possessed *hNF-L/hNF-L* cells, which were identified by labelling with DP-5. Among these chimeras, the frequency of swellings observed in RT-97-labelled sections appeared, by visual estimation, to be inversely correlated with the proportion of wild-type cells present. Thus, one chimera was dominated by the genotypically normal *hNF-L/hNF-L* cells, with axonal swellings seen only infrequently, while two were composed primarily of *dt/dt* cells, and contained numerous RT-97-labelled swellings. The remaining four chimeras possessed an intermediate number of axonal swellings and a genotype proportion that ranged from 18 to 38% *hNF-L/hNF-L* cells (Table 2; Figure 3). In three of the latter four chimeras, a detailed comparison of neuronal genotype and axonal phenotype was performed.

Analysis of Affected Chimeras The cervical and lumbar enlargements of the spinal cord contain both a large area of gray matter and abundant axonal swellings. Therefore, sections from these levels were analyzed to determine whether any swellings occurred on axons arising from the wild type *hNF-L/hNFL* neurons. For each chimera, serial sections of spinal cord were collected and

placed alternately into two groups. Immunolabelling of one group of sections with RT-97 allowed the total number of swellings on axons of both genotypes to be determined. This number served as an estimate of the total number of swellings present in the second group of sections, which were stained with DP-5 to label only the genotypically normal axons.

RT-97-immunolabelling revealed stained axonal swellings (and perikarya) distributed in a pattern typical of *dt/dt* mice (Figure 4). From the three chimeras, 202 RT-97-immunolabelled sections were prepared. In these, the number of swellings ranged from 3 to 64, and a total of 5,682 swellings were counted. In the alternate serial sections immunolabelled with DP-5, neuronal cell bodies, dendrites and axons, all with normal morphology, were observed, but not a single swelling was found (Table 2). The percentage of hNF-L-positive axons present in each chimera was estimated (see Experimental Procedures). According to their proportion, if the axons of the *hNF-L/hNF-L* neurons in chimeras had been as susceptible to developing swellings as those of *dt/dt* neurons, a total of 1477 DP-5-labelled swellings should have been present, and at least 90% of these would have been detectable. Therefore, only the axons originating from *dt/dt* genotype neurons, and not those of the *hNF-L/hNF-L* wild-type neurons, developed this characteristic feature of *dt* in chimeras.

Discussion

A fundamental step in understanding the pathogenesis of any inherited disease is to distinguish the primary targets of the mutation from other cell types that are secondarily affected. We have found that in the spinal cords of *dt/dt*↔+/+ chimeras, the axons of mutant, but not wild-type, neurons develop swellings. This indicates indicates that, in these chimeras, the expression of the normal *dt* gene product in neurons is both necessary and sufficient to prevent the development of swellings in their axons.

In chimeras, mutant and wild-type neuronal and non-neuronal cells could potentially interact with the neurons under study. Although there was no evidence that the presence of wild-type cells attenuated the mutant phenotype expressed by individual axons, we can not exclude the formal possibility that the development of swellings on the axons of mutant neurons requires a contribution from another neuronal or non-neuronal cell type that is also primarily affected by the mutation. However, our failure to observe any swellings in the axons of wild-type neurons demonstrates that if such an extrinsic deficiency exists, it is unable to act alone to produce this axonal pathology.

This result therefore rules out the possibility that a primary defect in glial cells is responsible for producing swellings in CNS axons, as has been proposed to occur in the PNS (Moss, 1981b). If a primary glial defect operated in the CNS, the results we have obtained could only be explained if, during development of the chimeras, the axons of wild-type neurons had been myelinated exclusively by wild-type oligodendrocytes, while those of mutant neurons were myelinated only by mutant oligodendrocytes. Since oligodendrocyte precursors migrate extensively within the developing mouse CNS (Lachapelle *et al.*, 1983,1990; David *et al.*, 1984; Baulac *et al.*, 1987), any such matching of glial and neuronal genotypes would appear extremely unlikely.

The proposal that a primary glial cell defect might induce axonal swellings in peripheral nerves was based on transplantation experiments in which some axons of a wild type host developed swellings after regenerating into a *dt/dt* nerve graft. Since many of the Schwann cells within the reinnervated graft were likely to have been of *dt/dt* genotype, an intrinsic action of the mutation within this cell type was inferred. However since the *dt/dt* donors could be identified only after they had developed a clinical phenotype, grafts were obtained from nerves in which axonal degeneration, inflammation, and possibly other secondary effects on the cellular and non-cellular milieu of the nerve had already occurred. Thus, in addition to the potential immune complications associated with tranplantation between the non-inbred mice used in that study, a number of additional environmental influences on regenerating axons were potentially present within the graft. Swellings are not pathognomonic to *dt*, but occur in response to a variety of influences (Jellinger, 1973); thus, the conclusions that can be drawn from these results regarding the specific influence of mutant Schwann cells in *dt* pathogenesis are limited. In contrast, the experimental intervention performed in the present experiment involved only the initial aggregation of preimplantation embryos, and therefore all axon-glial relationships arose by normal developmental processes. Moreover, the immune system in chimeras is fully tolerant of cells arising from both embryos in the initial aggregate (Mintz and Silvers, 1967). Therefore, we propose that the observations made in the nerve transplant preparations reflect the difficulties inherent in applying this technique to the study of axonal degeneration, rather than signifying the existence of a fundamentally different mechanism of *dt* pathogenesis in the PNS.

The definition of an intrinsic neuronal defect in *dt* restricts the possible nature of the *dt* deficiency that leads to axonal swellings and degeneration. In

this study, immunocytochemical staining methods have confirmed that most swellings contain neurofilament proteins and have, in addition, revealed that phosphorylated epitopes of NF-H and NF-M accumulate inappropriately in the perikarya of spinal cord neurons. In favourable sections, neurons exhibiting both of these features were observed, suggesting that they may arise from a common mechanism and that this may be central to the pathogenesis of *dt*. The chromosomal location of *dt* excludes a primary abnormality in any of the three neurofilament proteins (Green, 1981). However, the toxic agent β,β'-iminodipropionitrile (IDPN), which also leads to the development of axonal swellings and the perikaryal accumulation of phosphorylated neurofilament epitopes (Pashad *et al.*, 1986; Fiori and Lowndes, 1988; Gold and Halleck, 1989; Watson *et al.*, 1989), has been shown to cause increased phosphorylation of neurofilaments (Eyer *et al.*, 1989), and slowing of their axonal transport (Sickles, 1989). Therefore, the *dt* mutation might affect the activity of a kinase or phosphatase acting on NF-H and NF-M. As has been proposed for related diseases (Nixon and Sihag, 1991) an abnormally high degree of phosphorylation of neurofilament proteins within the axon could lead to a slowing of their rate of transport to the point where they formed focal accumulations.

The altered activity of a kinase or phosphatase could act directly within the perikaryon to prematurely or excessively phosphorylate newly-translated neurofilament proteins. However, perikaryal immunoreactivity for phosphorylated neurofilament epitopes has also been produced by experimental interventions that have no direct effect on neurofilament phosphorylation. These include axotomy (Goldstein *et al.*, 1987; Klosen *et al.*, 1990), deafferentation (Shaw *et al.*, 1988), depriving neurons of their targets (Doering *et al.*, 1991), and blockage of fast axonal transport by local application of colchicine (Gold and Austin, 1991). Despite the apparently diverse nature of these treatments, each would potentially

prevent the delivery of a target- or afferent-derived trophic factor to the neuron cell body. Thus, in *dt*, focally-accumulated neurofilaments might interact with other cytoskeletal components to cause an interruption axon transport. Perikaryal immunoreactivity for phosphorylated neurofilaments could therefore arise as a secondary response to this transport block, rather than as a direct manifestation of disordered neurofilament phosphorylation.

Regardless of the mechanism responsible for the perikaryal accumulation of phosphorylated neurofilament epitopes, their presence in the perikarya of spinal cord motor neurons was an unexpected finding. The axons of these neurons do not typically degenerate in *dt* and abnormalities in their perikarya have not been reported previously. In another component of the motor system, the red nucleus, the magnocellular neurons have been shown to possess a reduced ability to synthesize acetylcholinesterase (Stanley *et al.*, 1983); in some, but not all strains, this is accompanied by changes in the morphology of these neurons (Messer and Strominger, 1980; Sotelo and Guenet, 1988). It is therefore possible that in some neuronal or non-neuronal populations, the *dt* mutant causes a functional deficiency that that may contribute to the clinical phenotype but is not reflected by consistent or even detectable changes in cellular morphology. In particular, such a functional deficiency within motor neurons could account for the signs of partial motor denervation observed in older *dt* mice (Duchen *et al.*, 1964) and, if a similar deficiency were present in other neurons in the motor system, could contribute to the movement disorder that characterizes *dt* .

In conclusion, using a transgene-based genotype marking system in mutant↔wild-type chimeras, we have determined that the *dt* mutation causes axonal swellings through a mechanism intrinsic to affected neurons. A comprehensive understanding of *dt* pathogenesis will ultimately depend on

characterization of the *dt* gene: the results of this study strongly suggest that this gene is expressed by the neurons that are affected by the disease. Notably, the *dt* allele we are studying was derived from the genomic insertion of a transgene of known sequence (Kothary *et al.*, 1988). This has made feasible a strategy to clone the *dt* gene from the genomic sequences flanking the transgene's insertion site. Work to this end is in progress (Kothary, R., Brown, A., Rossant, J., and Mathieu, M. Insertional mutagenesis of the *dystonia musculorum* locus in a transgenic mouse line. 2nd Eastern Canadian Conference on Development and Cancer, Montreal, September 1991). Analysis of the *dt* gene's expression pattern will reveal whether other cell types express the gene and potentially contribute to the disease phenotype. Furthermore, characterization of the *dt* gene's protein product will allow its normal role in axonal maintenance to be precisely defined. The detailed understanding of *dt* pathogenesis this will afford should hold considerable promise for the understanding of similar inherited human neurological diseases. Finally, as demonstrated here, the genotype marking system based on the *hNF-L* transgene can be applied at the level of neuronal processes. Thus, the pathogenesis of other mouse mutants that cause axonal pathology will now be accessible to a similar chimera-based analysis.

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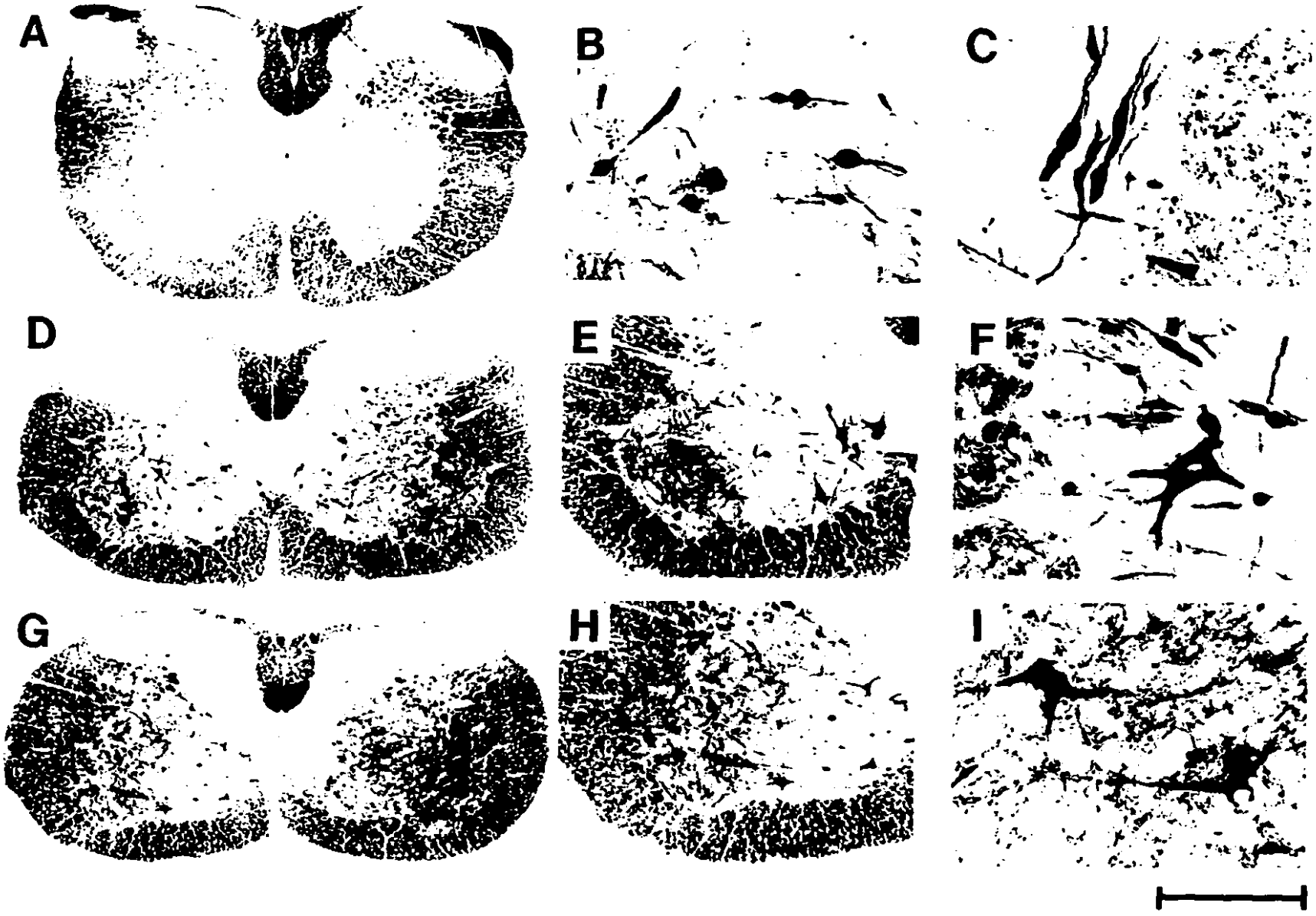
Figure 1. Neurofilament immunolabelling of wild-type and *dt/dt* spinal cord cross sections.

Spinal cord in A is from a +/+ mouse; all other sections (B to I) come from an affected mouse of *dt/dt; hNF-L/-* genotype .

A to F: Immunolabelling of spinal cord with RT-97, recognizing phosphorylated mouse NF-H. In spinal cord of wild-type mice, RT-97 labels axons in the gray and white matter, but not cell somata (A). In *dt/dt* mice (B to F) additional profiles are labelled in the gray matter (D), including both axonal swellings (B, C and F) and neuronal perikarya (F). Some of these labelled perikarya in the ventrolateral gray matter appear to be motor neurons.

G, H, and I: Immunolabelling with antibody RMO 55, directed against a phosphorylated epitope of NF-M. In *dt/dt* mice, this antibody also stains, in a similar distribution (G), axonal swellings (H) and neuronal perikarya (I). Note the severe distortion of the contour of the lower neuron in I, as a result of the markedly eccentric position of its nucleus.

Magnification: Calibration bar at lower right corresponds to: 100µm–C, F, and I; 377µm–B, E, and H; 667µm–A, D, and G.


A
B
C
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E
F
G
H
I

Figure 2. Immunolabelling of the human neurofilament-light protein with DP-5 antibody.

A: Spinal cord of +/+ mouse, showing that DP-5 does not immunolabel endogenous mouse neurofilaments. Note background labelling resulting from endogenous IgG remaining in dorsal roots, at upper right and left. (Punctate labelling within spinal cord corresponds to endogenous peroxidase activity within the few red blood cells not removed by perfusion.)

B to D: Spinal cord of *dt/dt; hNF-L/hNF-L* mouse. The hNF-L protein is immunolabelled by the DP-5 antibody in neurons and their axons throughout the gray and white matter (B). Specifically, axonal swellings in gray and white matter are strongly labelled (the small round profiles in C and the three diagonally-aligned round profiles in D)

Magnification: Calibration bar at lower right corresponds to: 580μm–A and B; 285μm–C; and 55μm–D

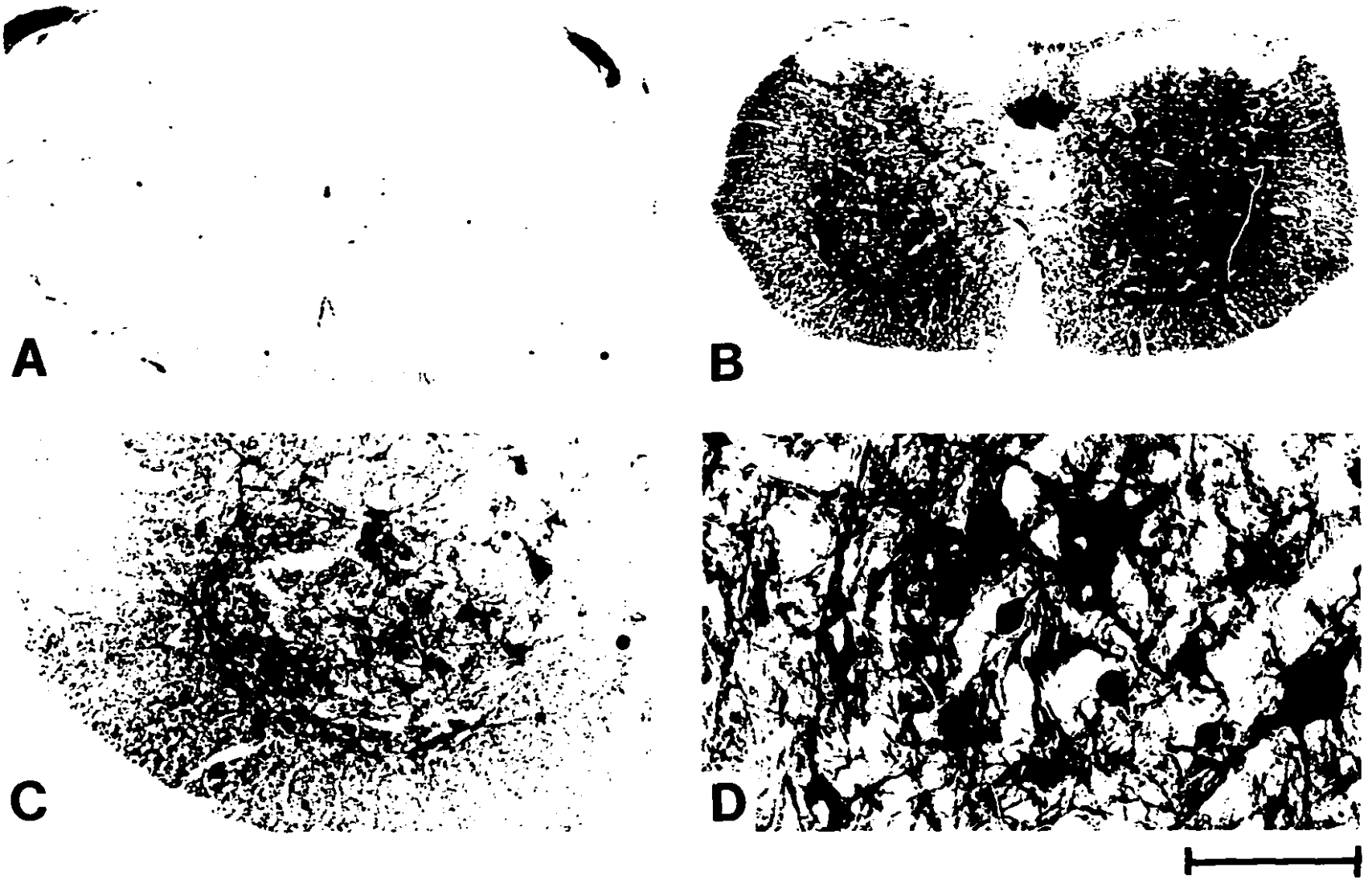

A
B
C
D

Figure 3. Neurofilament immunolabelling of *dt/dt↔hNF-L/hNF-L* chimeras. Sections pictured in left column are immunolabelled with RT-97; those on right with DP-5. A to D: chimera B29; E to H: chimera B17.

A and E: *dt/dt↔hNF-L/hNF-L* chimeras contain RT-97-immunolabelled axonal swellings and perikarya in a similar distribution but smaller number compared to that seen in *dt/dt* mice (compare 1D)

B and F: DP-5 labels a subpopulation of axons and cell bodies throughout the gray matter. These photomicrographs show the chimeric nature of the spinal cords (compare the density of labelling in the non-chimeric spinal cord shown in 2B). At this low magnification, some labelled profiles can not be distinguished from the axonal swellings labelled by RT-97. However, at higher magnification they are all revealed to be normal perikarya (see Figure 4).

C and D; G andH: Of the total axonal population labelled by RT-97 within a region of the ventral funiculus (C and G), DP-5 specifically immunolabels the sub-population of axons derived from the wild-type neurons expressing the hNF-L transgene (D and H).

Magnification: Calibration bar at lower right corresponds to: 800μm–A, B, E, and F; 100μm–C, D, G,H.

