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Lineage analysis in the vertebrate nervous system by retrovirus-mediated gene transfer

(retrovirus vectors/β-galactosidase/neural progenitors/retina)

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ABSTRACT **We describe a cell-lineage marking system applicable to the vertebrate nervous system. The basis of the technique is gene transfer using the retroviral vector system. We used *Escherichia coli* β-galactosidase as a marker gene and demonstrate a high level of expression of this marker from the viral long terminal repeat promoter, with simultaneous expression of the Tn*5* *neo* gene from the simian virus 40 early promoter. This expression has allowed us to detect individual infected cells histochemically. We applied this marking technique to the study of lineage relationships in the developing vertebrate nervous system, both *in vivo* and in culture. In the rat retina, we injected virus *in vivo* and histochemically identified clones of marked neural cells. In addition, we used this virus to infect cultures of rat cerebral cortex and have analyzed the clonal relationships of morphologically different neural cell types. The host range of the marking system extends to avian as well as mammalian species. Thus, this system should have broad applicability as a means of gene transfer and expression in the nervous system.**

Our understanding of vertebrate development would be greatly enhanced if the fate of individual cells was known. Single cell marking techniques have been applied to lineage mapping, but most of these techniques have limitations, such as dilution of the label or inaccessibility of target cells (1). Nonetheless, recent improvements in the injection of dyes have allowed observations of the development of individual cells in some species (2). Alternative methods involving the construction of genetic mosaics and xenoplastic transplants have provided information on the peripheral nervous system, cerebellum, and retina (3). These chimeras have been constructed using multicellular grafts or mixed blastocysts, preventing definitive conclusions regarding the potential of individual cells. In general, a greater degree of versatility in the development stages and types of cells that can be marked is desirable.

An ideal single cell marking system would be one in which the marker is indelible, innocuous, and does not spread to non-sibling cells. Also, it should be detectable histochemically so that individual cells can be identified. Certainly for "indelibility," a genetic marker would appear to be ideal. The gene transfer system that seems most capable of introducing genetic markers into embryos is the retrovirus vector system (reviewed in ref. 4). The replication-competent viruses are mostly innocuous, even when the virus is actively replicating. The vector systems that have been developed retain the ability of the retrovirus to integrate stably and precisely into a host cell chromosome and subsequently pass to all daughter cells as a normal, Mendelian gene. The vectors accept almost any foreign gene, and packaging systems for producing pure, replication-incompetent vector stocks have been established (5, 6). The packaging systems provide viral envelopes that allow infection of a broad range of host species. Thus, the features of stability, lack of spread, and the ability to carry genes for histochemical markers from virtually any source are inherent in the current vector system.

We have chosen *Escherichia coli* β-galactosidase (β-gal) as the histochemical marker for our studies. Several other groups working in other systems have also exploited this gene as a marker in a variety of cell types (for example, see ref. 7). The enzyme can be detected histochemically (8) and has also been successfully expressed in an avian retrovirus vector (9).

We have used a retroviral vector to mark cells of the nervous system by injecting virus into the rat retina. The retina is accessible, it can be stained as a whole mount, it has a variety of neuronal and glial cell types in an ordered morphology that permits the identification of labeled cells, and there are several cell types that are dividing at birth (10). We demonstrate here that retroviruses that encode the β-gal gene can be successfully introduced into the rat retina and mark cells such that they can easily be detected histochemically. In addition, we have used this marker system to infect primary cultures from the cerebral cortex and have studied the lineage relationships of the morphologically recognizable cell types.

MATERIALS AND METHODS

Plasmid Constructions. All procedures used in cloning were essentially as described (11). Enzymes and linkers were obtained from New England Biolabs or Collaborative Research (Waltham, MA) and were used according to manufacturer's specification.

Assays of β-gal. β-gal activity in cell extracts was assayed as described (9), using either *o*-nitrophenyl-β-D-galactoside or 4-methylumbelliferyl-β-D-galactoside (Sigma) as a substrate. Purified *E. coli* β-gal (Sigma) was used as a standard. For immunoblots, detergent extracts (0.1% Nonidet P-40/2 mM phenymethylsulfonyl fluoride/2 mM EDTA/15% glycerol/100 mM Tris, pH 6.8) were run on 7% $NaDodSO_4$/PAGE and transferred to nitrocellulose (12). The primary antibody was an anti-β-gal monoclonal antibody ascites fluid diluted 1:500 (13). For the ELISA, cells were homogenized in a Dounce homogenizer and serial 1:2 dilutions were incubated in 96-well microtiter wells. Each well was then incubated at room temperature for 1 hr with anti-β-gal monoclonal antibody at a constant dilution. The unbound antibody in each well was assayed by transferring the supernatants to

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Abbreviations: β-gal, β-galactosidase; cfu, colony-forming unit(s); SV40, simian virus 40; LTR, long terminal repeat; Mo-MuLV, Moloney murine leukemia virus; X-Gal, 5-bromo-4-chloro-3-indolyl β-D-galactoside; G418-R, G418-resistant; E15, embryonic day 15; BAG, β-gal-at-gag; SVX, pZIP-Neo SV(X)1.

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Immulon 2 Removawell strips (Dynatech, Alexandria, VA) coated overnight at 4°C with 1 μg of β-gal per well. The strips were washed and incubated with alkaline phosphatase-coupled goat anti-mouse immunoglobulin (Cappel Laboratories, Cochranville, PA). Finally, the strips were washed and allowed to react with 1 mg of *p*-nitrophenol per ml. This assay was standardized using homogenates of uninfected cells to which had been added a known amount of pure *E. coli* β-gal.

For 5-bromo-4-chloro-3-indolyl β-D-galactoside (X-Gal) visualization of β-gal activity in intact cells, the method of Dannenberg and Suga (8) was used, following fixation in 0.5% glutaraldehyde for 15 min.

Cell Lines and Viruses. Producer cell lines, ψ2 (5) and ψam (6), and NIH 3T3 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) and 10% calf serum. The following cell lines were used: neuroblastoma line 2A (14); N1E-115 (15); NG108 (16); C6 (17); PC12 (18); Y79-6 (19); QT6 (20); all lines were maintained as recommended in the cited references. Virus stocks were prepared by $CaPO_4$ transfection (21) of ψ2 cells. Virus stocks were concentrated by centrifugation (14,000 rpm in a Beckman J14 rotor at 4°C for 12–16 hr, followed by resuspension in 1% of the original volume in DMEM and 10% calf serum). Infections were performed in the presence of 8 μg of Polybrene per ml.

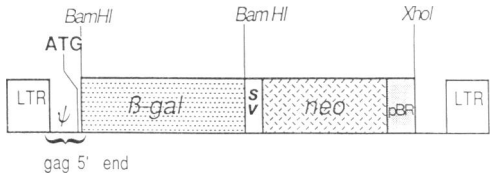
Primary Neural Cultures. Ninety-five to 98% pure astrocytes were prepared as described (22) on 12-mm glass coverslips. Onto these monolayers were plated a suspension of cells from embryonic day 15 (E15) Fisher rat cerebral cortices. The cortices were dissected from surrounding brain regions and meninges and dissociated with trypsin (0.025% for 30 min at 37°C), followed by gentle trituration with a Pasteur pipette. The resulting suspension was counted and plated at 3×10^4 cells per coverslip. Cells were infected on plating with 5 μl of β-gal-at-gag (BAG) virus stock [5×10^3 colony-forming units (cfu)] and 8 μg of Polybrene per ml.

Retina Injections and Histochemistry. Fisher rats were cryoanesthetized on the day of birth, an eyelid was surgically opened, and an incision was made in the front of the eyeball using a 27-gauge needle. A Hamilton 10-μl syringe with a blunt 31-gauge needle was then inserted to the rear wall of the eye and ≈1 μl of concentrated ψ2-produced BAG virus (2×10^7 cfu/ml) containing 0.05% trypan blue and 80 μg of Polybrene per ml was delivered. Mock injections were made with pZIP-Neo SV(X)1 (SVX) virus (23). Animals were killed at periods of 1–9 weeks of age; the retinas were removed, fixed in 0.5% glutaraldehyde, and processed for X-Gal histochemistry as described (8) except that the buffer used was phosphate-buffered normal saline and the concentration of the Fe compounds was raised to 35 mM. The retinas were examined as whole mounts, then frozen, and sectioned.

RESULTS

β-gal Vector Construction and Transmissability. We constructed a β-gal-transducing vector, BAG, by cloning the β-gal gene into the pDOL vector, which was derived from Moloney murine leukemia virus (Mo-MuLV) (Fig. 1). The wild-type Mo-MuLV LTR provided the promoter for the β-gal gene. The structure of the mRNA for β-gal, from the cap site through the most frequently used gag AUG (gag pr65), was identical to that of wild-type gag-pol mRNA. The SV40 early promoter and the Tn*5* *neo* gene, which transmits G418 resistance, were present downstream from β-gal to provide a selectable marker for *in vitro* experiments.