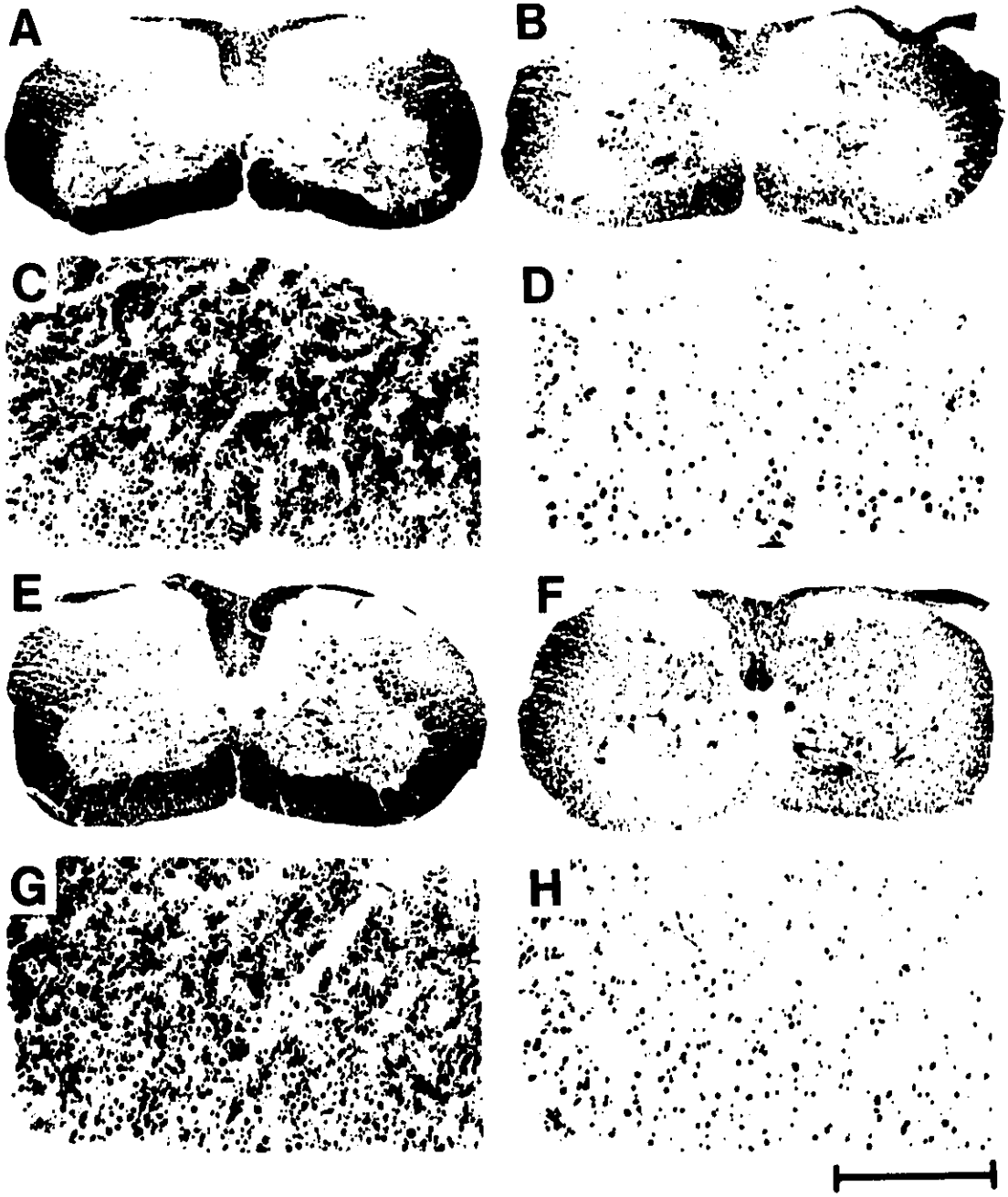
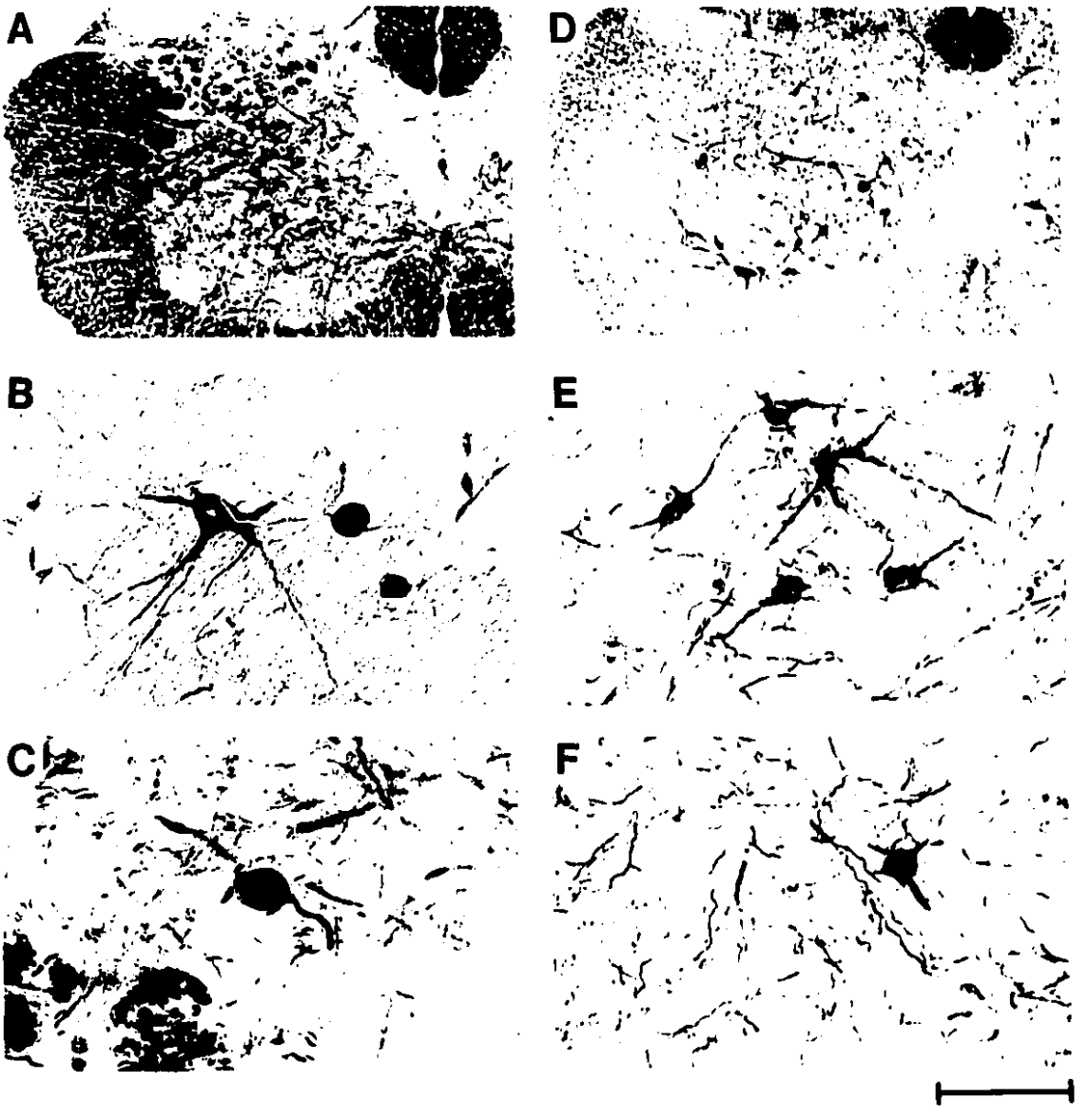

RT-97
DP-5
A
B
C
D
E
F
G
H

Figure 4. Axonal swellings are not found on DP-5-labelled axons in chimeras.
Chimera B17: sections on left are immunolabelled with RT-97; on right, immunolabelled with DP5 to label only wild-type neurons.

A-C: In chimeras, RT-97-immunolabelled cell bodies and axonal swellings have the same distribution (A) and appearance (B and C) as those seen in *dt /dt* mice. In B, an axon bearing a swelling originates from a stained neuronal perikaryon.

D-F: In corresponding sections of chimera spinal cord, DP-5 labels the subpopulation of neurons with wild-type genotype (D). Only cell bodies and axons with normal morphology are observed (E and F).

Magnification: Calibration bar at lower right corresponds to: 355μm–A and D; 89μm; B, E, C, and F.


RT-97
DP-5
A
D
B
E
C
F

Control Mouse	#Swellings/10 sections		% hNFL-positive. axons
	DP-5	RT-97	
C11	286	316	91%
C12	547	621	88%

Table 1: Labelling of human NF-L by DP-5 in axonal swellings of *dt/dt;hNF-L/-* mice. In these control mice, analyzed at 14 to 16 days of age, all axons belong to neurons carrying the *hNF-L* transgene. Swellings were counted in 10 sections of lumbar spinal cord (mouse C11) or cervical cord (mouse C12) immunolabelled with DP-5 or RT-97.

Chimera	#DP-5 / #RT-97	%hNF-L	Total #Swellings		
			RT-97	DP-5	DP-5 (expected)
B17	404 ±35 / 1073±48	38	2,028	0	(771)
B29	143±3 / 784±12	18	1,777	0	(320)
B30	160±5 / 778±42	21	1,877	0	(386)

Table 2: Genotype of axons bearing swellings in *dt/dt↔hNF-L/hNF-L* chimeras. "#DP-5" and "#RT-97" correspond to number of axons labelled with each antibody in a standard 125 µm x 125 µm region of the ventral funiculus. The results represent the mean ± standard error of the mean of counts of the right and left sides of three sections. The ratio of the two values provides the estimated proportion of axons of hNF-L genotype neurons (% hNF-L). The total number of axonal swellings detected by each antibody refers to the number detected in 45 spinal cord sections from chimera B17, 79 sections from B29, and 78 sections from B30. In each case, the product of the total number of swellings detected with RT-97 and the proportion of hNF-L-positive axons in the chimera yields the number of DP-5 labelled swellings expected if this axonal population had been as susceptible to developing swellings in chimeras as axons of *dt/dt* genotype neurons.

Chapter III Development of the Floor Plate in Transgenic Mice: β-galactosidase Histochemistry Reveals Cell Morphology and Evidence for Macromolecular Transfer to Commissural Axons

Preface

The preceding studies suggested that the floor plate is not involved in the pathogenesis of *dystonia musculorum*. However, the floor plate has been shown to play a number of roles in the development of the neural tube. One of these is the guidance of commissural axons, which, in the absence of the floor plate, are misrouted in the region of the ventral midline. Despite the clear requirement for the presence of floor plate cells, the nature of their contribution to axon guidance has remained undefined. The morphology of floor plate cells, and the way in which they interact with decussating commissural axons is clearly relevant to this issue. In the Tg4 line, the fortuitous expression of β-galactosidase in developing floor plate cells allowed them to be stained using histochemical methods. In addition, the transgene was found to be affected by an epigenetic modification process, which causes it to be expressed in mosaic fashion (McGowan *et al.*, 1989: Appendix II). Since this allows the morphological details of individual cells to be seen with great clarity, stained Tg4 neural tube preparations were studied with the objective of gaining further insight into the developmental role of the floor plate in commissural axon guidance.

Abstract

The trajectory choices made by commissural axons at the neural tube's ventral midline appear to require an interaction between local guidance cues and changes in the ability of their growth cones to recognize and respond to these cues. In the absence of the midline floor plate, commissural axons make errors in pathfinding, suggesting that this structure plays a critical role in their guidance. However, the precise nature of the floor plate's contribution remains undefined. In an effort to gain further understanding of the structure and function of floor plate cells, we have taken advantage of a line of trangenic mice in which floor plate cells express β-galactosidase. Histochemical preparations revealed that floor plate cells possess morphological features which may serve both to physically guide decussating commissural axons and to mediate an intimate interaction with them as they cross. Unexpectedly, β-galactosidase activity was also found within axonal segments adjacent to stained floor plate cells, raising the possibility that proteins synthesized by floor plate cells can be transferred to decussating axons.

Introduction

The floor plate is a column of non-neuronal cells that bridges the right and left halves of the neural tube at the ventral midline. Cells in this location are critically positioned to guide the growth of the axons that form the spinal cord's ventral commissure. In rodents, commissural axons originate from neurons in the dorsal neural tube and project ventromedially to converge upon, and then cross the floor plate. As their growth cones emerge from its contralateral margin, they make a rostral turn to project longitudinally within the adjacent ventral funiculus (Jessell *et al.*, 1989; Bovolenta and Dodd, 1990).

Both *in vitro* (Tessier-Lavigne *et al.*, 1988; Placzek *et al.*, 1990a) and *in vivo* (Placzek *et al.*, 1990b; Yaginuma and Oppenheim, 1991b), the axons of commissural neurons have been shown to change their trajectory to grow toward ectopically-positioned floor plate tissue. This suggests that their normal projection toward the ventral midline is guided by a diffusible chemoattractive factor released from the floor plate (Placzek *et al.*, 1990a, b). If this were the only cue to which their growth cones responded, commissural axons would be expected to converge upon the source of this factor at the midline and cease their outgrowth. That they continue to elongate suggests that local cues in the vicinity of the floor supersede its chemoattractive influence. This idea is supported by the observation that the midline trajectory of commissural axons is disturbed in mouse embryos carrying the *Danforth's short tail* (*Sd*) mutation, which lack the floor plate in the caudal portion of the neural tube (Dunn *et al.*, 1940; Theiler, 1959). In this region, commissural axons either fail to cross the midline or, if they succeed, continue to grow circumferentially up the contralateral side of the

neural tube, rather than turning into the ventral funiculus to project in the rostral direction (Bovolenta and Dodd, 1991).

One way in which the floor plate may contribute to local axon guidance is by providing a substrate with specialized adhesive properties. Floor plate cells have been shown to express a number of adhesive molecules that are absent from neighbouring neural tube regions, including p84 (Chuang and Lagenaur, 1990), polysialylated N-CAM (Bovolenta and Dodd, 1991; Griffith and Wiley, 1991), GP-90 (Moss and White, 1989; Ranscht and Dours, 1989) and F-spondin (Klar *et al.*, 1992). The adhesive characteristics conferred by these molecules might increase the floor plate's attractiveness as a substrate for commissural growth cones (Bovolenta and Dodd, 1990). However, this does not explain how the direction of growth cone extension across the midline is specified. Since axons from both sides of the neural tube cross the floor plate with directly opposing trajectories, a gradient in adhesion, or any other property, that guided axons from one side should oppose the growth of axons orginating contralaterally.

The failure of commissural axons to turn rostrally and join the ventral funiculus in *Sd* embryos indicates that the presence of the floor plate is also required for this trajectory change. Here also, the normal presence of a midline zone of special adhesive properties has been proposed to provide guidance cues: according to this hypothesis, axons encountering the contralateral margin of the floor plate would turn orthogonally to maintain contact with the floor plate, in preference to neighbouring regions of lesser adhesiveness (Bovolenta and Dodd, 1990). However, this leaves unexplained how the direction of rostral growth is specified. In normal embryos, commissural growth cones project rostrally-directed filopodia as soon as they encounter the contralateral ventral funiculus, suggesting that they respond to a specific signal within the funiculus indicating

the rostral direction. However, these axons earlier in their outgrowth show no response to the ipsilateral ventral funiculus, which they pass as they enter the floor plate (Bovolenta and Dodd, 1990). Thus, commissural axons from each side of the neural tube fail to respond to cues that elicit a clear and consistent response from those originating contralaterally. One explanation of this behaviour is that commissural growth cones gain the ability to respond to cues directing a rostral turn only after they have passed through the floor plate (Dodd *et al.*, 1988).

The possibility of such a midline shift in the responsiveness of growth cones to guidance cues is supported by the finding that the adhesive glycoproteins expressed by commissural axons change in response to contact with the floor plate (Dodd *et al.*, 1988). Commissural axons initially express the glycoprotein TAG-1, but its expression ceases when the growth cone reaches the floor plate, where it is replaced by the glycoprotein L1. L1, which has been shown to mediate axon fasciculation (Stallcup and Beasley, 1985), is restricted to the contralateral portion of the axon; since commissural axons contralateral to the floor plate grow by fasciculating within the ventral funiculus, their restricted expression of this protein may constitute one element of their ability to respond to this pathway (Dodd *et al.*, 1988). Where axons fail to encounter the floor plate in *Sd* embryos, expression of TAG-1 is prolonged, indicating a dependence for this transition on contact with the floor plate. Because commissural neurons in culture also make a transition from TAG-1 to L1 expression, the role of the floor plate in situ may be to regulate the timing of this switch with respect to the growth cone's position in the neural tube (Bovolenta and Dodd, 1991).

Based on the above evidence, the midline guidance of commissural axons appears to require an interaction between local guidance cues, some of which

may be supplied by the floor plate, and floor plate-dependent changes in the growth cones' responsiveness to these cues. However, the way in which the floor plate contributes to these guidance processes has not yet been resolved. To gain further insight into the structure and function of floor plate cells, we have taken advantage of an opportunity to study their morphology which arose in a line of transgenic mice. In the Tg4 line, a mouse heat shock protein *hsp68* promoter–*E. coli lac* Z construct inserted within chromosome 1, causing a recessive mutation of the *dystonia musculorum* (*dt*) gene (Kothary *et al.*, 1988). In the process, the transgene acquired a floor plate-specific expression pattern, which appears to be due to the influence of a tissue-specific enhancer adjacent to its insertion site (Kothary *et al.*, 1988; Gossler *et al.*, 1989). Subsequent experiments on *dt* pathogenesis suggest that the regulatory elements responsible are not those that normally control the expression of the *dt* gene, but rather those of a closely linked floor plate-specific gene (Campbell and Peterson, 1992).

Since *dt* heterozygotes exhibit no detectable abnormalitites, we have used histochemical staining techniques to examine the normal morphology of floor plate cells in these mice. A very useful feature of this transgenic line is that an epigenetic process acts on the transgene DNA to restrict its expression, on some strain backgrounds, to only a proportion of floor plate cells (McGowan *et al.*, 1989). The intense histochemical staining afforded by β–galactosidase substrates, in combination with the mosaic distribution of expressing cells, has allowed the morphological details of individual cells to be appreciated with unparalleled clarity. We describe in this report the expression pattern of this transgene and the details of floor plate cell morphology this has revealed. In addition, we report the unexpected presence of β-galactosidase activity within commissural axon segments neighbouring these stained floor plate cells, which, we propose,

may reflect the existence of a general process of macromolecular transfer from floor plate cells to decussating axons.

Materials and Methods

β-galactosidase Histochemistry.

Bluo-gal. Embryos were removed from the uterus and dissected free of fetal membranes under PBS (0.15M NaCl, 0.1M phosphate buffer [pH 7.3]). Incisions were made along either side of the neural tube to increase penetration of fixative and stain. Embryos were fixed by immersion, with gentle agitation, in a mixture of 0.5% paraformaldehyde and 0.5% glutaraldehyde in PBS for 2 hours at 4°C. After a brief rinse in PBS, tissue was stained by incubating at 37° overnight in Bluo-gal (Bethesda Research Laboratories), 0.5mg/ml in PBS containing 10mM potassium ferricyanide, 10mM potassium ferrocyanide and 2mM KCl.

CMFDG. Embryos were dissected from fetal membranes under PBS. The neural tube was transected at the level of the developing hindlimbs. By cutting rostrad from this point with scissors along the lateral margins of the neural tube, it was freed from adjoining tissue and the overlying epithelium was removed. A second longitudinal cut divided the roof plate. To free the neural tube from its pial membranes, which impeded penetration of the CMFDG substrate, closed scissors were inserted, in the region of the hindbrain, between these membranes and the neural tube's ventral face. The neural tube was transected at the level of the midbrain and peeled from the underlying membranes by applying gentle traction to the freed rostral end with forceps.

To osmotically load the substrate, the neural tube tissue was incubated for 2 minutes in a solution of 1/2 strength PBS containing 1mM CMFDG at 37°C. The tissue was then incubated at 37°C in PBS for 5 minutes to allow enzymatic

cleavage of the substrate. To minimize U. V.-induced fading of the fluorescence, tissue was incubated in 5 mg/ml paraphenylenediamine in PBS at 4°C for a further 5 minutes. Because fixation weakened the signal, the floor plate was viewed and photographed unfixed, in the "open book" configuration, its ventral face up.

Cryostat Sections

Stained tissue was first embedded in acrylamide by incubating at 4°C for 6-24 hours in 10% acrylamide monomer (acrylamide:bis = 30:1) with 0.24% TEMED, as well as 1.5% glycerol to improve sectioning properties. The tissue was placed on a pad of polymerized acrylamide in one well of a 6-well tissue culture plate and covered with 5 ml of fresh monomer which had been pre-cooled to 4°C and to which had been added 100µl 10% NH_4SO_4 and 12µL TEMED. Polymerization was allowed to proceed for 4-6 hours at 4°C. After blocks were trimmed they were frozen in isopentane at -40°C, stored in isopentane at -80°C, and sectioned in a cryostat at 15-25µm.

Electron Microscopic Sections

Bluo-gal-stained neural tubes were rinsed in 0.1M phosphate buffer (pH 7.4) and postfixed for 4 hours to 2 days at 4°C in Webster's fixative (2.5% glutaraldehyde, 0.5% paraformaldehyde in phosphate buffer). Following 1 hour fixation in 1% OsO_4 and dehydration in graded methanol, tissue was processed through propylene oxide (30 minutes), equal parts propylene oxide: epon (3-4 hours), and epon (overnight), and polymerized at 60°C for 48 hours.

Blocks were either sectioned for E.M. analysis or, to identify stained features of interest, 3µm sections were first cut on an ultramicrotome using glass

knives. Sections were dried onto glass slides for 30 minutes, then mounted in glycerol and coverslipped. Those sections selected for further analysis on the basis of stained features were photographed. After removal of the coverslip and rinsing of the slide with distilled water, sections were recovered by scraping them off the slide with a razor blade under a drop of water. Sections were transferred using a 26 guage needle to a drop of water placed on a blank epon block that had been previously been faced off to a smooth surface. Removal of the water droplet with filter paper caused the section to flatten onto the block surface. Blocks were then incubated at 60°C overnight to ensure adherence to the block (Campbell and Hermans, 1972).

Following further trimming, ultrathin sections were cut on a diamond knife and floated onto copper slot grids that had been coated with collodion film (1% in amyl acetate) then carbon coated. Sections were stained in 4% uranyl acetate followed by 0.4% lead citrate in 0.1 N NaOH. They were viewed and photographed on a Philips CM-10 electron microscope.

Results

Developmental expression pattern of the Tg4 transgene.

The initial focus of this investigation was to investigate the morphology of floor plate cells. In order to provide a developmental context for this morphological study, we first examined the spatial and temporal expression profile of the transgene. β–galactosidase expression was detected by incubating fixed embryos as whole mounts in the indigogenic β–galactosidase substrates X-gal and Bluo-gal (BRL). Both substrates produced blue labelling of cells expressing the enzyme, but, because the staining produced by Bluo-gal was considerably more intense, it was used for all of the preparations shown here.

β–galactosidase expression was detected in the floor plate from E10.5 to E14.5 (Fig. 1). In litters examined at E10.5, the less developmentally mature embryos lacked staining in a variable proportion of the caudal spinal neural tube, suggesting that a rostral to caudal gradient in the onset of transgene expression existed. Subsequently, staining extended the full length of the floor plate as defined morphologically (Kingsbury, 1930), reaching from the base of the developing tail to the hindbrain/midbrain border. The level of β–galactosidase activity appeared, by visual estimation, to be maximal on E12.5-13.5. After E14.5, the staining intensity diminished considerably, although detectable activity persisted in the caudal neural tube until E18.5, and in the hindbrain into the first postnatal week.

The onset of transgene expression at E10.0-E10.5 corresponds precisely to the time at which the first axons arrive at the floor plate in the spinal neural tube (Wentworth, 1984). At this stage, the earliest labelled cells lay within the lateral

floor plate, extending obliquely from the ventral tip of the central canal (Fig. 1). Due to the paucity of decussating axons at this stage, the cell bodies of stained cells occupied all but the most ventral portion of the floor plate, each cell having only a short basal process joining it to the external limiting membrane. Stained transversely oriented profiles similar to those seen at later stages (see below) were sometimes associated with the basal processes, but the level of β-galactosidase expression was too low to allow their morphological details to be clearly resolved.

At subsequent developmental stages, the floor plate increased in thickness as a result of both an increased number of cell bodies, which assumed a pseudostratified arrangement, and an increase in the thickness of the marginal layer of decussating axons. At these later stages, less-intensely stained cells surrounding the floor plate were also present (Fig. 1). Those cells immediately dorsal to the floor plate adjoined the central canal and tended to be bipolar and radially oriented; between the floor plate and the developing motor column, bipolar and multipolar cells, some aligned with the trajectory of commissural axons traversing this region, were also observed. Staining was also seen within the mesenchyme immediately ventral to the floor plate; however, in this region it did not appear to outline individual cell bodies, but rather to label portions of a number of cells. This pattern of expression within and around the floor plate, including that within ventral mesenchyme, corresponds to that recently reported for the secreted adhesive protein F-spondin (Klar *et al.*, 1992).

It was notable that the staining extended rostrally through the developing hindbrain, since this region contains a commissure analogous to that in the spinal cord, formed by the axons of interneurons arising from cranial nerve sensory ganglia. The developing hindbrain contained a distribution of stained cells

within and lateral to the floor plate that was similar to that observed within the spinal neural tube. However, as defined by the arrangement of stained cells, the floor plate had a slightly different shape in this region. Whereas in the spinal neural tube it appeared in cross section as a triangle, whose apex lay at the ventral tip of the central canal, in the myelencephalon the floor plate was separated into paired oblique bands. Each of these bands intersected the central canal dorsolateral to its ventral tip. Rostral to the pontine flexure, the separation of these bands increased further. Ventral midline staining terminated at the fovea isthmus, the point marking the morphological limit of the floor plate, although pale staining of some cells aligning the central canal dorsal to the floor plate continued through the midbrain.

Morphology of stained floor plate cells.

The embryos used for these initial developmental studies were those in which a high proportion of floor plate cells expressed the transgene. As a result, the intensity of staining within the marginal region of the floor plate obscured the details of individual cells. However, in some regions it was evident that, in addition to the expected radially aligned profiles, linear profiles with a mediolateral orientation were present. These transverse profiles were particularly apparent where they projected laterally from the floor plate, or, in the developing hindbrain, where the midline region of the floor plate was otherwise unstained (Fig. 1E). The appearance of these labelled profiles was that of extended lateral branches of floor plate cell basal processes, but the density of staining did not permit their relationship to the floor plate cells to be resolved with certainty.

A solution to the problem of visualizing these details arose in the course of breeding Tg4 mice, when it became apparent that the proportion of floor plate cells that expressed the transgene varied considerably when the transgene was crossed into different strain backgrounds (McGowan *et al.*, 1989). While on some backgrounds no cells expressed the transgene, and on others most or all appeared to stain (as pictured in Fig. 1), on many backgrounds an intermediate proportion of cells were labelled (Fig. 2). This variability in staining did not appear to result from any inter-strain differences affecting the floor plate itself, since no variability in the histological appearance of the floor plate and neural tube was seen. Rather, the proportion of β-galactosidase-expressing cells was inversely correlated with the degree of transgene methylation in each embryo, indicating that an epigenetic modification of the transgene DNA itself was responsible (McGowan *et al.*, 1989). While the mechanism conferring this modification has not been defined, its fortuitous occurrence provided a unique opportunity to observe the morphological details of individual floor plate cells unobscured by the labelled processes and cell bodies of adjacent cells.

Figs 2A and B show stained neural tube cross sections from a subline in which an intermediate proportion of floor plate cells expressed the transgene. The majority of stained cells lay in the lateral floor plate, although some were seen in the central midline region. In general, the morphology of individual cells resembled that of radial glia: extending from each cell body were an apical process, which terminated on the central canal, and a basal process, which projected toward the basal lamina. While the apical processes appeared unremarkable, the basal processes possessed a number of specialized features. First, many of these processes did not appear to reach the basal lamina, but instead terminated within the marginal layer of decussating axons. They

occupied a wider profile in cross sections than did the apical processes, their width often equalling that of the cell body. Their profile was narrower in sagittal than in transverse sections, indicating that they were relatively flattened in the rostrocaudal axis. The ventral ends of many basal processes deviated either medially or laterally from the radial alignment of the more proximal portion of the process. Finally almost all of the labelled basal processes were embellished with a brushwork of fine, short processes. These were less evident in sagittal sections, suggesting that many were extended in alignment with decussating commissural axons.

In these mosaic floor plates, the stained transverse profiles and their relationship with stained floor plate cells could be seen with much greater clarity (Fig. 2). The profiles were aligned with commissural axons, whose initially oblique trajectory changed, as they crossed the floor plate, to a more mediolateral alignment. Transverse profiles were of variable length, some spanning the entire width of the floor plate and many extending a short distance beyond its margins. They more frequently projected laterally from the ventral marginal zone, in alignment with axons leaving the floor plate, than dorsolaterally, in alignment with arriving axons. Like commissural axons, stained profiles crossed the floor plate perpendicular to its longitudinal axis. Most of these extended only a short distance beyond the floor plate, although infrequently they were observed running longitudinally within the ventral funiculus.

Although, in cross section, the labelled transverse profiles frequently crossed in close proximity to one or more floor plate cell basal processes, the relationship of the two did not suggest that they were continuous. Instead, transverse profiles either overlay a basal process, in close but different planes of focus, or a basal process terminated within the marginal layer at the point where

it intersected a transverse profile (Figs 2A and B). In many cases a space clearly separated profiles in the two orientations. Since the transverse profiles were not associated with labelled perikarya outside the floor plate, they appeared to represent linear segments isolated from cell bodies.

The incongruous appearance of these profiles raised the possibility that they represented an artefact of the staining procedure rather than the genuine distribution of the enzyme. The floor plate of non-transgenic embryos was not stained by Bluo-gal, indicating that the transverse profiles did not result from the presence of endogenous mammalian galactosidases. However, since the enzymatic cleavage and subsequent precipitation of indigogenic substrates occur as separate steps (Cotson and Holt, 1958), the possibility existed that Bluo-gal cleaved in floor plate cells had diffused before non-specifically precipitating as artefactual linear profiles. To investigate this possibility, transgenic neural tubes were incubated in a fluorescent β-galactosidase substrate, chloromethylfluorescein di-β-D-galactopyranoside (CMFDG), whose localization within the tissue does not depend on precipitation. Whereas the indigogenic substrates are applied to fixed tissue, CMFDG must be loaded into living cells, where it becomes linked to glutathione by the enzyme glutathione S-transferase. In cells containing β-galactosidase activity, the fluorescein di-β-D-galactopyranoside (FDG) moiety is cleaved to yield fluorescein, which remains bound to glutathione and is therefore retained within the cell (Zhang *et al.*, 1991). We reasoned on this basis that the distribution of the CMFDG reaction product would not be subject to any of the artefacts potentially affecting deposition of the Bluo-gal reaction product. When unfixed transgenic floor plate tissue was incubated in CMFDG, typical radially-aligned floor plate cells were labelled, and, in addition, transverse profiles of identical morphology and alignment to those

observed in Bluo-gal-labelled preparations were seen (Fig. 2E). Therefore, this indicated that the transverse profiles represented membrane-bound structures containing transgene-encoded β–galactosidase.

Stained profiles appear to represent midline segments of commissural axons.

To determine whether or not these profiles were branches of floor plate cells, we examined floor plate tissue, either unstained or stained with Bluo-gal, by electron microscopy. First, a preliminary survey of the floor plate was conducted to observe the appearance of its components when fixed appropriately for the Bluo-gal histochemical reaction. The fixation protocol optimized for histochemistry preserved ultrastructural detail sufficiently to enable floor plate cells to be clearly distinguished from decussating axons (Fig. 3). The ultrastructural appearance of each these components corresponded to those described in previous studies: floor plate cells possessed an electron-dense cytoplasm containing numerous single or clustered, electron-dense glycogen granules, while, in contrast, axons were uniformly more electron lucent (Sturrock, 1981; Uehara and Ueshima, 1984; Yoshioka and Tanaka, 1989).

The ultrastructural appearance of floor plate cells and their relationship to axons confirmed and extended the impression of their morphology that had been gained by observations at the light microscopic level (Fig. 3). Basal processes appeared to be rostrocaudally flattened and also to undulate in and out of the plane of section, since they often appeared as short discontinuous segments. Each of these individual segments had an irregular profile, which appeared to result from an alteration of the contour of the floor plate cell basal process to conform to that of axons adjacent to it. Where axons crossed a basal process they

often appeared to be embedded in its surface: axons cut in longitudinal section were frequently interposed between the divided segments of a basal process whose margins followed the axon's dorsal and ventral contours. Extending laterally from the basal processes were many fine processes of variable calibre. As has been observed in the rat (Yoshioka and Tanaka, 1989) and the chick (Yaginuma and Oppenheim, 1991a) these processes were extended along commissural axons and in some cases appeared to wrap around them. The net impression that these features provided was that floor plate cells, through the conformation of either their basal processes or the lateral processes these in turn gave rise to, had insinuated themselves into all available space in order to envelop, as completely as possible, the axons in their vicinity.

The fine lateral branches of the floor plate cell basal processes possessed the electron-dense cytoplasm and glycogen granules that were seen elsewhere in the cell. This suggested that if the long stained profiles were also extensions of floor plate cells they would be easily identified as such on the basis of these features. To identify such processes for E. M. analysis, 3 µm plastic sections were surveyed under the light microscope; those containing stained transverse profiles of suitable length were re-sectioned at the ultrathin level. Bluo-gal cleaved by cytoplasmic β-galactosidase is typically deposited on the nuclear or plasma membrane rather than within the cytoplasm. In these preparations, Bluo-gal reaction product outlining the long transverse profiles was readily detected. Unexpectedly all such profiles appeared to be commissural axons; none displayed any of the features typical of floor plate cell cytoplasm (Figs 4 and 5).

All Bluo-gal-stained floor plate cells had a typical ultrastructural appearance (Fig. 5), indicating that the long stained transverse profiles were not branches originating from a group of transgene-expressing floor plate cells with

an atypical cytoplasmic composition. Therefore, unless floor plate cells gave rise to a population of branches having previously unrecognized axon-like cytoplasm, this result strongly suggested that β–galactosidase was, instead, present within a restricted segment of some commissural axons.

This finding was not anticipated. β–galactosidase activity had not, either with Bluo-gal or with the much more sensitive fluorescent substrate FDG (data not shown) been detected in either the cell bodies giving rise to these axons or in the intervening axonal segments proximal to the floor plate. Although the possibility existed that, like L1, the transgene product was targeted by the neuron to a restricted axonal segment, the β–galactosidase expressed by these mice is encoded by a bacterial gene, which would not be expected to encode signal sequences capable of mediating such a specific subcellular localization. Therefore, any axonal targeting of the enzyme within the neuron would require that β–galactosidase be expressed as a fusion protein containing the necessary signal sequences. In contrast to the series of theoretical but highly unlikely integration events that would be required to achieve this, the construct employed in this experiment as well as its derivatives have been observed to express authentic β-galactosidase in response to enhancer elements adjacent to multiple sites of insertion (Gossler *et al.*, 1989).

Another factor strongly arguing against the possibility of neuronal expression of *lac* Z was the invariable proximity of stained axonal segments to stained floor plate cells. In mosaic neural tubes, floor plate cells expressing the transgene were scattered seemingly at random along the length of the floor plate (Fig. 2D). If the transgene were expressed in commissural neurons and transported to the midline segments of their axons, these stained segments should also be distributed randomly along the length of the floor plate.

However, their distribution should show no intimate relationship to that of stained floor plate cells. We reasoned that the presence or absence of such a relationship would be particularly evident in neural tubes containing a low proportion of staining floor plate cells. Therefore, from two such mosaic neural tubes a series of 50 sections of 3 μm each were analyzed, and each section was scored for the presence or absence of stained floor plate cells and of stained axonal profiles (Fig. 6). The majority of sections containing stained floor plate cells also contained stained axonal profiles. Conversely, in one region a gap of 50 μm contained no stained floor plate cells and within it not a single stained axon was found. In only three cases were labelled transverse profiles seen in sections not containing stained basal processes; in each case, stained floor plate cells were found in the adjacent section, indicating that these axons had been isolated by the plane of sectioning. This result was consistent with the impression gained from earlier observation of thicker sections that the transverse profiles tended to be close to, although not continuous with, stained floor plate cells. Furthermore, this indicated that the presence of β–galactosidase activity within a midline axonal segment depended absolutely on the expression of the enzyme in an immediately-adjacent floor plate cell.

Discussion

The guidance requirements of developing commissural axons in the region of the neural tube's ventral midline are potentially complex. In this small space, axons originating bilaterally converge and must grow past one another with directly opposing trajectories. Moreover, between the time they enter the floor plate and the time they leave it, commissural growth cones must relinquish their chemotropic attraction to floor plate cells and gain the ability to initiate and follow a rostral trajectory within the ventral funiculus. Since the adhesive glycoproteins expressed on commissural axonal membranes change in register with the floor plate, the normal trajectory changes made by these axons may require a coordinated interplay between the external guidance cues encountered by their growth cones and floor plate-dependent shifts in their ability to respond to these cues (Dodd *et al.*, 1988). The importance of the floor plate for local axon guidance is highlighted by the disturbances in commissural axon trajectory that occur when it is absent (Bovolenta and Dodd, 1991). However, the nature of the guidance cues floor plate cells may provide, or how these cells might interact with growth cones to change their pathfinding characteristics remains largely unresolved.

The morphological features of floor plate cells reported here suggest several ways in which these cells may guide and otherwise interact with growing commissural axons. The basal processes of stained floor plate cells appeared flattened in the rostrocaudal axis. A similar morphology has been observed for the basal processes of chick floor plate cells, which commissural growth cones contact closely as they cross the midline (Yaginuma *et al.*, 1991a). In combination with their specialized adhesive properties, the large surface area this morphology

would provide could serve as an attractive substrate for commissural growth cone extension. In addition, the aggregate effect of many floor plate cells with this architecture might be to present a physical barrier to rostrocaudal growth within the floor plate, such that growth cones would be channeled into following a strictly transverse trajectory (Yaginuma *et al.* 1991a). Neuroepithelial cells in more lateral regions of the neural tube also appear flattened, and have been proposed to constrain the growth of neuronal processes to the transverse plane in an analogous manner (Holley, 1982). The combination of such physical channelling with adhesive characteristics supporting growth cone extension might be sufficient to ensure that axons arriving at the floor plate continue to grow straight through it. This would constitute a solution to the problem of guiding similar axonal populations bidirectionally across the same space: since both adhesion and physical channelling would function equally in either direction, the direction in which axons extended within the floor plate would be determined solely by the direction from which they approached it.

Other features of floor plate cell morphology appear to be directed toward an interaction with the axon segments lying behind growth cones. The basal processes of floor plate cells had a highly irregular morphology in cross section, which appeared to be due to an alteration in their shape to conform to the contour of the axons crossing them. Their many fine lateral processes extended further along these axons and in many cases appeared to enwrap them. This arrangement appeared to produce a large area of apposition between floor plate cells and axons. Since the growth cones of these axons would already have grown past these regions of apposition, this would not serve any apparent purpose in supplying physical or other guidance cues to the growth cone surface. However, it might serve to separate axons within the floor plate from one

another; since at later stages L1 is expressed on axonal segments lying within the floor plate (Dodd *et al.*, 1988), contralaterally originating axons meeting in this region might otherwise fasciculate inappropriately on one another and be misrouted.

In addition, such an extended region of membrane apposition between floor plate cells and axons might be of direct relevance to the unexpected observation made here that β-galactosidase activity was localized within linear profiles traversing the floor plate. When they were initially observed, the most plausible explanation for these profiles was that they represented extended lateral branches of floor plate cells. However, in E. M. preparations, their ultrastructural appearance did not resemble that of floor plate cells, but instead was indistinguishable from that of commissural axons. While this immediately suggested that the transgene was expressed by commissural neurons, it is difficult to account for the restricted distribution of the enzyme on this basis. Although little is known about the relative stability of β-galactosidase in different regions of growing axons, it would be unlikely that neuronally synthesized enzyme would be detectable only in axonal segments lying within the floor plate. Furthermore, even in the unlikely event that the transgene-encoded β-galactosidase had acquired the signal sequences that would be required to mediate such a specific midline localization, the observed co-distribution of stained axonal segments and stained floor plate cells along the length of the neural tube would not be expected. The remaining formal possibility that floor plate cells extend lateral branches of extemely dissimilar cytoplasmic composition from the remainder of the cell can not be ruled out. However, it appears unlikely in view of the homogenous distribution of cytoplasmic features,

such as glycogen granules, throughout other parts of these cells, including their fine lateral processes.

Thus, while the association of the reaction product with the floor plate suggests that it is derived from expression of the transgene within floor plate cells, the ultrastructural appearance of the labelled transverse profiles suggests equally strongly that they are segments of commissural axons. The most parsimonious explanation is that β-galactosidase is expressed by floor plate cells and transferred to adjacent commissural axons. This possibility has several attractive theoretical features with respect to the present results. First, it may be an explanation of why floor plate cells establish a high degree of membrane apposition with decussating axons, as this would be an expected requirement of any mechanism mediating direct intercellular transfer. In addition, it could explain the observation that although stained profiles were invariably close to stained floor plate cells, the two often appeared discontinuous. If the mechanism of transfer were patent only transiently, and if β-galactosidase was relatively stable within the axon, shifts in tissue architecture occurring as development continued could separate stained axons from the floor plate cell from which they received the enzyme. Such transient contact would further explain why each floor plate cell, although in proximity to many axons, was associated with only a few stained axons at a time. Furthermore, in many cases stained axon segments had, at one end, stained profiles with the appearance of growth cones, raising the intriguing possibility that molecules normally expressed by floor plate cells could become functional components of growth cones.

Due to the foreign nature of β-galactosidase, any transfer of this protein from floor plate cells to axons would require it to have been passively included in a process that normally operates within floor plate cells for some other reason.

Although floor plate cells possess ultrastructural features suggesting that they are active at secretion (Tanaka *et al.*, 1988; Yoshioka and Tanaka, 1989), the uptake by growth cones of extracellularly secreted β-galactosidase would not readily account for our observations. Instead, the restricted number of stained axons per stained floor plate cell and their comparable intensity of staining suggests that the mechanism of transfer involves a specific interaction between floor plate cells and axons. Because the transfer process would have to be relatively non-specific with respect to the molecules involved, other soluble molecules within floor plate cytoplasm would be expected to be distributed in the same way. Consistent with this prediction, the EGF receptor tyrosine kinase substrate p35 has been found to be expressed in floor plate cells and also to be distributed along axons lateral to the floor plate, but not in other axonal segments or neuronal perikarya (McKanna and Cohen, 1989).

The existence of a process capable of accommodating macromolecular transfer from floor plate cells to axons would appear initially to be at odds with current concepts of how growth cones interact with their environment for the purposes of pathfinding (e.g. Goodman *et al.*, 1984; Dodd and Jessell, 1988). However, evidence from other systems has demonstrated that intercellular protein transfer may be an important component of mature axon-glial relationships as well as intercellular signalling processes in development.

A number of studies on the squid (Gainer et al, 1977; Lasek et al, 1977; Lasek and Tytell, 1981; Tytell and Lasek, 1984), crayfish (Andersson *et al.*, 1969; Meyer and Bittner, 1978; Peracchia, 1981) and newt (Singer and Salpeter, 1966; Singer and Green, 1968) have suggested that macromolecular transfer between glial cells and mature axons is possible and in certain cases may be a common process. One role hypothesized for this transfer is in response to the separation

of axon segments from the protein synthetic machinery of the neuron cell body: in the case of an urgent demand for particular proteins within an axon segment, either for physiological regulation or in response to injury, the protein synthetic capacity of the adjoining glial cells could be exploited (Tytell *et al.*, 1986). Studies of the squid giant axon have shown that as many as 80 different proteins, representing a wide range of molecular weights and charge may be transferred (Tytell and Lasek, 1984). Although this would require that the transfer process be relatively non-discriminatory with respect to the proteins involved, the kinetics of transport suggest that it involves direct transfer of cytoplasmic components from glia to axons (Gainer *et al.*, 1977). Fingerlike protrusions of the glial cell membrane may be engulfed pinocytotically by the axon (Gilbert *et al.*, 1982; Tytell and Lasek, 1984); alternatively, regions of membrane fusion may permit direct communication of axonal and glial cytoplasm (Peracchia, 1981). If either of these mechanisms operated between floor plate cells and commissural axons, they would appear capable of mediating transfer of β-galactosidase without the need for special targeting of the enzyme.

The process operating between floor plate cells and commissural axons might not be intended to transfer protein per se, but rather to transmit a specific signal. In the developing Drosophila retina, the normal differentiation of the R7 photoreceptor cell requires stimulation of the sevenless tyrosine kinase receptor on its membrane by the boss ligand present on the adjacent membrane surface of the R8 cell (Van Vactor *et al.*, 1991). The boss ligand is an integral membrane protein, having seven transmembrane domains as well as extracellular N-terminal and intracellular C-terminal domain (Hart *et al.*, 1990). Surprisingly, stimulation of the sevenless receptor with boss was found to be accompanied by internalization of the entire boss protein within the R7 cell; the presence of boss-

immunoreactive vesicles inside sevenless-expressing cells suggested a mechanism in which R7 cells phagocytotically engulf microvillar R8 membrane processes by R7 cells (Cagan *et al.*, 1992). This process closely resembles that proposed for glial-axonal transfer in the squid and would similarly appear capable of transferring cytoplasmic β-galactosidase.

The normal transfer of either cytoplasmic proteins or a specific boss-like signal from floor plate cells to commissural growth cones clearly could be of significance to the way in which changes in the growth cones' pathfinding properties are effected in this region. The machinery by which growth cones navigate to the floor plate by homing in on a diffusible chemotropic molecule is likely to be quite different from that by which they are steered within the ventral funiculus, where they fasciculate on other axons. The differences potentially include not only the membrane receptors involved, but the way in which these interact with the cytoskeletal elements that are directly involved in generating motility. As evidenced by the distribution of L1 and TAG-1, changes in pathfinding ability appear to require correctly-timed shifts in the synthesis and transport of proteins from the neuron cell body, as well as distribution of these molecules in register with the floor plate. Molecules transferred from floor plate cells could directly interact with a number of intra-axonal processes, affecting the processing or insertion of new membrane receptors or directly engaging or disengaging their interaction with the motile cytoskeleton. Moreover, traversal of the floor plate appears to endow growth cones with the ability to respond to an as yet undefined rostral signal; molecules transferred from floor plate cells might actively mediate this change in sensitivity. Whether they were cytoplasmic or membrane proteins, the effects of transferred floor plate molecules would be locally restricted and would not require a delay for either

retrograde transmission of a signal to the neuron or subsequent axonal transport of newly synthesized proteins. In addition, their effects would be absolutely dependent on the presence of the growth cone within the floor plate, thereby eliminating the possibility of an inappropriately early response to guidance cues, particularly those of the ipsilateral ventral funiculus.