To test transmissability, BAG plasmid was transfected into the ψ2 packaging line and the transiently produced virus was assayed by infection of NIH 3T3 cells, followed by quantitation of the number of G418-resistant (G418-R) colonies. Virus produced by stably transfected G418-R ψ2 clones was also tested for the ability to transduce simultaneously the



BAG

FIG. 1. Structure of the β-gal-transducing Mo-MuLV vector. The pDOL vector of A. Korman and R. Mulligan (Massachusetts Institute of Technology) was modified to encode the gag pr65 ATG. A *Pvu* I to *Hae* III fragment of Mo-MuLV (nucleotides 423–625, ref. 24) was linked with *Bam*HI linkers (12-mer linkers, Collaborative Research) at the *Hae* III site. This fragment (containing the ATG at Mo-MuLV nucleotide 621) was ligated into pDOL in place of the preexisting *Pvu* I to *Bam*HI fragment to create a translation initiation codon for the bacterial β-gal fragment. A 3-kilobase *Bam*HI fragment encoding *E. coli* β-gal (from pMC1871, ref. 25) was ligated into the *Bam*HI site. The simian virus 40 (SV40) early promoter and *neo* gene were from pSV2neo (26); the pBR322 origin of replication was from SVX virus (23); and the remaining Mo-MuLV sequences at the 3′ end of the vector were from wild-type Mo-MuLV, nucleotides 7195 (former *Hpa* I site) to the end of the viral long terminal repeat (LTR). The polyoma early region (polyoma *Bam*HI to *Hin*cII fragment) was ligated into the plasmid outside of the viral LTRs and flanking sequence.

β-gal and *neo* genes. The titers produced transiently after transfection of pDOL and BAG were ≈10^4 G418-R cfu/ml. The stable titers produced by ≈25% of the BAG-transfected or pDOL-transfected ψ2 clones were 10^6 G418-R cfu/ml.

Stable ψ2 producers of BAG virus, BAG-infected NIH 3T3 clones, and NIH 3T3 populations infected either from transiently produced virus or with supernatants from stable ψ2 producers were analyzed by Southern blotting and cos cell-fusion rescue (23) to determine the structure of the integrated viral genomes. The viral genome exhibited the structure of the original BAG plasmid, and a single copy of the virus was observed in all infected clones examined.

Expression and Detection of β-gal. β-gal enzymatic activity was assessed by colorimetric and fluorimetric assays performed on total cell extracts. Transfected ψ2 and infected NIH 3T3 cells expressed $3–5 \times 10^5$ copies of β-gal protein per cell. This estimate was made by comparing the amount of activity in these cells with that of authentic *E. coli* β-gal added to uninfected cell extracts. As a second means of estimating the level of β-gal protein in infected cells, antibodies against β-gal were used to develop an ELISA. This assay can detect any immunologically reactive protein that might be enzymatically inactive. The ELISA results were in agreement with those of the enzyme assay, indicating that most of the immunologically reactive β-gal was also enzymatically active. An immunoblot using a monoclonal antibody against *E. coli* β-gal showed that infected and transfected cells contain an immunoreactive band not present in uninfected cells, which comigrated with authentic *E. coli* β-gal (Fig. 2).

Histochemical Detection of β-gal Enzymatic Activity. The X-Gal technique proved to be a sensitive and reliable method for the identification of individual BAG-infected cells. Fig. 3*A* shows blue, X-Gal-stained cells among white uninfected cells, after X-Gal staining.

Host Range of BAG Viruses. To produce a virus stock capable of infecting a broad host range (rodent, human, dog, cat, mink, and monkey), the ψam packaging line (6) was infected with virus from a ψ2 BAG producer. Clonal ψam BAG producer lines were titered, giving, in the best instances, 10^5 G418-R cfu/ml.

The ψam-produced BAG virus was infectious on chicken embryo fibroblasts, in agreement with a previous observation (28). The canine line D17, human retinoblastoma line Y79-6, and primary cultures of chicken retina were also infectable.