The role of floor plate cells might therefore exceed that of a supportive substrate or signpost, encompassing the active modification of commissural axon pathfinding properties. In an analogous way, by secreting the slit protein, midline glia in the developing Drosophila nervous system have been proposed to actively modify the pathfinding properties of axons which, like commissural axons, assume a longitudinal trajectory after crossing the midline (Rothberg *et al.*, 1988, 1990). The slit protein is found not only on the surface of the glial cells but also on the midline and longitudinal portions of the axons. However, neither *slit* gene expression nor the protein itself can be detected in the corresponding neuron cell bodies or proximal axon segments, indicating that the protein is transferrred to the axons by the glial cells. The characteristics of slit indicate that it may bind to both axonal membrane receptors and the extracellular matrix via different domains, suggesting that its application to the axonal membrane may change the membrane's adhesive properties and thus the adhesive cues the growth cone follows (Rothberg *et al.*, 1990).

In conclusion, the expression of transgene-encoded β–galactosidase has provided a unique opportunity to view the morphology of floor plate cells. This has indicated that floor plate cells possess morphological features which may provide a directional scaffold for decussating axons and, in addition, permit an intimate association between floor plate cells and axonal membranes. The function of the latter role, as suggested by the additional localization of β-

galactosidase to midline commissural axon segments, may be to mediate a more general process of macromolecular transfer. The chacterization of further proteins synthesized by floor plate cells should provide additional opportunities to evaluate such floor plate-to-axon transfer. In addition, the regulatory sequences of floor plate specific genes may be useful in targeting the expression of further foreign proteins to the floor plate. This approach could yield additional evidence for or against a generalized process of macromolecular transfer and may in addition lead to direct manipulation of the pathfinding mechanisms involved. If the present observation can be substantiated, macromolecular transfer may be found to play a significant role in those regions of the developing nervous system where growing axons choose among different guidance cues to make trajectory changes in the presence of intermediate targets.

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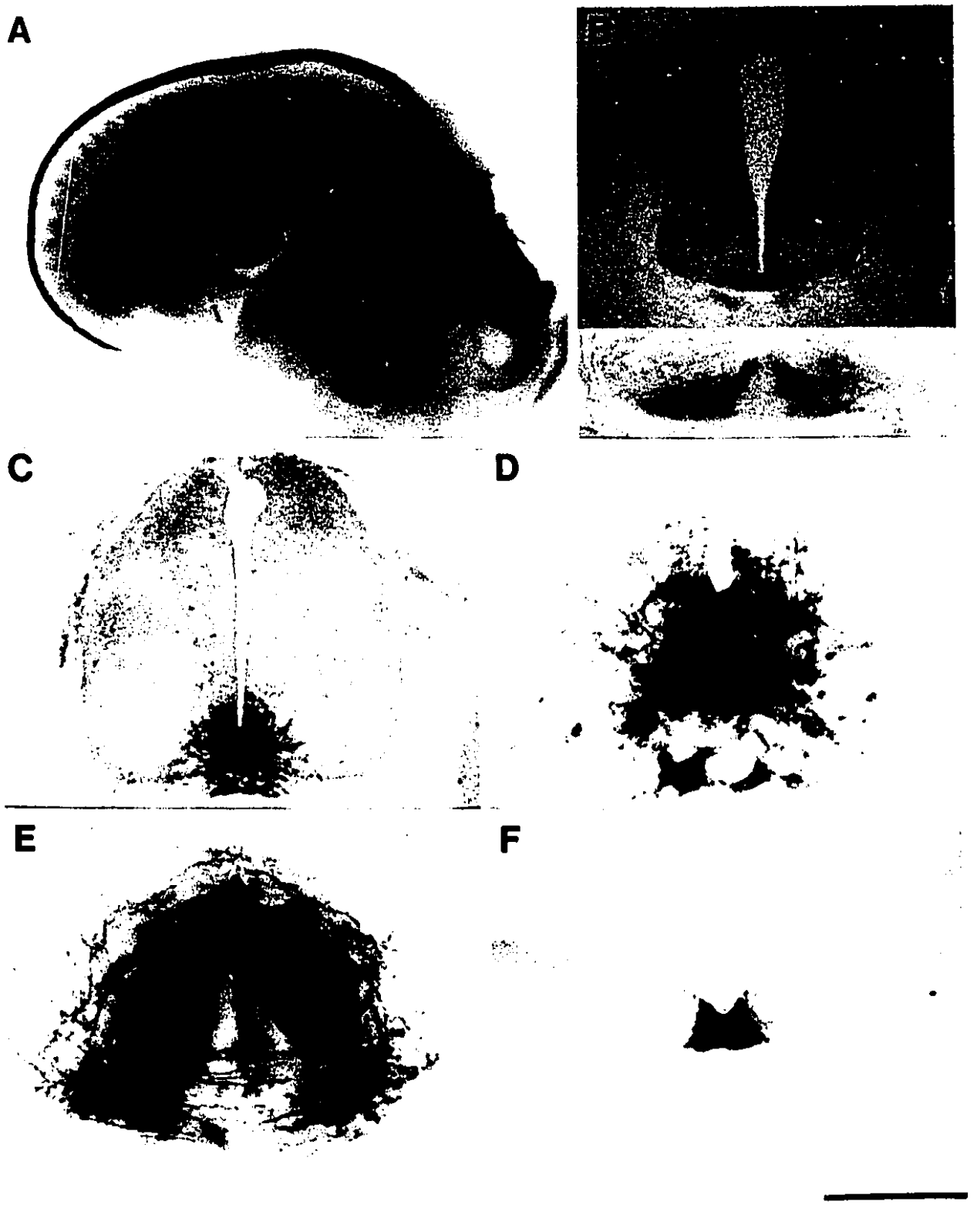
Fig. 1. The floor plate of Tg4 transgenic embryos is stained by the β-galactosidase substrate Bluo-gal. (A) A sagittally-hemisected E12.5 embryo, showing that the transgene is expressed throughout the rostrocaudal extent of the floor plate. (B) Cross section of E10.5 neural tube. At this stage, soon after the onset of transgene expression, few axons have crossed the floor plate. The stained cells lie in the lateral floor plate; at higher magnification (inset) they can be seen to have only short basal processes, their cell bodies occupying most of the floor plate's thickness. (C, D) At E12.5 cells within and surrounding the floor plate are labelled, including indistinctly-stained tissue in the mesenchyme ventral to the floor plate. (G, H) E11.5 floor plate in the caudal hindbrain (G) and pontine flexure (H). In this region, the stained floor plate has a different shape, revealing, in addition to radially oriented floor plate cells, stained profiles traversing the floor plate mediolaterally.

Calibration bar: A=2.3mm; B, C and G=316μm; B (inset) and E=50μm; D=79μm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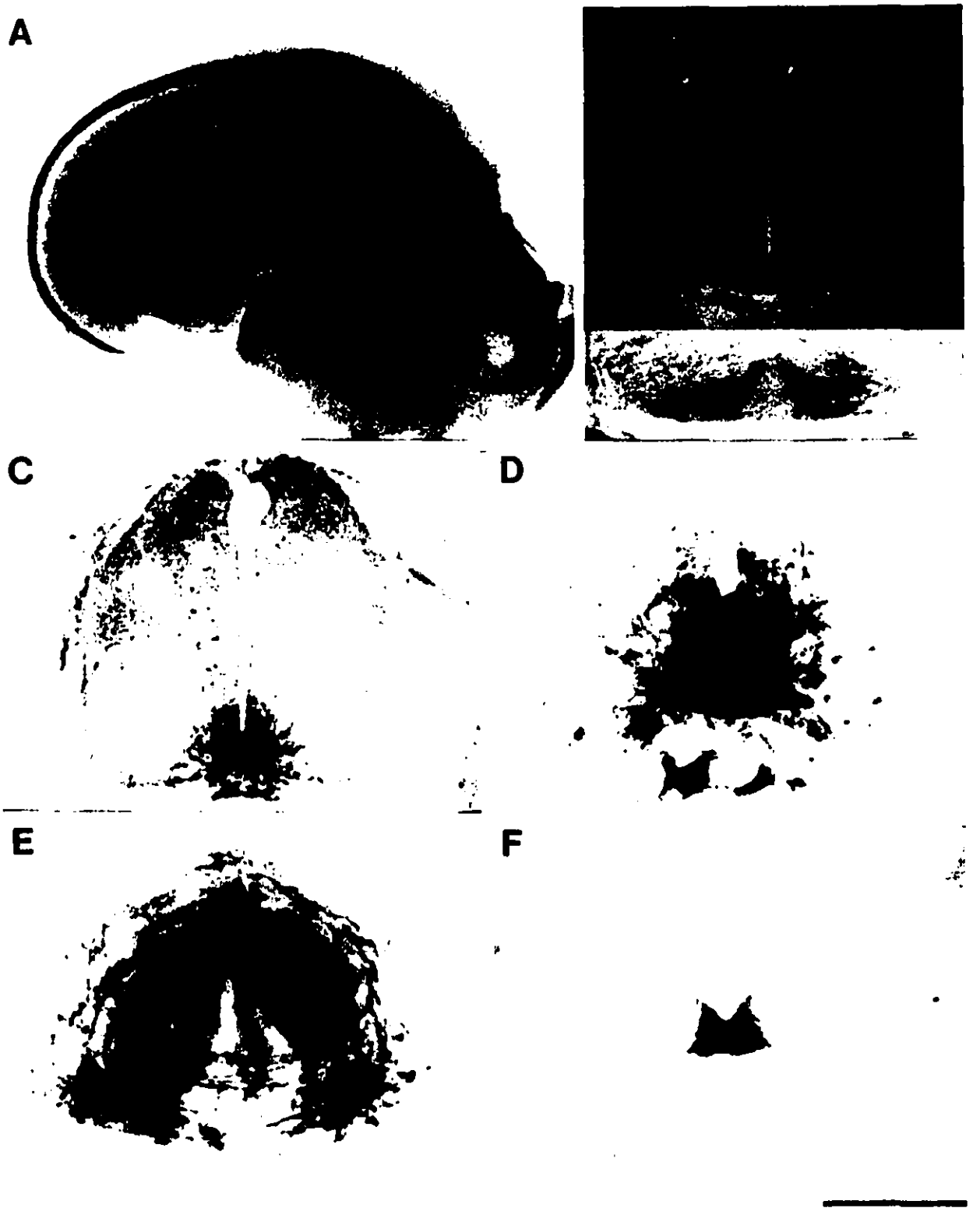
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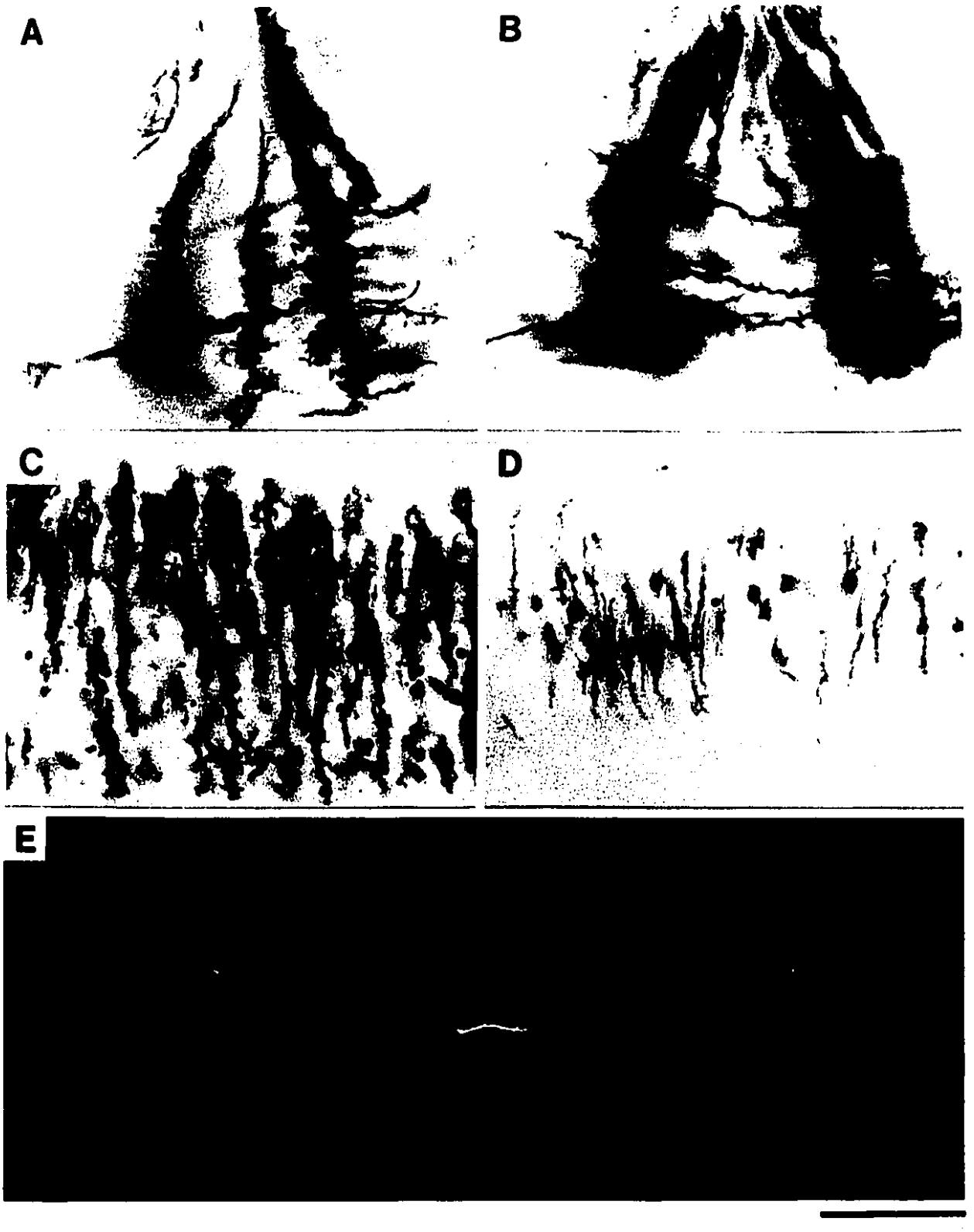
Fig. 2. Epigenetic modification of the Tg4 transgene DNA results in mosaic expression. E12.5 embryos. (A, B) Cross sections of Bluo-gal-stained floor plate, showing the clarity with which morphological details can be observed. The basal processes of floor plate cells are widened transversely and possess a brushlike layer of fine lateral processes (A). In addition, longer stained transverse processes are clearly evident (B). Transverse profiles often do not appear to arise from floor plate cells–in this photomicrograph, showing several transverse profiles, the nearest stained floor plate cells are in a different plane of focus. (C) Sagittal section of floor plate; ventral is down. The basal processes of floor plate cells are not as wide as they appear in cross sections (compare A), suggesting that they are rostrocaudally flattened; the dots interspersed with them are cross sections of transverse profiles. (D) Frontal section taken through the marginal layer of the floor plate. Here radial floor plate processes appear in cross section as dots and linear transverse profiles can be seen to be oriented transversely to the floor plate's longitudinal axis. (E) Ventral view of unfixed floor plate whole mount, in which cells have been loaded with the fluorescent β-galactosidase substrate CMFDG. Stained floor plate cell basal processes appear foreshortened. As seen with Bluo-gal labelling, transversely oriented linear profiles are also present.

Calibration bar: A and B=32μm; C=50μm; D and E=79μm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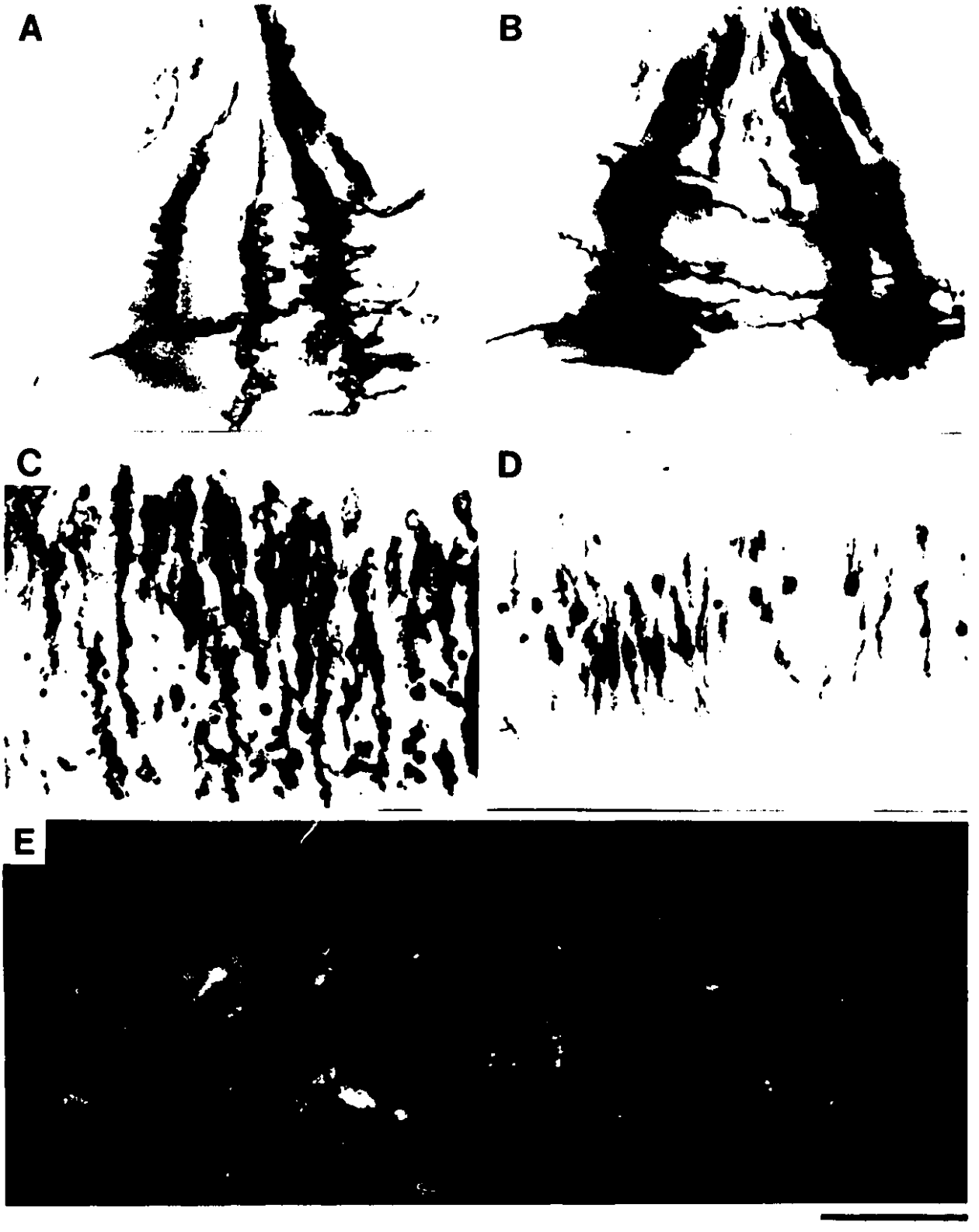
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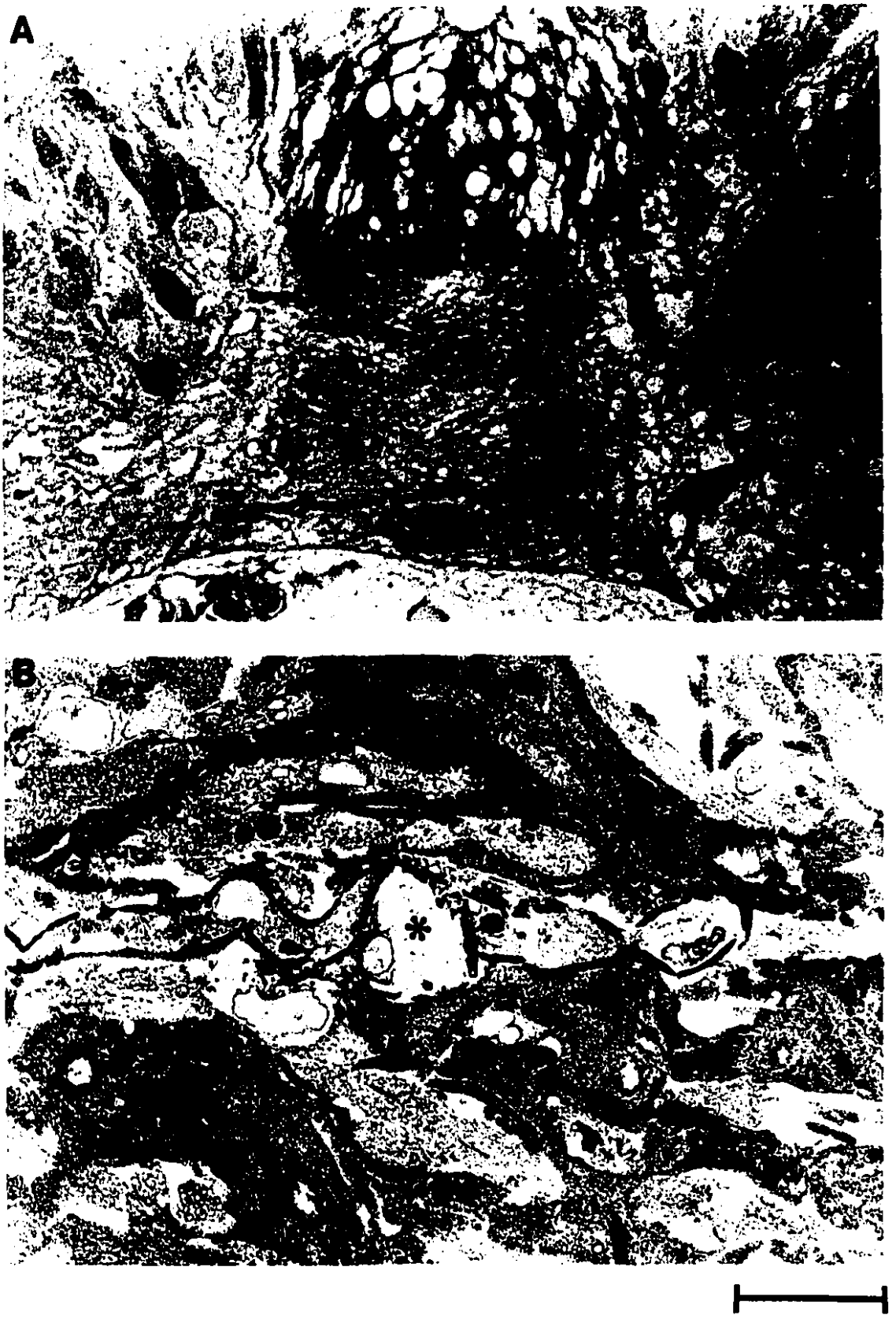
Fig. 3. The ultrastructural appearance of the floor plate suggests a high degree of membrane apposition between floor plate cells and decussating axons. Three radial floor plate cell processes can be seen. A portion of the nucleus of the cell on the extreme right is also visible. Floor plate cell cytoplasm is relatively electron dense compared to that of transversely oriented axons. Basal processes often appear in sections as short segments (e.g. middle process); they have an irregular contour which appears to result from structural modifications directed toward increasing membrane apposition with axons. Included in these alterations are transverse processes of fine calibre extended along axons (particularly evident arising from basal processes at left). A small amount of highly electron dense Bluo-gal reaction product, associated with another floor plate cell out of the plane of section, is visible at right.

Calibration bar = 3.4μm

Fig. 4. Stained transverse profiles have the ultrastructural appearance of axons, and not floor plate cells (A) Light micrograph of Bluo-gal stained floor plate, showing labelled transverse profile. The label at the left end of the profile in a manner consistent with its being a growth cone. Resectioning and viewing under electron microscope (B) shows that Bluo-gal, which typically deposits on cell membranes of β-galactosidase-containing cells, clearly outlines a profile with electron-lucent cytoplasm that is indistinguishable from other commissural axons. The region marked by the arrowhead in A corresponds to that marked by the asterisk in B. Note the difference in the cytoplasmic appearance of this process to that of floor plate cells, which are electron dense and contain numerous glycogen granules. Note also the high degree of membrane apposition between floor plate cells basal processes with axons.

Calibration bar: A=31μm, B=2μm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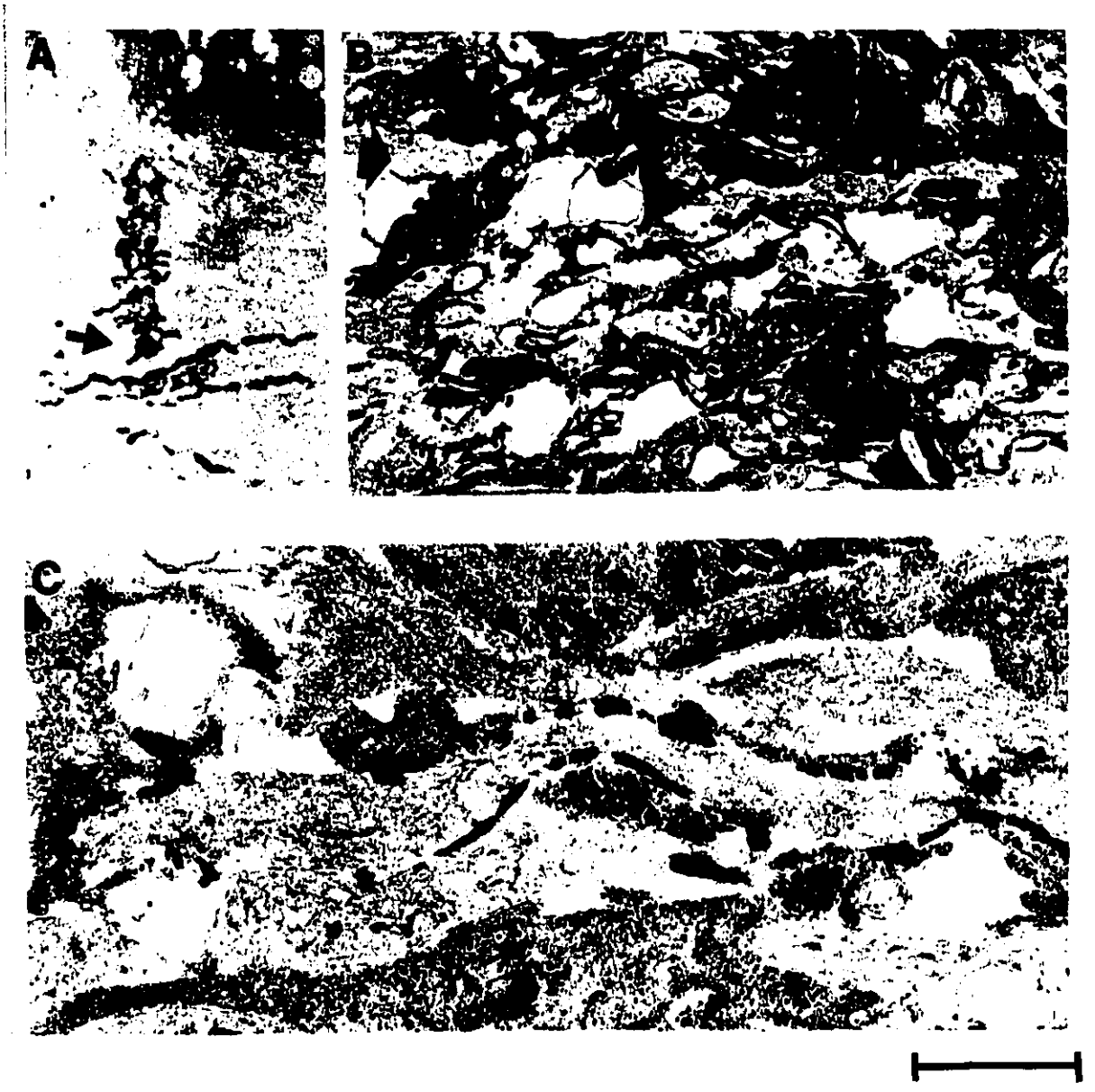

Fig. 5. Stained floor plate cells have a typical ultrastructural appearance which is distinct from that of stained axons. (A) Light micrograph of stained basal floor plate process and nearby transverse profiles. (B) Re-sectioning for E. M. analysis shows ventral portion of floor plate cell (arrow, corresponding to region marked with arrow in A) to have typical cytoplasmic features. In contrast, transverse profiles have homogeneous, more electron lucent cytoplasm. (C) Ultrastructural features of a further transverse profile are consistent with the suggestion that these represent stained axonal segments.

Calibration bar: A=63μm, B=3μm, C=1.6μm

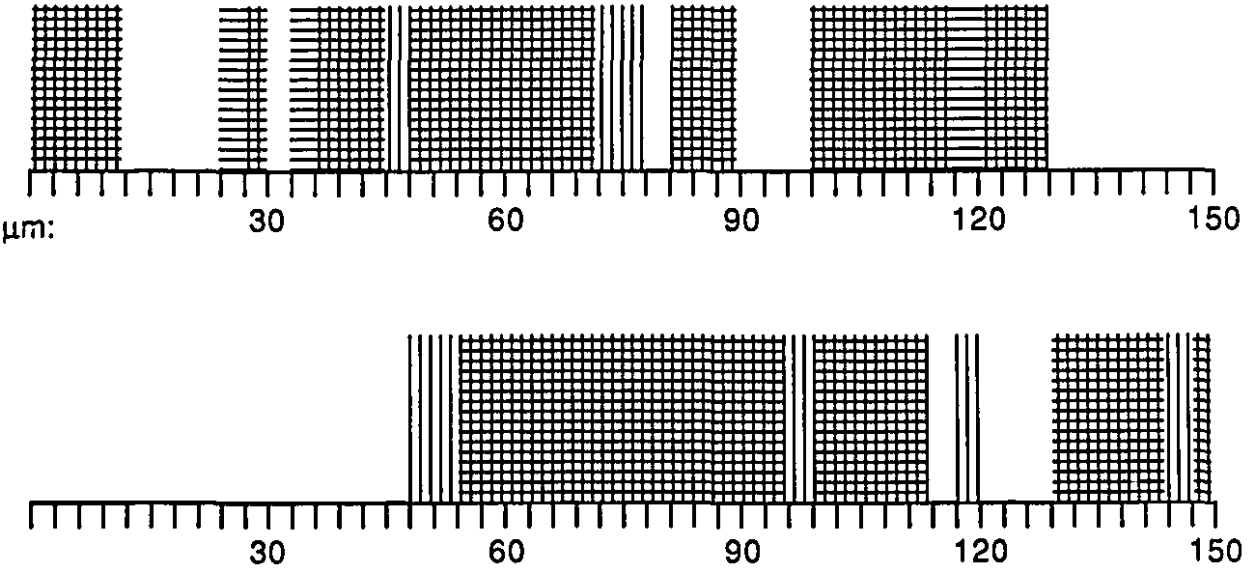

Fig. 6. In each of 50 serial 3µm-thick sections from two mosaic neural tubes, stained transverse profiles were found only near stained floor plate cells. the vertical lines indicate the presence of stained floor plate cells, while the horizontal lines denote the presence of transverse profiles. In all cases, transverse profiles were found either within sections containing the stained floor plate cells or in the adjacent section.

Chapter 4 General Discussion

Preface

In the Tg4 line, the conferral of a floor plate-specific expression pattern on a transgene inserted at the *dt* locus raised the possibility that floor plate cells normally express the *dt* gene and might thus be the primary target of the *dt* mutation. To investigate this possibility, *dt/dt*↔+/+ chimeras were constructed, in which the axons of wild-type neurons were identified by the expression of an *hNF-L* transgene. In these chimeras, the axons of mutant, but not wild-type spinal cord neurons developed characteristic swellings, defining the existence of an intrinsic defect within the affected neurons. In addition, this study revealed that phosphorylated neurofilament epitopes accumulate inappropriately in the perikarya of affected neurons, suggesting that a disorder of neurofilament processing is present in *dt* and may play a role in its pathogenesis.

The intrinsic neuronal action of the *dt* mutant defect was inconsistent with the hypothesized location of the defect within the floor plate, and suggested that the expression of the transgene in these cells occurred in response to the regulatory elements of a gene related to the *dt* gene only by linkage. Despite its novel origin, the expression of the histochemical marker β-galactosidase in floor plate cells, and in particular its mosaic character, presented a unique opportunity to examine their morphology throughout development. Using this marker, I observed that floor plate cells possess a number of morphological features consistent with their suggested role as a directional scaffold for decussating commissural axons. These cells also appeared to establish a high degree of membrane apposition with nearby axons. Unexpectedly, β-galactosidase activity appeared to be localized within midline segments of commissural axons adjacent to stained floor plate cells. This raised the intriguing possibility that a process involving macromolecular transfer might normally operate between floor plate cells and commissural axons.

2. Localization of the *dt* mutant defect by chimera analysis.

In *dt/dt*↔+/+ chimeras, axonal swellings, a characteristic pathological feature of *dt*, were not found in the subpopulation of axons originating from wild-type neurons. However, swellings were present within the total axonal population, indicating that they occurred on the axons of mutant neurons. Control studies showed that the expression of the *hNF-L* transgene did not prevent the development of the mutant phenotype in *dt/dt* mice. Therefore, the restriction of axonal swellings to neurons of mutant genotype defined the existence of an intrinsic neuronal defect in *dt*. Although technical limitations prevented the inclusion of DRG neurons in this analysis, the similarity in the appearance and timing of the pathological features exhibited by their axons suggests that they also are affected by an intrinsic *dt* mutant defect.

In the chimeras studied, the environment of the neurons under analysis would be expected to consist of both mutant and wild-type cells of all neuronal and non-neuronal types. The results indicate that such wild-type cells were not able to rescue mutant neurons expressing the *dt* defect; conversely, mutant cells were not able to impose the mutant phenotype on the axons of wild-type neurons expressing the normal *dt* gene product. However, the formal possibility exists that the development of axonal swellings by mutant neurons requires the additional contribution of another mutant cell type. For example, swellings might have occurred only where the axons of mutant neurons were myelinated by mutant Schwann cells. Similarly, it is formally possible that wild-type neurons, if placed in an entirely mutant environment, would develop the mutant phenotype. However, as it would be expected that the immediate environment of some wild-type neurons in chimeras would be enriched in mutant cells, in this

case some swellings, of the thousands observed, should have been detected on the axons of wild-type neurons.

In spite of such formal considerations, the observed requirement for mutant genotype in affected neurons rules out previous hypotheses of *dt* pathogenesis which postulated the development of axonal swellings via influences extrinsic to the neuron. The variety of such extrinsic theories highlights the utility of chimera based analysis in cases where observation of the affected cell types leads to no clear conclusion regarding the primary target. These include those involving peripheral sensory receptor defects (Sotelo and Guenet, 1988), growth factor deficiencies (Ebendal and Lundin, 1984), and primary defects in myelinating cells (Moss, 1980, 1981a, 1981b).

In those cases where a given cell type is involved in the disease pathology via an an extrinsic influence, complementary chimera analyses can be used to identify the other cell type responsible, thus providing a great deal of information as to the pathogenetic process. In contrast, the finding, as in the current study, of an intrinsic defect does not by itself provide any further clues as to the pathogenetic process. The intrinsic neuronal action of the *dt* mutant defect is consistent with hypotheses involving a primary disturbance of axonal flow (Janota, 1972; Hanker and Peach, 1976), but does not provide any further support for this specific possibility. However, the additional observations yielded by neurofilament immunohistochemistry in the present study provide additional details of dt histopathology which may be significant to the pathogenetic process.

3. Neurofilaments in *dt* pathogenesis.

Neurofilaments have been noted to be a prominent component of the axonal swellings of *dt* (Janota, 1972; Sotelo and Guenet, 1988). The use of neurofilament immunocytochemistry provided a means of directly examining the distribution and phosphorylation state of specific neurofilament epitopes in affected *dt/dt* neurons. Immunoreactive hNF-L as well as phosphorylation epitopes of NF-M and NF-H were detected in spinal cord axonal swellings in both affected *dt/dt* mice and *dt/dt*↔+/+ chimeras. In addition, this study revealed that a length of more moderately increased calibre frequently adjoined focal axonal swellings, and that these segments were also strongly immunoreactive for neurofilament epitopes. Finally, these experiments revealed that neuronal perikarya, which in favourable sections were observed to be associated with swollen axonal segments, were inappropriately immunoreactive for phosphorylated neurofilament epitopes.

The accumulation of neurofilaments in association with their inappropriate phosphorylation in neuronal perikarya raises the possibility that a disturbance of neurofilament handling is present in *dt* and may be important to its pathogenesis. While the normal function of neurofilaments is not completely understood, one role appears to be a structural one, in particular the maintenance and regulation of axonal calibre (Lasek *et al.*, 1983; Hoffman *et al.*, 1984, 1987; Monaco *et al.*, 1989). Particularly in large myelinated axons, a correlation is seen between the number of neurofilaments and the diameter of the axon (Friede and Samorajski, 1970; Lasek *et al.*, 1983). In conditions where neurofilament synthesis is diminished, as in the period following axotomy, the decline in neurofilament content in the axon is accompanied by parallel decreases in its diameter

(Hoffman *et al.*, 1984, 1985; Oblinger and Lasek, 1988). The observed correlation between neurofilament content and axon diameter is consistent with the possibility that accumulation of neurofilaments might be responsible for the disturbances of axonal calibre in affected *dt/dt* neurons.

The *dt* locus does not correspond to the site of any of the neurofilament genes. However, a potential site where the transport of neurofilament proteins could be affected by the primary or secondary effects of a mutation is the phosphorylation of neurofilament tail domains, which has been proposed to affect their rate of transport (Schlaepfer, 1987). NF-M and NF-H normally begin to be phosphorylated in their carboxy-terminal tail regions as they enter the axon, and they continue to be phosphorylated throughout their transport through the axon (Sternberger and Sternberger, 1983; Nixon *et al.*, 1987). The principal sites of phosphorylation are on serine residues residing within conserved amino acid sequences containing the sequence lysine-serine-proline; the tail domain of NF-H contains over 200 such sites (Lee *et al.*, 1988). These phosphorylation epitopes are recognized specifically by monoclonal antibodies such as SM1-31 (Sternberger and Sternberger, 1983; Shaw *et al.*, 1986). Neurofilament phosphorylation has been proposed to mediate the normal regulation of axonal calibre via the rate by which neurofilaments are transported through the axon; slowing the rate of transport relative to the rate of synthesis would increase the axonal neurofilament content (Lewis and Nixon, 1988; Nixon and Sihag, 1991). One proposed mechanism is that phosphorylation of NF-H and NF-L tail domains, by increasing charge density on the sidearms, causes these to protrude further from the neurofilament core (Carden *et al.*, 1987; Lee *et al.*, 1987, 1988). In addition to directly increasing axonal diameter (de Waegh *et al.*, 1992) this could cause steric drag to transported neurofilaments, as well as uncovering

sites by which neurofilaments could interact with stationary elements of the axon: the combination of these two effects would be predicted to slow the transport of neurofilaments and favour their retention within the axon (Nixon and Sihag, 1991). It has been further proposed that in disease states, excessive phosphorylation might slow neurofilament transport to the point where it was arrested completely, leading to their accumulation (Nixon and Sihag, 1991).

Study of animals exposed to certain toxic agents supports a correlation between excessive neurofilament phosphorylation, accumulation of neurofilaments, and disturbances in axon calibre. One such model is the long term intrathecal administration of the toxic agent β,β'-iminodipropionitrile (IDPN). Within a few days of IDPN administration, swellings containing neurofilaments appear on the proximal portions of large sensory and motor axons (Chou and Hartmann, 1965). Over the next month, these enlarge to up to 100μm in diameter, but do not extend to more distal portions of the axon. IDPN intoxication has been shown to selectively impair the transport of neurofilaments, carried by slow transport, without impairing fast transport or other components of slow transport, the tubulin and tau proteins (Parhad *et al.*, 1986; Griffin *et al.*, 1978). As a consequence, neurofilaments accumulate in the proximal portion of the axon and are depleted in distal regions (Carden *et al.*, 1987). This slowing of neurofilament transport is correlated with increased phosphorylation of Lys-Ser-Pro epitopes on NF-H within axons (Watson *et al.*, 1989). A defect in intra-axonal transport, rather than elevated neurofilament synthesis by the cell body, is supported by experiments in which direct supperineurial injection of IDPN causes a focal disruption of the axoskeleton (Griffin *et al.*, 1983).

Intrathecal administration of aluminum salts to rabbits also leads to blockage of neurofilament transport, and is accompanied by perikaryal accumulation of phosphorylated epitopes. In this disease, a block in neurofilament transport within proximal axonal segments leads to the massive accumulation of neurofilaments within perkarya, dendrites, and proximal axonal segments (Klatzo *et al.*, 1965; Bizzi *et al.*, 1984; Troncoso *et al.*, 1985). Furthermore, treatment with aluminum increases the incorporation of inorganic phosphate into NF-H (Johnson and Jope, 1988); and phophorylated NF-H epitopes accumulate in proximal axonal segments and perikarya of affected neurons (Bizzi and Gambetti, 1986; Troncoso *et al.*, 1986; Pendlebury *et al.*, 1988).

Animals intoxicated with acrylamide also exhibit increased neurofilament phosphorylation (Howland and Alli, 1986), slowing of their axonal transport, and neurofilamentous axonal swellings (Gold *et al.*, 1988). For each of these intoxication syndromes, it has been proposed that a specific defect in neurofilament phosphorylation precipitates a blockage of their transport and thus their accumulation (Schlaepfer, 1987; Griffin *et al.*, 1978, 1983). Thus, one possibility in the case of the *dt* mutation is that it affects a kinase to increase its activity within the axon.

The perikaryal accumulation of phosphorylated neurofilaments in aluminum (Bizzi and Gambetti, 1986; Pendlebury *et al.*, 1988) and acrylamide (Gold *et al.*, 1988) intoxication would appear to support the presence of disordered neurofilament phosphorylation and to suggest a straightforward pathogenetic sequence that would apply also apply to *dt*. For example, a generally disordered kinase activity might be excessive both in axons and in perikarya; alternatively, axonally accumulated phosphorylated neurofilament epitopes might 'back up' into the perikaryon. However, data from other sources

do not support such a simple relationship. In IDPN intoxicated neurons, despite the presence of a severe and specific block in the transport of neurofilaments and their accumulation in proximal axonal segments, the accumulation of phosphorylated neurofilament epitopes in perikarya is not reported. In addition, high doses of acrylamide, which produce the greatest block in neurofilament transport, do not lead to perikaryal phosphorylated neurofilament accumulation, while chronic doses, which impair transport less, do lead to perikaryal immunoreactivity (Gold *et al.*, 1988). It has thus been proposed that immunoreactivity of perikarya to phosphorylated neurofilament epitopes represents a response of the neuron to axonal injury, rather than a specific manifestation of the interruption of neurofilament transport (Gold *et al.*, 1988).

This hypothesis is supported by the finding that a variety of treatments that do not appear to primarily affect neurofilaments nonetheless result in the perikaryal accumulation of phosphorylated neurofilament epitopes. These include axotomy (Drager and Hofbauer, 1984; Goldstein *et al.*, 1987; Klosen *et al.*, 1990) or axon crush (Rosenfeld *et al.*, 1987), transplantation of CNS neurons into ectopic CNS (Doering *et al.*, 1991) or PNS (Doering and Aguayo, 1987) locations, and deafferentation (Shaw *et al.*, 1988; Mansour *et al.*, 1989). Most surprisingly, application of colchicine, via a protocol leading to specific impairment of fast axonal transport, but little axon degeneration, leads to abnormal accumulation of phosphorylated neurofilaments in the perikarya of DRG neurons (Gold and Austin, 1991). However, despite the diversity of the conditions leading to its appearance, perikaryal neurofilament immunoreactivity does not appear to be a non-specific response of neurons to injury, as transection of ventral roots causes only a subpopulation of neurons to express abnormal reactivity (Mansour *et al.*, 1989). One possibility is that such treatments interfere with the delivery of a

target or afferent-derived trophic factor to the perikaryon, and that this through an unknown mechanism, is the cause of perikaryal neurofilament accumulation (Gold and Austin, 1991).

These studies therefore suggest no simple relationship between perikaryal accumulation of phosphorylated neurofilaments and neurofilament transport abnormalities *per se*. Furthermore, despite extensive study of both IDPN and aluminum intoxication, it has not yet been resolved whether primary neurofilament hyperphosphorylation leads to a block of neurofilament transport, or whether stagnant neurofilaments, whose transport is blocked for another reason, are secondarily hyperphosphorylated because of prolonged or inappropriate exposures to kinases (Troncoso *et al.*, 1986; Schlaepfer, 1987; de Waegh, 1992). The presence of the same features in *dt* provides a promising opportunity for their relationship to be determined. While not addressed here, double labelling of *dt/dt*↔+/+ chimeras would reveal whether motor neurons, which show little axonal pathology, develop perikaryal phosphorylated neurofilament immunoreactivity due to an intrinsic or extrinsic mutant defect. More generally, cloning of the *dt* gene and identification of its normal product will potentially allow the primary events in *dt* pathogenesis to be distinguished from secondary consequences. Such an understanding will be of great potential signficance not only to an appreciation of disease pathology but also to an understanding of normal neurofilament physiology.

4. Role of *dt* as a model for human neurodegenerative diseases.

On the basis of similarity in the distribution of affected fibers and the presence of an ataxic phenotype, *dt* has been considered an animal model for the

human disease Friedreich's ataxia (Sotelo and Guenet, 1988). However, in view of the neurofilamentous accumulation in *dt/dt* mice, an understanding of *dt* pathogenesis may also be of relevance to a number of human neurodegenerative diseases which involve the pathological accumulation of cytoskeletal components (Schlaepfer, 1987). For example, a hallmark of Alzheimer's disease is the accumulation, in the perikarya and degenerating neurites of affected brain neurons, of neurofibrillary tangles, consisting of accumulations of paired helical filaments, which, although different in structure from the normal neuronal cytoskeleton, bear antigenic similarity to it, particularly to neurofilaments (Cork *et al.*, 1986). Some monoclonal antibodies to phosphorylated neurofilament epitopes recognize the perikaryal neurofibrillary tangles in Alzheimer's disease, suggesting that they may arise via a common mechanism to the animal models discussed (Perry *et al.*, 1985; Sternberger *et al.* 1985; Cork *et al.*, 1986; Miller *et al.*, 1986; Coleman and Anderton 1990). Monoclonal antibodies recognizing phosphorylated neurofilament epitopes similarly label the proximal neurofilamentous swellings and perikaryally accumulated neurofilaments of motor neurons in amyotrophic lateral sclerosis (Manetto *et al.*, 1988; Munoz, 1988; Mizusawa *et al.*, 1989). Similar abnormal staining for perikaryal neurofilaments is found in a number of other neurodegenerative disease, including Parkinson's disease, Pick's disease and others (Dickson *et al.*, 1986; Cork *et al.*, 1987; Ulrich *et al.*, 1987). Thus, cloning of the *dt* gene, and characterization of the molecular consequences of its mutation may lead to a parallel understanding of the pathogenesis of these human diseases, or at least some aspect of it, and so provide a rational basis for their treatment.

5. Relationship of Tg4 transgene expression to *dt* mutation.

Does the expression of the Tg4 transgene in the neural tube floor plate indicate that the *dt* gene is also expressed in these cells? The experiments undertaken in this thesis allow this question to be addressed via a comparison of the inferred site of *dt* gene expression, derived from the localization of the mutant defect, with the observed sites of transgene expression. The finding of an intrinsic mutant defect argues strongly against the possibility of any involvement of the floor plate in *dt* pathogenesis. As discussed above, the theoretical requirement for combined expression of the mutant defect in both neurons and other cells, including floor plate cells, can not be formally ruled out. However, in contrast to cells such as myelinating cells, which establish a close and continuing relationship with axons, it is difficult to conceive of a pathogenetic relationship being established between neurons and floor plate cells: neuronal degeneration begins postnatally, after apparently normal development and myelination, while the floor plate is a transient structure which is absent by the time of birth and whose identified functions are all restricted to the mid-gestational period (Jessell *et al.*, 1989).

Another possibility consistent with the results of chimera analysis is that the *dt* gene is expressed in both neurons and the floor plate, but that its function in the floor plate is different from that in neurons, and expression of the *dt* defect in floor plate cells has no bearing on the phenotype of neurons. However, with the exception of a small number of unidentified cells observed in some developing dorsal root ganglia (Kothary *et al.* 1988), expression of the transgene could not be detected in any of the neurons that degenerate. Therefore, a

comparison of *dt* pathogenesis and transgene expression provides no indication that the expression of the transgene reflects that of the *dt* gene.

An alternative possibility is that expression of the transgene reflects the influence of regulatory elements belonging not to the *dt* gene but to an adjacent gene on chromosome 1. The most likely explanation for the conferral of a floor-plate specific expression pattern on the transgene in the Tg4 line is as a result of enhancer elements neighbouring its genomic site of insertion. In both Drosophila (O'Kane and Gehring, 1987; Ghysen and O'Kane, 1989) and mice (Allen *et al.*, 1988; Gossler *et al.*, 1989), foreign DNA constructs have been shown to acquire different tissue-specific expression patterns in developing tissues depending on their site of integration within the genome. Where the genomic regions neighbouring the insertion sites in these cases have been analyzed, they have been shown to contain elements with the properties of enhancers: they activated expression of an associated gene either upstream or downstream, in either orientation, and were able to do so at a distance from the gene (Hamada, 1986a, 1986b). The *hsp68-lacZ* construct appears to contain, by chance, the characteristics of constructs found elsewhere to cause effective enhancer trapping: these constructs have consisted of a reporter gene that is paired with a minimal promoter that is able to promote transcription, but, alone, does so at levels too low to be detectable (Gossler *et al.*, 1989). The flexibility with respect to the relative orientation and distance with which enhancers can influence such constructs may explain the frequency with which ectopic expression patterns have been acquired by randomly-integrated enhancer trapping constructs: in one case, a derivative of the *hsp68-lacZ* construct was expressed in one out of seven lines in which it was integrated (Gossler *et al.* 1989), while, in another study employing an *HSV-TK-lacZ* hybrid construct, unique developmental expression

patterns were detected in 25% of insertion events (Allen *et al.*, 1988). In contrast, gene trapping experiments, which require the construct to insert within the intron of an expressed gene, and in the correct orientation and reading frame, show a much lower frequency of expression (Gossler *et al.*, 1989; Skarnes *et al.*, 1992).

The possibility that the regulatory elements driving transgene expression belong not to the *dt* gene but to a gene closely linked to it is supported by preliminary molecular genetic analysis of the Tg4 insertion site (Kothary *et al.*, 1991). This has shown that the transgene's insertion was associated with a 50 kilobase deletion, a finding consistent with previous observations that transgene insertion sites are often associated with extensive alterations in the DNA into which they insert (Covarrubias *et al.*, 1985, 1986, 1987; Mahon, 1988). Probes obtained from this deleted region hybridize to mRNA from postnatal dorsal root ganglia, spinal cord and brain (Kothary *et al.*, 1991), consistent with the hypothesized neuronal site of *dt* gene expression. The absence of detectable transgene expression in these tissues within postnatal mice supports the hypothesis that its expression pattern in the Tg4 line reflects that of another gene normally expressed in the developing floor plate. The presence of such a deletion would also be expected to bring adjacent chromosomal regions in closer proximity to the transgene; while enhancers can affect transcription from a distance, a deletion would increase the chance that the enhancer of a gene linked to *dt* could be brought into effective range to influence transgene expression.

6. Investigation of floor plate cell morphology using β-galactosidase histochemistry.

The analysis of chimeras suggested that involvement of the floor plate in *dt* pathogenesis was unlikely. However, the floor plate is of considerable interest in its own right in view of its postulated role in commmissural axon guidance. Although its presence appears to be required for the normal guidance of axons at the ventral midline neural tube, the precise nature of the floor plate's contribution remains unresolved. In addition to its role in providing a chemotropic signal for commissural axons, the floor plate appears to be involved in two aspects of local guidance. First, since many fewer axons cross the midline in the absence of the floor plate, this suggests a supportive role for growth cone extension. Second, examination of the changes in regionally restricted membrane epitopes expressed by commissural axons in the presence or the absence of the floor plate suggests that the floor plate is required for switches in these epitopes and possibly for their spatial restriction. In the case of L1, such a switch might enable axons to extend within the ventral funiculus by fasciculating on pre-existing axons. Finally, the observation that commissural axons do not respond to the ipsilateral ventral funiculus, and show a response to the contralateral one only if the floor plate is present, suggests that contact with the floor plate is required for growth cones to respond to an as yet undefined rostral directional signal.

One characteristic that would be clearly relevant to the nature of their interaction with commissural axons is the morphology of floor plate cells. Few studies have directly addressed this subject, and floor plate cells have usually been portrayed as archetypal radial glia, having simple radially aligned

processes extending from ventricle to basement membrane (Placzek *et al.*, 1990). The expression of β-galactosidase, and in particular the restriction of this expression to only a proportion of floor plate cells to form a mosaic, provided a unique opportunity to visualize the contour and morphological details of individual floor plate cells, which, using conventional histological staining methods, would ordinarily be obscured by staining of adjacent cells. Histochemical staining of Tg4 embryos showed the basal processes of floor plate cells to be flattened in the rostrocaudal plane and to be relatively wider in their transverse diameter. Many of these basal processes did not reach the basal lamina. A brushwork of fine lateral processes extend from each basal process. Electron microscopic examination showed that these processes, and modifications of the main radial process, appeared to establish a high degree of membrane apposition with axons.

These findings are in agreement with electron microscopic observations in the chick embryo (Yaginuma *et al.*, 1991), in which the basal processes of floor plate cells were found to be wider in their transverse than their rostrocaudal diameter. That study also described transverse processes which were found to wrap around commissural axons. The combination of rostrocaudally flattened floor plate cells and little extracellular space resulted in narrow channels, through which correspondingly flattened growth cones extended, in contact with floor plate cells and the basal lamina.

One consequence of this architecture may be to constrain commissural growth cones to a transverse trajectory (Yaginuma *et al.*, 1991). These growth cones normally cross the midline strictly tranversely, then make an abrupt right angled turn as they exit the floor plate (Bovolenta and Dodd, 1990). In the absence of the floor plate, commissural axons in Xenopus cross the midline with

abnormally oblique trajectories (Clarke *et al.*, 1991). The orthogonal turn made by growth cones as they exit the floor plate might represent their first opportunity to respond to the rostral signal, as they escape this constraining influence.

7. Transfer of β-galactosidase from floor plate cells to commissural axons.

Unexpectedly, β-galactosidase activity was also detected in midline segments of what appear to be commissural axons. It was not found in other axonal or neuronal sites and was present only in those axons adjacent to staining floor plate cells. The most parsimonious explanation of the distribution of β-galactosidase within commissural axon segments was its transfer from floor plate cells. Because β-galactosidase would not be expected to contain any specific targeting sequences, transfer of this enzyme would require its passive inclusion in a normal constitutive transfer process intended for some other normal function. Study of other systems has yielded two precedents for intercellular macromolecular transfer via postulated processes which would appear capable of including cytoplasmic β-galactosidase. First, as has been proposed to operate in the squid giant axon (Lasek and Tytell, 1981), a process might exist which is intended specifically for the intercellular transfer of macromolecules. Second, recent study of an inductive signal in the developing Drosophila retina (Cagan *et al.*, 1992) suggests that a novel mechanism of signal transduction could, if it operated in floor plate cells, also lead to the transfer of unmodified β-galactosidase.

Macromolecular transfer from glial cells to the squid giant axon. Study of the squid giant axon has provided both a precedent for the existence of

intercellular macromolecular transfer and a system whereby the parameters and possible mechanisms of such transfer are accessible for investigation. The principal advantage of the squid giant axon is its size, which allows its axoplasm to be extruded, and thus analyzed separately from the glial cells surrounding the axon. When squid giant axons were separated, along with their associated glial cells, from their neuronal cell bodies, and incubated in (^{3}H)-Leucine, labelled proteins were found not only in the glial cytoplasm but also in the extruded axoplasm. That the finding of labelled proteins was not an artefact of the extrusion process was shown by radioautography of axons and glia in tissue sections, in which silver grains were found within axons and within glial cells. Although squid giant axons had previously been shown to lack ribosomal RNA (Lasek *et al.*, 1973), the possibility that the labelled proteins resulted from intraaxonal protein synthesis was ruled out by injecting RNAse into the axon: this did not interfere with axoplasmic appearance of labelled proteins (Lasek *et al.*, 1977). In addition, isolated axoplasm removed from squid giant axons was shown to be incabable of incorporating radiolabelled amino acids into protein (Lasek *et al.*, 1977). This was further tested by perfusing isolated axons, which were similarly incubated in extracellular (^{3}H)-Leucine, with a synthetic axoplasm, and the appearance of labelled proteins in the perfusate monitored. Labelled proteins appeared in the perfusate for up to 8 hours. Inclusion of RNAse in the perfusate did not impair the appearance of labelled proteins in the perfusate, but extracellularly applied protein synthesis inhibitor puromycin did, further supporting the hypothesis that the proteins did not result from the translation of intraaxonal RNA but rather transfer from surrounding glia (Gainer *et al.*, 1977).

The nature of the proteins transferred suggests a mechanism compatible with the transfer of unmodified β-galactosidase. Transferred, labelled proteins ranged in size from 12,000 to greater than 200,000 daltons (Lasek *et al.*, 1974, 1977), and represented up to 40% of the protein synthesized by the glial cells. Two-dimensional polyacrylamide gel electrophoresis showed the labelled proteins to represent a subset of glial proteins, but that those not included were in general those associated with the cytoskeleton, such as neurofilament proteins or tubulins (Lasek and Tytell, 1981; Tytell and Lasek, 1984). Despite the variety of proteins included, the mechanism of transfer appeared to be specifically established between glial cells and axons. Although labelled BSA was found to be taken up by squid giant axons in vitro, it appeared in axons with a completely different kinetics than did glially produced proteins, primarily over the first 60 minutes of incubation, while transfer of glial proteins was linear over up to 8 hours (Gainer *et al.*, 1977). In addition, although glia were found to release protein into the media, the profiles of these proteins differed considerably from those transferred to the axon (Lasek *et al.*, 1977). This suggested a specific mechanism of transfer to exist between glia and axons, although because these proteins represented a wide variety of sizes and charge it would have to be relatively non-discriminatory with respect to the characteristics of these proteins (Tytell and Lasek, 1984). The authors postulated, first, a modified pinocytosis mechanism, whereby fine processes consisting of outpouchings of glial cytoplasm, were pinched off and engulfed by the axon (Tytell and Lasek, 1984). This was supported by observations that the transferred proteins within the axoplasm appeared to be in particulate form, consistent with their being within a vesicle, and that such vesicles were visualized using the fluorescent stain acridine orange (Gilbert *et al.*, 1982). Alternatively, they proposed direct

cytoplasmic communication as hypothesiszed by Perrachia, who observed membrane-spanning pores on E.M. sections (Peracchia, 1981).

A rationale for the transfer of such proteins was suggested by one prominent member of the transferred proteins, traversin. This protein has an M_r and isoelectric point similar to those of the major heat shock proteins (Tytell *et al.*, 1986). Synthesis of this protein in glia and transfer into the axon were stimulated by subjecting the axon preparation to heat stress. Since these proteins have been shown elsewhere to function in the repair of proteins damaged by heat stress, this suggested that the role of transferred proteins might be to assist in repair of injury or regulation of axonal processes. The availability of the protein synthetic capacity of the immediately adjacent glia would eliminate the delay that would otherwise be associate with the necessity of sending a retrograde signal to the neuronal cell body and awaiting the axonal transport of the synthesised protein (Tytell *et al.*, 1986)

In an analogous manner, growth cones at a distance from their cell body could utilize the protein synthetic capacity of floor plate cells at a time when they must alter their growth properties and sensitivity to external guidance cues. Floor plate derived molecules could directly participate in the signal transduction mechanism, or act as a switch in preexisting growth cone machinery. The requirement for a specific membrane to membrane transport mechanism would increase the specificity of the floor plate derived signal, and would, for example, safeguard against the ipsilateral rostral turning of commissural growth cones.

The fact that a heat shock gene is expressed in glia for transfer to axons appears coincidental in view of the floor plate cells' expression of a construct

containing an *hsp68* promoter. However, it is unlikely that the expression of the transgene in these cells is in response to trans-acting factors normally regulating their developmental expression of heat shock genes: in six other lines expression of the *hsp68-lacZ* construct was induced by heat shock, but in none of these other lines could spontaneous developmental expression of the transgene be detected (Kothary *et al.*, 1988).

bride of sevenless. A more recent finding suggests an alternative type of intercellular transfer in a developing system which would also potentially accomodate cytoplasmic β-galactosidase. In the developing Drosophila retina, the R8 photoreceptor cell induces the differentiation of the adjacent R7 cell through the interaction of its membrane-bound ligand, the *bride of sevenless* (*boss*) gene product, with the *sevenless* tyrosine kinase receptor on the R7 membrane (Rubin, 1991). The *boss* ligand is a transmembrane protein with a large cytoplasmic domain and is restricted to the portion of the R8 membrane that apposes the R7 cell. Surprisingly, immunocytochemical labelling showed that transmission of the R8 signal involved the internalization of the entire boss protein, including its cytoplasmsic domain to the interior of the R7 cell (Cagan *et al.*, 1992). The presence of vesicles within R7 cells possessing *boss* immunoreactivity suggested that one mechanism of this transfer was the modified uptake of outpouching of R8, boss-containing membrane, similar to that hypothesized to operate in the case of the squid giant axon (Tytell and Lasek, 1984).

If a similar process operated between floor plate cells and commissural axons or growth cones, cytoplasmically distributed β-galactosidase could conceivably be included in the process, even though it would be normally directed at transferring membrane-bound, not cytoplasmic molecules. Such a

process would provide an alternative way in which the properties of growth cones could be altered in response to floor plate contact. Membrane bound signals activating second messenger signals within growth cones have been proposed as a means by which cells along their pathway may alter growth cone structure and motility (Dodd and Jessell, 1988). As in the developing Drosophila retina, in the floor plate a boss-like signal which could be localized and whose transmission would depend on membrane apposition would increase the specificity with which a signal could be transmitted to commissural axons.

The ability of floor plate cells to spatially restrict the distribution of such a molecular signal, in view of the existence of a rostral and caudal face to their basal processes, provides a potential means by which the rostral direction could be signalled to growth cones. Although such a rostral signal has been hypothesized its nature remains unknown: it appears to function the length of the neural tube, a distance that would exceed the effective range of a single gradient of a diffusible or even substrate localized molecule. However, evidence from the present study and previous electron microscopic analyses (Yaginuma *et al.*, 1991) suggests that commissural growth cones may travel through the floor through narrow channels, whose walls are formed on one side by the caudal faces of floor plate cells, and on the other by the rostral faces. If each floor plate cell localized a signal only to the membrane of its caudal face, the growth cone would be exposed to the signal on its own rostral, but not caudal, face throughout its growth within the floor plate. The magnitude of the gradient established by such a local signal could be great, as it would act over a short range and, due to the requirement for membrane-to-membrane contact for its transmission, would be prevented from acting inappropriately on adjacent rostral axons to cause them to turn caudally. The polarized distribution of the

hypothesized signal could be established in individual floor plate cells in response to the rostrocaudal gradient of floor plate cell differentiation (Wentworth, 1984).

Thus, rather than floor plate cells enabling growth cones with the ability to respond to an external rostral signal, the observed dependence of commissural axons on the floor plate for their rostral projection may be because the signal is intrinsic to the floor plate itself. The observation that commissural growth cones make an abrupt orthogonal turn upon exiting the floor plate might thus represent a tendency to turn that is gained in the floor plate, and is promptly executed as the growth cone escapes the physical constraints imposed by floor plate cell geometry.

Yaginuma and Oppenheim (1991) performed an experiment of direct relevance to this hypothesis, in which they rotated a segment of chick neural tube 180 degrees, such that its rostral and caudal ends were reversed. They wished to determine whether commissural axons within the segment would project caudally, according to the segments original polarity, or would grow in the rostral direction despite the rotation, which would indicate that they followed more global cues that were extrinsic to their local environment. Almost all axons grew rostrally, which would argue directly against the present hypothesis of locally embedded directional cues. As these authors acknowledged, the rotations were done at an early stage, which left open the possibility that cues intrinsic to the graft were respecified following rotation in response to the surrounding tissue, a phenomenon that has been observed in the retinotectal system (Yaginuma and Oppenheim, 1991). However, in view of this result, support for the present hypothesis will require identification of a floor plate specific molecule this is expressed in a rostrally or caudally polarized manner.

The expression of β-galactosidase in the Tg4 line has raised the intriguing possibility that a process involving the transfer of cytoplasmic macromolecules, or possibly membrane-bound signal operates between floor plate cells and commissural axons. However, the low level of the transgene expression in this line, and our limited knowledge regarding its mechanism, limit the utility of this line to allow a more definitive evaluation of this hypothesis. A definitive determination of whether such a process occurs will require, first, assessment of other proteins expressed in floor plate cells. The reported distribution of the p35 protein within both floor plate cells as well as midline commissural axon segments (McKanna and Cohen, 1989) has already provided support for the hypothesis that a mechanism of transfer operates during the development of normal mice. If further such examples are obtained, targeting the expression of exogenous molecules to the floor plate in transgenic mice, using floor plate specific promoters such as that of the recently cloned F-spondin gene (Klar *et al.*, 1992), would allow the parameters of such a process to be defined. If a process for the transfer of membrane-localized or cytoplasmically distributed molecules is confirmed, this will allow the further definition of the complex ways in which axons are guided in their outgrowth from source to target.

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Claims to original research

1. Development of an immunohistochemical staining method for specific *in situ* detection of the human neurofilament protein hNF-L.

2. Identification of an intrinsic neuronal defect in *dystonia musculorum*.

3. Identification of the presence of phosphorylated neurofilament epitopes in the perikarya of affected neurons in *dt/dt* mice.

4. Description of floor plate cell morphology in mice.

5. Evidence obtained for the occurrence of macromolecular transfer from floor plate cells to commissural axons.

Appendix I

Kothary, R., Clapoff, S., Brown A., Campbell, R., Peterson, A. and Rossant, J. (1988). A transgene containing *lac Z* inserted into the *dystonia* locus is expressed in neural tube. *Nature* **335**, 435-437.

Contributions of coauthors:

Members of Janet Rossant's laboratory (R. Kothary, S. Clapoff and A. Brown) constructed the Tg4 transgenic mouse line and identified both the presence of transgene expression in hemizygous embryos and the existence of a movement disorder in homozygous mice. My supervisor, Alan Peterson, determined that the movement disorder corresponded to that of *dystonia musculorum*, and established the allelic nature of the insertional and spontaneous mutations. Using histochemical staining for β-galactosidase I localized the site of transgene expression to the ventral medial neural tube as well as participating in the preparation of the manuscript.

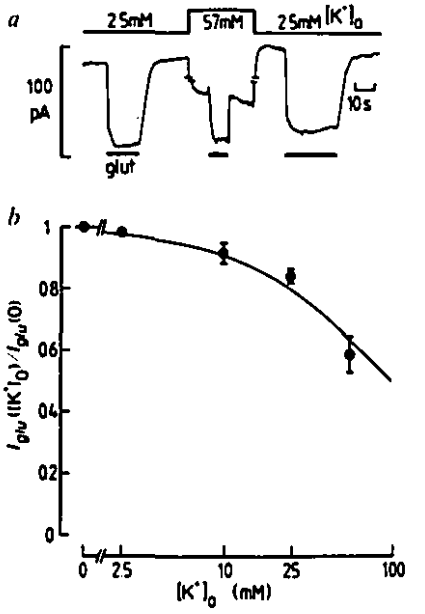

Fig. 3 External potassium inhibits electrogenic glutamate uptake. *a*, Current evoked by 30 μM glutamate (black bars) at −40 mV in a cell that was initially in a solution containing 2.5 mM K^+, then in a solution containing 57 mM K^+ (which produced an inward current shift of 220 pA through the small fraction of K^+ channels remaining unblocked in 6 mM barium), then again in 2.5 mM K^+. *b*, Dependence on $[K^+]_o$ of the glutamate-evoked current from experiments like those in *a* (mean data ± s.e.m. from five cells for $[K^+]_o$ = 0, 10, 57 mM and six cells for 25 mM) normalized to the current measured on the same cell with 2.5 mM $[K^+]_o$ (the value of which was set at 0.98 so that the mean value obtained for 0 mM $[K^+]_o$ was unity). External solution contained (in mM): KCl, *x*; choline chloride, 57 − *x*; NaCl, 50; $CaCl_2$, 3; $MgCl_2$, 0.5; glucose, 15; HEPES, 5; $BaCl_2$, 6; *p*H adjusted to 7.3 with 2 mM NaOH; $[K^+]_o$ was *x* = 0, 2.5, 10, 25 or 57 mM. Pipette solution contained (in mM): KCl, 95; NaCl, 5; HEPES, 5; $MgCl_2$, 7; Na_2 ATP, 5; $CaCl_2$ 1; K_2 EGTA, 5; *p*H adjusted to 7.0 with 14 mM KOH. Smooth line has the form $K_m/([K^+]_o + K_m)$, with K_m = 100 mM. With a near-saturating glutamate dose (1 mM), the current in 57 mM $[K^+]_o$ was 65.2% (±2.1% s.e.m., five cells) of its size in 2.5 mM $[K^+]_o$, similar to the value in Fig. 3*b* for 30 μM glutamate (59.6 ± 5.8%), ruling out the possibility that high $[K^+]_o$ reduces the current by greatly decreasing the carrier's affinity for external glutamate. Reducing $[Na^+]_o$ from 52 to 27 mM (replaced by choline) reduced the current evoked by 30 μM glutamate at −40 mV by 62.6% (±1.9% s.e.m., 12 cells) in 2.5 mM $[K^+]_o$ and by 61.2% (±2.5%) in 57 mM $[K^+]_o$, implying an unchanged apparent affinity for external Na^+ in high $[K^+]_o$. If high $[K^+]_o$ reduced the glutamate-evoked current by K^+ competing for the Na^+ binding sites on the carrier, the apparent affinity for Na^+ would be lowered in high $[K^+]_o$ (and reducing $[Na^+]_o$ would give a greater fractional reduction of the current).

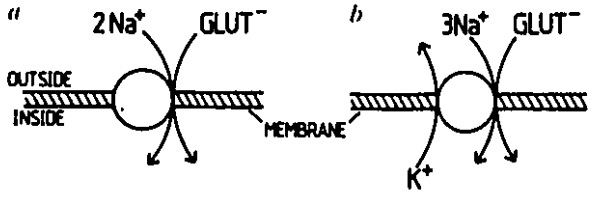

Fig. 4 Stoichiometry of the glutamate uptake carrier. *a*, Stoichiometry based on radioactive tracer studies[2,3]. Glutamate uptake is driven by the co-transport of two sodium ions into the cell. *b*, Stoichiometry based on the data in this paper. Glutamate uptake is coupled to the transport of one potassium ion out of the cell, as well as to an influx of sodium. We postulate that three sodium ions enter the cell on each carrier cycle: if only two were transported, there would be no current generated. Chloride, calcium and magnesium are not transported (see text). We considered the possibility that protons might be co-transported (so the carrier could transport two Na^+ and one H^+ into the cell instead of three Na^+). At external *pH* values of 5 or 9 (when 83% of the glutamate exists in the form with one net negative charge, in which >99% of it exists at *p*H = 7.3) the glutamate-evoked current was ~25% the size of that at a *p*H of 7.3. The increase in uptake between *p*H = 9 and *p*H = 7.3 could in principle result from the carrier transporting H^+ ions into the cell. But it is equally likely that this *p*H dependence (and the subsequent decrease on further acidification to *p*H 5) reflects the titration of charged groups at the binding sites on the carrier for sodium and glutamate.

mate concentration to toxic levels, in two separate ways. First, the raised $[K^+]_o$ will depolarize glia and thus inhibit uptake because of its strong voltage-dependence[4]. Secondly, the rise in $[K^+]_o$ will directly reduce uptake by preventing the loss of K^+ from carrier (Fig. 3). For a rise of $[K^+]_o$ from a physiological 2.5 mM to a pathological 50 mM (as can occur in anoxia[7]) and a consequent glial cell depolarization from around −90 mV to around −20 mV, these mechanisms will reduce uptake by 66% and 35% respectively, a combined reduction to less than one quarter of the physiological rate.

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A transgene containing *lacZ* inserted into the *dystonia* locus is expressed in neural tube

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The site of integration of transgenes in the host genome can affect levels of expression[1] and occasionally confer ectopic patterns of expression on otherwise tissue-specific genes[2]. We describe here a line of mice in which an *hsp68-lacZ* transgene is expressed in unstressed developing neural tissue and where the transgene insertion has caused a mutation of a neural tissue-specific gene, *dystonia musculorum* (*dt*). This coincidence suggests that expression of the *hsp68-lacZ* construct may be controlled directly by *cis*-acting regulatory sequences that normally control the developmental expression of the *dt* gene. Such constructs may serve as useful tools for identifying new tissue-specific enhancers and their associated genes[3,4].

We have generated seven lines of transgenic mice carrying a mouse *hsp68* promoter-*Escherichia coli lacZ* hybrid gene for studies of heat-shock gene regulation. In six of these lines the *lacZ* gene was silent unless tissues were subjected to heat shock or arsenite treatment (R.K., S.C., M. Perry, L.A. Moran and J.R., unpublished results). But one line, designated Tg4, expressed the *lacZ* gene spontaneously. This line contained

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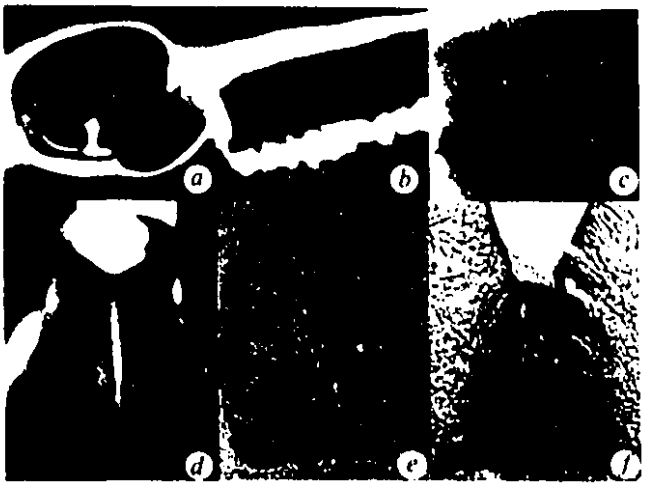

Fig. 1 Transgene expression in Tg4/− fetuses. Whole fetuses were stained for β-galactosidase activity. *a*, Day 12.5. Staining is visible as a thin blue line inside the dorsal midline surface of the fetus. *b*, Neural tube and associated DRG from same embryo. An intense band of staining follows the ventral margin of the neural tube. Arrow points to one DRG containing an intense spot of reaction product. *c*, Higher magnification of DRG identified by arrow in *b*. *d*, Day 10.5. Roof of hindbrain vesicle has been removed to reveal twin bands of staining traversing its floor. *e*, Cross-section of day-12.5 neural tube. On either side of the midline the most prominent staining forms a diagonal band between the neural tube's ventral surface and the ventral edge of the developing central canal. A thinner line of staining continues dorsally along the margin of the central canal. *f*, Higher magnification of *e*. In this preparation, most of the reaction product does not appear to lie within cell bodies. At this level of resolution it is not possible to distinguish between staining of cell membranes, fine processes, or extracellular material.
Methods. Whole fetuses were fixed for 1 h in 2.5% glutaraldehyde and 0.5% paraformaldehyde in 0.1 M phosphate buffered saline, *p*H 7.3 (PBS). They were then incubated from 12–24 h at 37 °C in a solution of 1 mg ml^{-1} X-gal in PBS (ref. 9). Tissue was viewed as whole mount, or frozen in OCT medium (Tissue-Tek) for cryostat sectioning at 10 μm.

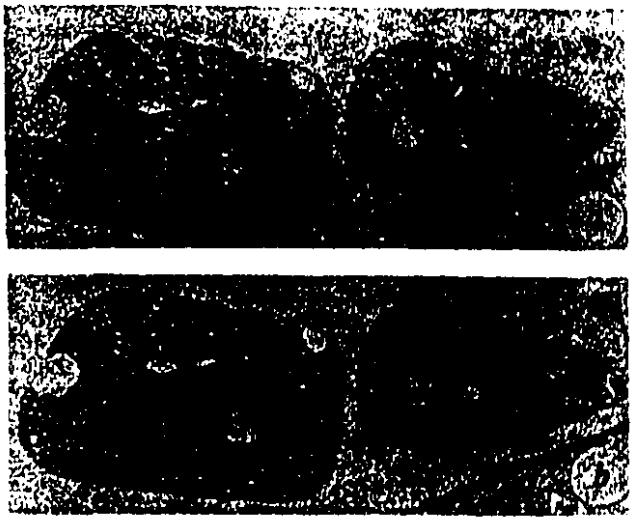

Fig. 2 Cross-sections of spinal roots from the left thoracic 10 (T10) level of dt^{tg4}/dt^{J} mouse. Normal ventral root is on the left and affected dorsal root is on the right. Serial cross-sections were stained with paraphenylenediamine (*a*) to specifically label myelinated fibres[10] and with toluidine blue (*b*) to reveal general histological features. The small dorsal root contains few myelinated fibres and a large number of apparently denervated Schwann cells. In contrast, the ventral root is of normal size and is composed of myelinated fibers of unremarkable histological appearance.
Methods. Mice were anaesthetized, then perfused through the heart with 0.1 M phosphate buffer (*p*H 7.2), followed by 2.5% glutaraldehyde and 0.5% paraformaldehyde in 0.1 M phosphate buffer (*p*H 7.2). Spinal roots were embedded in epon, sectioned at 1 μm and stained. Final magnification ×510.

≈15–20 copies of the transgene inserted as a single head-to-tail concatamer (data not shown). When reacted with X-gal (5-bromo-4-chloro-3-indolyl β-D-galactopyranoside), one-half of the fetuses obtained from normal CD-1 females mated to hemizygous Tg4/− males revealed a column of β-galactosidase activity running along the dorsal midline. Southern blotting of placental DNA confirmed that these embryos were Tg4/− hemizygotes (not shown).

The column of staining was situated along the medial ventral edge of the neural tube and had a triangular shape in cross-section (Fig. 1). Staining was detected first in the rostral-most region of the neural tube in day-9.5 fetuses and was most intense at day 13.5, when staining occurred along the entire length of the neural tube. Also, developing dorsal root ganglia adjacent to labelled levels of the neural tube sometimes expressed one or a few discrete spots of reaction product (Fig. 1). Neonatal mice no longer revealed *lacZ* activity in either spinal cords or ganglia.

When hemizygous Tg4/− mice were mated together, litters of average number were born, but at around 10–12 days after birth one quarter of the offspring began to show signs of limb incoordination. Affected mice progressively lost coordination and most died before weaning or shortly thereafter. Southern blot analysis showed that such affected pups were homozygous for the transgene (data not shown), indicating a recessive insertional mutation. Histological investigation revealed a severe and apparently specific loss of dorsal spinal root sensory axons at all levels of the spinal cord (Fig. 2). Ventral motor roots were remarkably unaffected considering the severe motor symptoms of affected pups. In cross-section, dorsal roots were small, contained few axon profiles, a dramatic paucity of myelinated fibres, and an abundance of apparently denervated Schwann cells. In addition, the occasional giant axon profile was observed.

Both the clinical course of the disease and the histopathological phenotype of this mutant were identical to those described for a spontaneous mutation of the mouse, *dystonia musculorum* (*dt*)[5,6]. Mice heterozygous for the *dt* allele were obtained from the Jackson Laboratory and crossed with Tg4/− hemizygotes. One quarter of the resulting offspring expressed the typical *dystonia musculorum* phenotype at both the clinical and histopathological levels (Fig. 2), demonstrating that the Tg4 insertional mutant and *dt* are allelic. Generation of a new insertional mutation at the *dt* locus should allow the molecular cloning of the *dt* gene and ultimately lead to the characterization of the *dt* gene product. Further, *dystonia musculorum* has been compared to a group of inherited human neurological diseases, including Friedreich's ataxia, in which an analogous movement disorder is accompanied by sensory neuronal degeneration[7,8]. Characterization of the mouse *dt* gene may clarify the aetiology of these specific diseases, or provide a more general molecular

model for genetic diseases involving loss of specific neuronal populations.

Insertional mutation of the *dt* gene and the simultaneous appearance of a nervous-system-specific expression profile in the Tg4 transgene line raises the possibility that the two events are directly related. Expression of the *hsp lacZ* construct could be activated by *cis*-regulatory elements of the *dt* gene. The *lacZ* expression profile would, therefore, define the normal developmental and cell type-specific expression pattern of the endogenous *dt* gene. The rare labelled cells we have observed within the developing dorsal root ganglia (DRG) of Tg4 mice may indicate that lack of *dt* gene expression within sensory neurons themselves is directly responsible for the observed neuropathy. Alternatively, the sensory neuropathy could be a secondary consequence of lack of *dt* gene expression within the ventral neural tube where staining was most intense. It is surprising that expression of the *lacZ* gene is obvious only during mid-gestation, whereas degeneration of sensory neurons in *dt/dt* mice apparently occurs post-natally. This may reflect failure of the transgene to respond to all of the elements that normally drive *dt* expression, or it may define a real difference between the time of *dt* gene expression and the time at which deficiency in *dt* products causes an obvious phenotype. There is relatively little information available on the phenotype of *dt/dt* mice before onset of clinical symptoms. The Tg4 insert into the *dt* locus provides a means of identifying homozygotes much earlier in development and it will be interesting to see if subtle phenotypic effects can be detected in the developing neural tube of *dt/dt* mice.

It remains possible that the neural-tube-specific expression of *lacZ* is activated by sequences that are close to the *dt* gene but are otherwise unrelated. In this case, the pattern of *lacZ* expression could identify sequences controlling an unknown gene that could also be important for neural development. Further, as this gene is presumably close to the *dt* locus, the transgene may have inserted into a group of closely linked, neural-specific genes. All these issues will be resolved when the genomic region around the Tg4 insertion is cloned and its products are identified. To this end we are in the process of isolating probes to the flanking sequences of the Tg4 insertion from a genomic library of Tg4/−DNA.

We have clearly shown that a normally inactive promoter, such as *hsp68*, can respond to host regulatory elements in the mammalian genome. It may therefore be possible to design strategies to identify other regulatory elements and their associated genes, exploiting the random insertion of a transgene whose expression can be easily detected *in situ*. Approaches of this sort have begun in both *Drosophila*[3] and mice[4] and could be extremely useful in identifying those genes that participate in the early and fundamental events controlling mammalian embryogenesis.

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Appendix II

McGowan, R., Campbell, R., Peterson, A. and Sapienza, C. (1989). Cellular mosaicism in the methylation and expression of hemizygous loci in the mouse. *Genes Dev.* **3**, 1669-1676.

Contributions of coauthors:

Ross McGowan, a member of Carmen Sapienza's laboratory, performed DNA hybridization analyses. I performed all histochemical staining procedures in addition to participating in preparation of the manuscript.

Cellular mosaicism in the methylation and expression of hemizygous loci in the mouse

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Proposed models for the inheritance of locus-specific methylation phenotypes in somatic cells include those in which there is stable inheritance of a methylation pattern such that all cells contain a similarly methylated locus, as well as models in which the inheritance of methylation can be variable. We investigated these possibilities by examining the methylation and expression of hemizygous loci in the mouse. Our results demonstrate that differences in both methylation and expression can exist between apparently identical cells and that such mosaicism is genetically controlled.

[*Key Words*: Methylation; mosaicism; transgene]

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The modification of mammalian DNA most commonly involves the addition of a methyl group to position 5 of cytosine, usually when that base precedes a guanosine (Vanyushin et al. 1970). Cytosine methylation within or adjacent to many loci is thought to affect, or be affected by, several biochemical processes involved in the control of gene expression, as well as mutation and genomic imprinting: Actively transcribed sequences tend to be undermethylated relative to those that are not being transcribed (Doerfler 1983); DNA sequences that are highly methylated, such as satellite DNA (Selig et al. 1988) or those on the inactive X chromosome (German 1962), tend to be late-replicating; the methylation of cytosine increases the rate of C-to-T transitions, resulting in point mutations at those sites at an increased frequency (Wang et al. 1982); and the methylation of some transgenes has been shown to be affected by gamete of origin and therefore to show a genomic imprint (Hadchouel et al. 1987; Reik et al. 1987; Sapienza et al. 1987; Swain et al. 1987).

Because the demonstration that the methylation of CpG sites is heritable through DNA replication and cell division (Wigler et al. 1981), it has been generally assumed that once the methylation pattern of a locus has been established in an embryonic cell, all of its descendants should faithfully reproduce the pattern such that they all remain identical with respect to the methylation of any particular CpG site. We demonstrated previously that the methylation phenotype of the 379 quail troponin I transgene locus could be affected by genetic and epigenetic factors to produce three different phenotypes in different individuals (Sapienza et al. 1989a). These different phenotypes could reflect methylation differences either between or within the transgene array.

To distinguish between these possibilities, we analyzed the methylation phenotype of hemizygous ecotropic virus loci and a *lacZ*-containing transgenic line. The expression characteristics of the *lacZ*-containing transgene were also analyzed using an in situ histochemical assay. The evidence we obtained suggests that cells sharing the same developmental and phenotypic lineage can express different DNA methylation phenotypes within an individual. Furthermore, such otherwise identical cells can differ in the expression of a transgene locus, resulting in a mosaic phenotype.

Results

In an attempt to identify whether methylation differences could exist between cells within a tissue, we assayed the methylation state of a *SmaI* site adjacent to an endogenous ecotropic virus locus. Because different inbred mouse strains contain such loci at different chromosomal sites (Jenkins et al. 1982), F_1 hybrids between strains that carry different ecotropic virus loci are hemizygous at each locus. This situation is analogous to that of hemizygous transgene loci but can be interpreted more easily because the ecotropic virus locus consists of only a single copy of the provirus.

The DDK inbred strain contains a single ecotropic virus locus (designated *Emv-DDK* in Fig. 1), whereas the RF/J strain contains three such loci (designated *Emv-1*, *Emv-16*, and *Emv-17* in Fig. 1). Genetic analyses indicate that *Emv-16* and *Emv-17* are tightly linked (Jenkins and Copeland 1985), whereas *Emv-1* (Jenkins and Copeland 1985) and Emv-DDK (C. Sapienza, unpubl.) segregate independently. F_1 hybrids between DDK and RF/J are thus hemizygous at each locus. The single copy of the *Emv-*

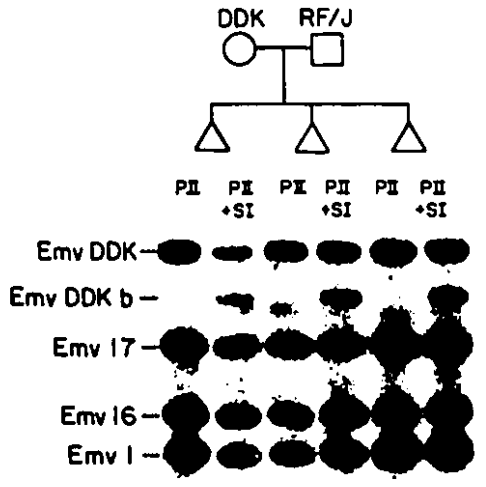

Figure 1. Tail DNAs from the F_1 progeny of a cross between the DDK and RF/J inbred strains, digested with *PvuII* (lanes PII) and with *PvuII* plus *SmaI* (lanes PII + SI) and probed with a 300-bp ecotropic virus-specific fragment (Jenkins et al. 1982). The *PvuII* digests distinguish four ecotropic virus loci (*Emv-1*, *Emv-16*, and *Emv-17* from RF/J) (Jenkins et al. 1982), and *Emv-DDK* from DDK. *PvuII* plus *SmaI* digestions demonstrate the methylation mosaicism evident at the Emv-DDK locus (bands Emv-DDK and Emv-DDKb).

DDK provirus contained within each cell was contributed by the DDK female through an ovum.

Any particular methylation-sensitive restriction endonuclease cleavage site in or around those hemizygous loci must have been either methylated or unmethylated in the single gamete of origin. Because we are examining only a single restriction site within each cell, a somatically stable inheritance model predicts that the site should either be methylated in all cells and, therefore, completely insensitive to cleavage by *SmaI* or unmethylated in all cells, and, therefore, completely sensitive to cleavage by *SmaI*. However, if this site is methylated differently in different cells, such a tissue mosaic should give rise to a DNA sample that is partially sensitive to cleavage by *SmaI*.

The *PvuII* and *SmaI* cleavage patterns of the *Emv-DDK*, *Emv-1*, *Emv-16*, and *Emv-17* loci in DDK-RF/J hybrids are shown in Figure 1. Whereas *Emv-1*, *Emv-16*, and *Emv-17* appear to be completely insensitive to cleavage by *SmaI*, the *PvuII* fragment corresponding to the Emv-DDK locus becomes reduced in intensity and a new band of ~8 kb appears.

Because each cell carries only one copy of each provirus, the partial *SmaI* sensitivity of DNA isolated from whole tissue must reflect differences at the level of individual cells. Furthermore, the same cleavage pattern is found in a variety of somatic tissues whose last common developmental ancestor was primitive ectoderm (data not shown). Apparently identical proportions of methylated and unmethylated cells therefore exist in all tissues, implying either that methylation differences between cells are established early in development and are then faithfully propagated or that these differences are generated continuously.

Because such evidence for methylation differences at the level of individual cells is indirect and could conceivably be due to different cell types within a tissue, we sought to define an assay system capable of resolution at the level of the single cell. Such a system was available in the Tg4 transgenic line of mice. The Tg4 transgene locus consists of 15–20 copies of a mouse *hsp68–Escherichia coli lacZ* construct inserted at the *dystonia musculorum* locus on chromosome 1 (Kothary et al. 1988). In this particular line, the *lacZ* transgene is expressed in the floor plate of the neural tube during days 10–14 of development. However, *lacZ* expression was absent in the neural tubes of some transgenic fetuses. Therefore, we investigated whether the methylation of *MspI* sites at this transgene locus correlated with the expression of *lacZ* in the neural tube. Figure 2 shows that day-12 fetuses containing a hypomethylated transgene express the *lacZ* gene in the neural tube, whereas fetuses containing a hypermethylated transgene do not. As has been observed with other transgene loci, the methylation phenotype of this transgene was identical in all nonexpressing tissues, with the exception of testes and extraembryonic membranes (Reik et al. 1987; Sapienza et al. 1987; Swain et al. 1987).

Furthermore, within this transgenic line, as in the troponin I line described previously (Sapienza et al. 1989a), individuals can not only carry the transgene locus in high or low methylation states but also in intermediate states. The observed correlation between methylation and expression of this transgene (Fig. 2) allows us to distinguish between the two possible ways in which such an intermediate methylation phenotype could arise. If the intermediate transgene methylation phenotype observed in these mice is shared by all the cells in the neural floor plate, *lacZ* expression should also be identical in all cells. However, if the intermediate methylation phenotype were a composite of both hyper- and hypomethylated cells, the staining pattern should reveal this mosaicism. The data presented in Figure 3 support the latter possibility. In these individuals the intermediate methylation phenotype was associated with a reduced number of expressing cells in this region, which we interpret to represent a mosaic population of expressing and nonexpressing cells. Moreover, the proportion of cells that expressed *lacZ* was inversely correlated with the degree of methylation of the transgene. The intensity of staining appeared to be equivalent in all fetuses stained on the same occasion (whether mosaic or not), although the staining intensity did vary between trials.

In previous experiments with the 379 troponin I transgene, we observed the methylation phenotype of the transgene in an individual to be affected by the genotype of the nontransgenic female parent (Sapienza et al. 1989a). To determine whether the Tg4 transgene locus was similarly affected, we analyzed fetuses derived from crosses of transgenic males to females of different

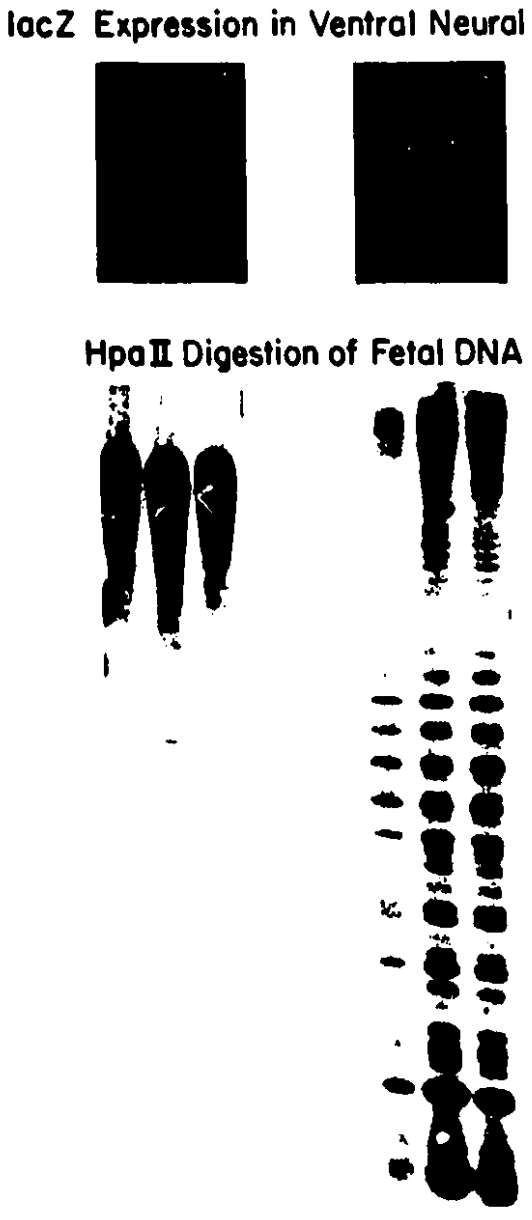

Figure 2. *lacZ* expression in neural tubes and restriction endonuclease digestion of head DNAs from 12-day Tg4 mouse fetuses. A ventral view of a representative neural tube stained for *lacZ* expression (as detailed in Materials and methods) is shown at *top*, showing either expression (*right*) or nonexpression (*left*) of the transgene. Beneath each neural tube is *Hpa*II-cleaved DNAs from three individual fetuses displaying the staining pattern shown above them, probed with a 4.5-kb sequence representing all of the transgene insert (Kothary et al. 1988). Nonexpressing individuals carry a hypermethylated transgene, whereas expressing individuals carry a hypomethylated transgene.

strains. Figure 4 shows that offspring of the same male may exhibit different transgene methylation phenotypes. Matings to a C57BL/6 female produced a hypomethylated phenotype and 'complete' staining, whereas offspring of the BALB/c female cross were mosaic for *lacZ* expression and had an intermediate transgene methylation phenotype. Similar results have been obtained from matings involving five different Tg4 transgene-carrying males which produced a total of 52 transgenic progeny (22 from C57BL/6 females and 30 from BALB/c females). Only a single exception was found in which one fetus in a litter of completely staining siblings from a cross to a C57BL/6 female showed incomplete staining in its neural tube. These findings demonstrate that the methylation and expression of the transgene are under genetic control and that differences exist between common inbred strains of mice. Furthermore, this level of regulation must involve a *trans*-acting factor because the maternal genetic effect is realized after fertilization.

Discussion

We have previously described a transgene locus, the 379 troponin I gene (Sapienza et al. 1989a), that could display three different somatic methylation phenotypes. A similar situation has been identified for another transgenic locus, the Tg4 gene. These three phenotypes could reflect one or both of the situations illustrated in Figure 5.

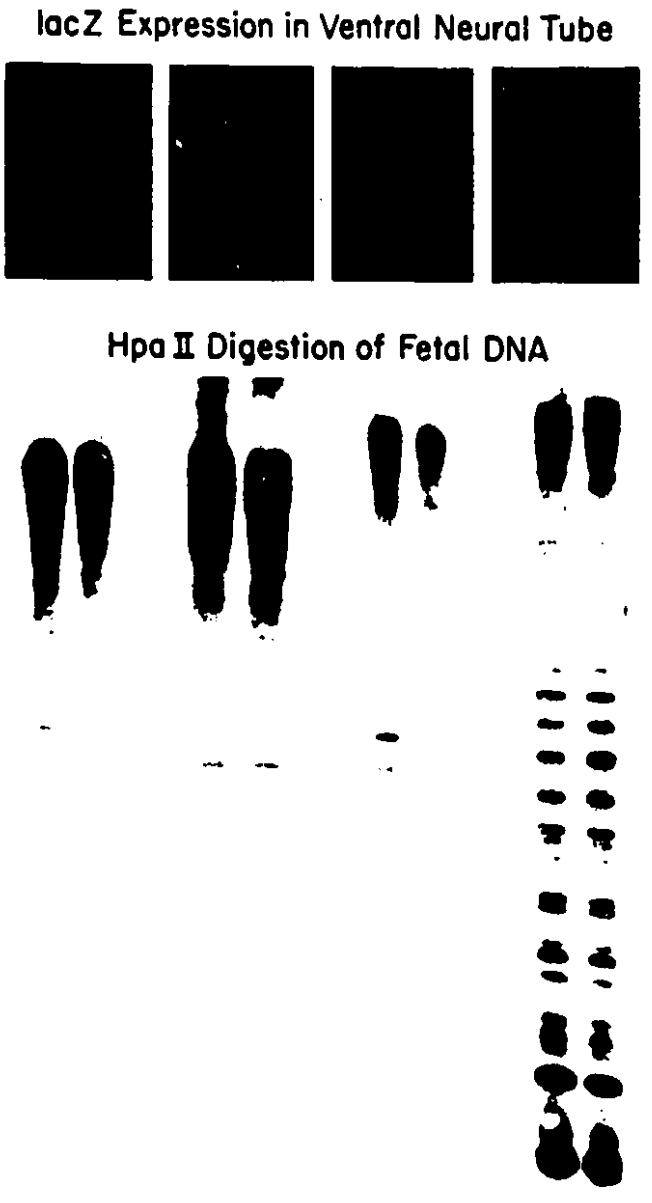

Figure 3. *lacZ* expression in neural tubes and restriction endonuclease digestion of head DNAs from 12-day Tg4 mouse fetuses. As in Fig. 2, a ventral view of the neural tube is shown at *top* and *Hpa*II-cleaved DNAs are presented beneath them. Again, hypermethylation of the transgene is associated with nonexpression in the neural floor plate and hypomethylation with expression in those cells, but intermediate levels of methylation are shown to be associated with an intermediate number of expressing cells. The higher the methylation, the lower the proportion of expressing cells.

Either (1) differences exist in the methylation of *Msp*I sites among different copies within a transgene array, but within an individual all the arrays are alike, such that each cell within a tissue carries a transgene array which is identically methylated (Fig. 5A); or (2) differences in the methylation of the transgene array exist between cells, such that some cells carry an array with one methylation pattern and others carry an array with a different methylation pattern (Fig. 5B).

The model in Figure 5A assumes that the methylation pattern of the array is established only once and that, once established, the pattern is stably propagated in all of the cells that give rise to somatic tissues. The model in Figure 5B assumes that cells with at least two different methylation phenotypes are established after fertilization and that both types of cells may contribute to somatic tissue (Sapienza et al. 1989b).

Analyses of a single methylatable site, the CpG dinucleotide, contained in a *Sma*I recognition sequence adjacent to a hemizygous ecotropic virus locus identified both a methylated and nonmethylated *Sma*I site in the same tissue, consistent with the idea that different cells within the same tissue can be methylated differently. If the data in Figure 1 were an isolated case, one might explain these results as being due to a random failure to methylate the affected *Sma*I site in a single descendant of a progenitor cell that contained a methylated *Sma*I site. Such epigenetic 'mutations' are well known and relatively common (Holliday 1987). However, the relative amounts of the two bands, methylated and non-

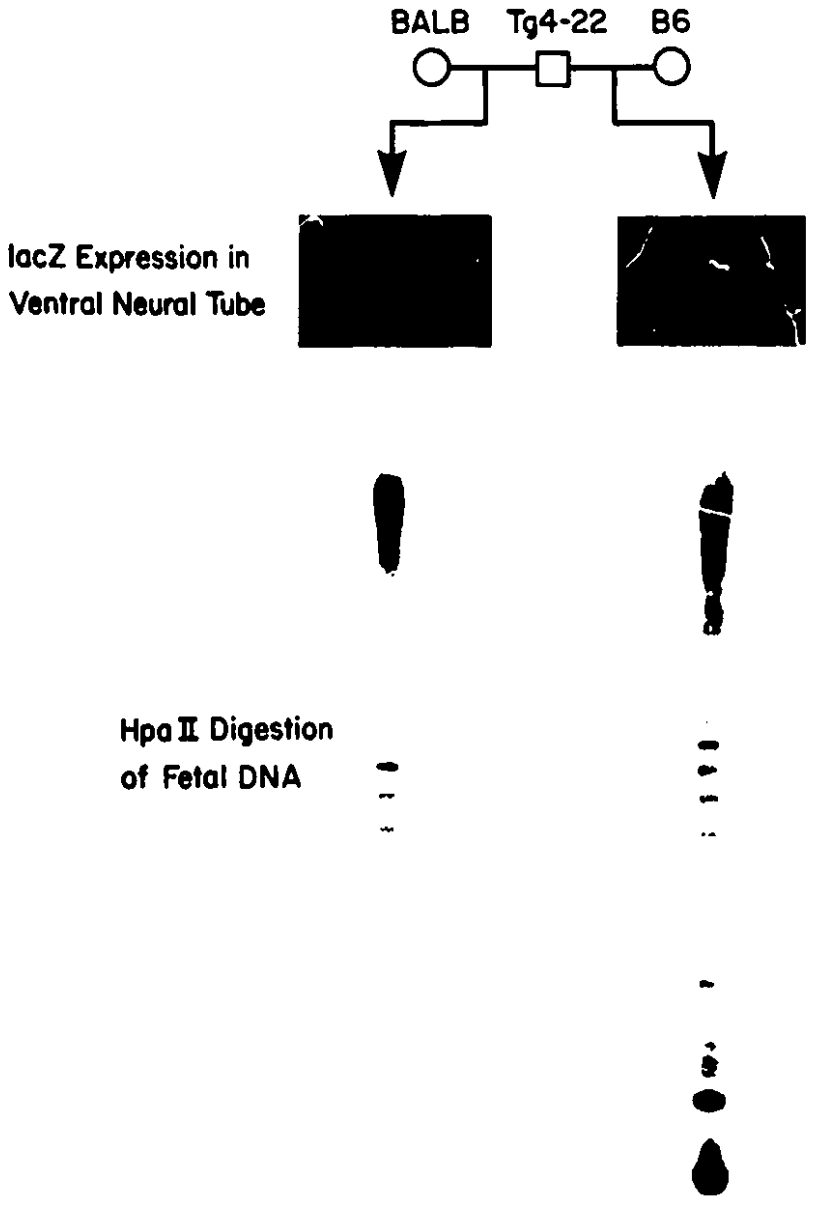

Figure 4. (*Top*) A pedigree is presented and shows the mating of a single transgenic male to different inbred females. The ventral portion of the neural tube stained for *lacZ* expression and *Hpa*II-cleaved DNAs of 12-day fetuses are shown beneath the pedigree and demonstrate that the same male can give rise to progeny with different methylation and expression phenotypes, depending on the genotype of the nontransgenic female. Matings to C57BL/6 females produce progeny showing extensive *lacZ* expression throughout the neural tube floor plate, whereas matings to BALB/c females produce progeny that show mosaic expression.

methylated, is constant in all individuals and, by densitometric analyses, is roughly 7 : 1, suggesting that the event consistently took place by the time there were about 8 progenitor cells, i.e., the same event must have occurred independently at the same time in development in all 21 F_1 progeny examined (Fig. 1 and data not shown). Furthermore, the failure of the *Emv-1*, *Emv-16*, and *Emv-17* loci to be cleaved by *Sma*I provides an internal control to demonstrate that this event is not random.

Histochemical staining for the presence of *lacZ* in the Tg4 transgenic line allowed for an analysis of expression at the level of single cells and demonstrated a correlation between the expression and methylation phenotype. Because the intermediate expression phenotype in some transgenic animals reflects changes in the number of cells expressing *lacZ* rather than the level of *lacZ* expression in all cells, it seems likely that the intermediate methylation phenotype reflects changes in the number of hypermethylated versus hypomethylated

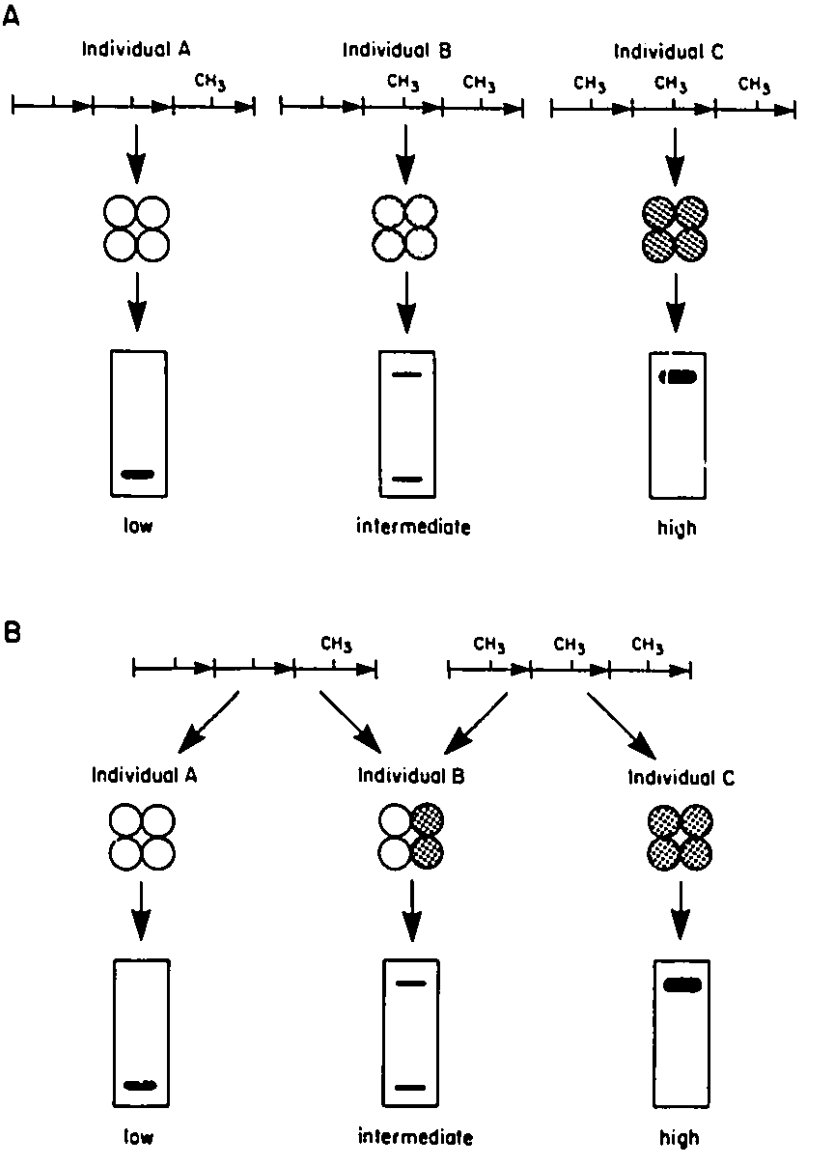

Figure 5. Two models demonstrating the possible means of producing the three methylation phenotypes observed for the two transgene loci (line 379-TNI and line Tg4). In both models the transgene array is represented along the *top*, the cell configuration in the *center* [(open circles) cells that contain a hypomethylated allele; (hatched circles) cells that contain a partially methylated allele; (cross-hatched circles) cells that contain a hypermethylated allele] and the methylation-sensitive restriction endonuclease hybridization pattern along the *bottom*. (*A*) One possibility in which all cells within an individual have the array methylated similarly but different individuals have a different degree of transgene array methylation; (*B*) the other possibility in which different cells within an individual are methylated differently.

cells (Fig. 5B). The methylation of the 379 troponin I transgene also displays different methylation phenotypes (Sapienza et al. 1989a) similar to that of the Tg4 transgene and is therefore also consistent with the model presented in Figure 5B.

This model requires the establishment of at least two cell-specific methylation patterns. The mechanism by which such differences are established between apparently identical cells is unknown, but data presented here (Fig. 4), as well as previously published data (Sapienza et al. 1989a), indicate that this process is under genetic control. Because tissues of ectodermal, endodermal, and mesodermal origin from each individual have the same overall transgene methylation phenotype, we presume that the same relative numbers of cells with each methylation phenotype are present in each tissue. These observations suggest the possibility that the methylation mosaicism is already established in the last common ancestor of these tissues, i.e., by primitive ectoderm formation at day 4.5 of development (Hogan et al. 1986). However, we cannot say whether the methylation pattern is established only once and then propagated faithfully or is generated continuously. Either possibility will produce the observed pattern.

The genetic influence of one inbred mouse strain (C57BL/6) on the methylation phenotype of the two transgenic lines we studied appears to be different. In the 379-TNI line (Sapienza et al. 1989a), the transgene becomes more methylated under the influence of this genotype, whereas the converse is true in the Tg4 line. This is presumably a result of either the site of insertion or inherent differences between the two sequences and demonstrates that different domains or gene structures can be treated differently in the same genetic background.

It is interesting to note that several other transgenes

have been reported to exhibit cellular mosaicism in expression when maintained in a hemizygous state, even though the tissue-specific site of expression is correct. Among these are a myelin basic protein antisense cDNA (Katsuki et al. 1988), a liver fatty acid-binding protein–human growth hormone construct (Sweetser et al. 1988a) and an intestinal fatty acid-binding protein–human growth hormone construct (Sweetser et al. 1988b). In addition, another *lacZ*-containing transgene shows expression differences in preimplantation embryos, apparently as a function of genetic background (M.H.A. Surani, pers. comm.). The expression phenotype we see with the Tg4 transgene, therefore, is not a unique consequence of sequence or site of insertion. In this regard it is noteworthy that the Tg4 insertion disrupts the *dt* locus and, therefore, lies within or adjacent to an endogenous locus (Kothary et al. 1988).

Allele-specific methylation differences have been demonstrated in cultured cells of human origin (Chandler et al. 1987) and heritability of locus-specific methylation patterns have been demonstrated in human pedigrees (Silva and White 1988) but neither of these observations shows an effect of genotype on either the methylation or expression pattern.

Somatic mosaicism has been implicated in the establishment or progression of a variety of clinical disorders (for review, see Hall 1988). Such mosaicism has been interpreted as arising from random events that occur in developing somatic tissue such that daughter cells of the affected somatic cell carry a mutation. Our analysis of the methylation phenotypes associated with transgenes, as well as those of an ecotropic retroviral locus, suggest that methylation mosaicism may arise normally during development. Furthermore, the different cells in the methylation mosaics are apparently not identical in their ability to express all of the genetic information they contain. Such expression differences may reflect true mutations rather than epigenetic changes, but if this were the case somatic mutation rates must be very high, and either the creation or allocation of cells containing such mutations must be under genetic control.

If the allele inactivation process we observe at hemizygous transgene loci also operates at endogenous loci containing two alleles, one predicts that some individuals will be functionally hemizygous at affected loci in some fraction of cells (Scrable et al. 1989). The genetic consequences of such mosaicism (Sapienza 1989) would be that mutant alleles at loci subject to methylation and inactivation would behave in a dominant fashion but would exhibit incomplete penetrance and variable expressivity. These possibilities are being investigated.

Materials and methods

Mice

Inbred strains of mice were obtained from The Jackson Laboratory (Bar Harbor, Maine). The two transgenic lines of mice used have been described previously. 379-TNI is a quail tropoin I fast-fiber muscle-specific gene (Hallauer et al. 1988); Tg4 is a *hsp68–lacZ* construct (Kothary et al. 1988). Timed matings were accomplished by placing estrus females with transgenic males and identifying copulatory plugs on the following morning (day 0 of pregnancy). Females were killed at 12 days gestation, and fetuses were removed. For the Tg4 line, fetal heads were used for DNA extraction, and the bodies were fixed and stained for β-galactosidase activity.

Southern hybridization

DNA was prepared from tail biopsies or fetal tissues, as described previously (Sapienza et al. 1987), and cleaved with the indicated restriction endonucleases, as suggested by the manufacturer (Pharmacia). The extent of digestion was tested by the addition of control plasmid DNA to a sample of the experimental digest. Digestion was considered to be complete when the internal control gave a digestion pattern identical to the plasmid alone when digested with the same enzyme. Gel electrophoresis, transfer of DNA to nylon membranes (Nytran, Amersham Corp.), preparation of ^{32}P-labeled probes, hybridizations, and autoradiography were also described previously (Sapienza et al. 1987).

E. coli *β-galactosidase assays*

β-Galactosidase activity was determined using a modification of the method described by Kothary et al. (1988). Fetal tissues were fixed in 0.5% paraformaldehyde, 0.5% glutaraldehyde in PBS (0.07 M Na_2HPO_4, 0.03 M NaH_2PO_4, 0.5 M NaCl) for 1 hr at 4°C. The tissues were rinsed in PBS and incubated in a staining mixture consisting of 3.1 mM K^+ ferricyanide, 3.1 mM K^+ ferrocyanide, 10 mM $NaPO_4$ (pH 7.2), 0.5 M NaCl, 1.0 mM $MgCl_2$, and 0.4 mg/ml halogenated indoyl-β-D-galactoside (Bluo-gal, BRL) for 12–18 hr at 37°C.

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