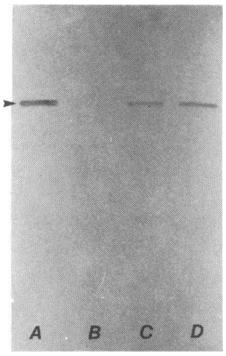


FIG. 2. Immunoblot of cell extracts from cells carrying the BAG virus. Nonidet P-40 extracts of cells were run under reducing conditions on a 7% $NaDodSO_4$/polyacrylamide gel, transferred to nitrocellulose, and allowed to react with a monoclonal antibody to *E. coli* β-gal. Lane A, *E. coli* β-gal from Boehringer Mannheim; lane B, uninfected chicken embryo fibroblasts; lane C, chicken embryo fibroblasts infected with ψam-produced BAG virus, selected in G418, and grown for several passages as a polyclonal population; lane D, a stably transfected clone of ψ2 cells transfected with BAG plasmid. In lanes B–D, equal amounts of protein (50 μg) were loaded per lane. An arrowhead indicates the position of β-gal at ≈100 kDa.

The level of β-gal enzyme activity in chicken embryo fibroblasts was as high as that observed in mouse NIH 3T3 cells (3×10^5 molecules per cell) and the enzyme comigrated with authentic β-gal on $NaDodSO_4$/PAGE (Fig. 2). Thus, the two mammalian promoters, the viral LTR and the SV40 early promoter, seem to express quite effectively in chicken cells.

To assess the tissue specificity of Mo-MuLV vector infection and expression in the nervous system, several neural cell lines and primary cultures were infected with ψ2-produced BAG virus. Neuroblastoma lines 2A (a derivative of C1300), N1E-115, and NG108, pheochromocytoma line PC-12, and a glioma line (C6) yielded 1–10% as many colonies on infection as NIH 3T3 cells. Expression of β-gal was approximately as high as that found in NIH 3T3, when quantitated. Primary rat and mouse olfactory bulb, cerebral cortex, and retina were successfully infected, although efficiency varied among different cultures (data not shown).

***In Vitro* Lineage Marking.** Primary cultures of E15 rat cerebral cortex were exposed to BAG virus and later stained with X-Gal. Pilot experiments indicated the amount of virus necessary to yield only five or six infected clones per experiment, a situation in which clones could easily be distinguished from one another. The probability that a cluster of blue cells, well isolated from other clusters of blue cells, descended from more than one infected parental cell was quite low.

Three types of clones were observed. In one type, all of the marked cells had a fibroblastic morphology and were probably all type 1 astrocytes (using the nomenclature of Raff *et al.*; ref. 29). However, without applying more definitive criteria than morphology, one cannot exclude the possibility that they were fibroblasts, endothelial cells, or meningeal cells. This was the only type of clone that contained just one morphological cell type. A second type of clone (Fig. 3*B*) in which the majority of the cells were also fibroblastic also included cells that had a single, flat, extensive process. The morphology of these cells is reminiscent of that of radial glia. All such clones had numerous (≈10–20) fibroblastic cells and either four or six of the radial glia-like cells.

A third type of clone contained process-bearing cells, most of which were either neurons and/or type 2 astrocytes (Fig. 3*C*). We know this from parallel cultures that were immunohistochemically stained with antibodies against either glial fibrillary acidic protein or neurofilament (data not shown). At the present moment we are unable to say whether such clones contain both neurons and glia, but all such clones contained process-bearing cells and fibroblastic cells (perhaps type 1 astrocytes). No clone that we have seen has been comprised of process-bearing cells alone.

Clearly, morphological criteria are inadequate to settle such lineage questions definitively; immunological markers are required. Unfortunately, we have found that immunofluorescence is incompatible with the X-Gal-staining procedure.

Lineage Mapping *in Vivo*. Concentrated BAG virus was injected into the retina of rat pups on the day of birth. The intended site of delivery of virus was the mitotic zone between the pigment epithelium and neural retina. From observations of the spread of dye coinjected with the virus, as well as the distribution of infected cells over the entire retina, it appeared that the injected virus spread between these two layers. After injection, the animals were allowed to develop for up to 2 months. Fig. 3*D* shows whole mounts of infected and control retinas. The infected retina was injected with $\approx 10^4$ cfu of BAG virus and shows a large number of blue clones. The possibility of staining whole mounts in this fashion and therefore identifying clones prior to sectioning is an advantage of the retina as an experimental model. Infection and expression of BAG was very efficient in the retina, as judged by the number of blue clusters in the whole mount preparations; in retinas with the highest amount of infection, several thousand clusters were observed. In experiments in which less virus was injected, fewer clusters were observed.

As shown in Fig. 3 *E* and *F*, the labeled cells were primarily rod photoreceptors, as judged by morphology and cell location. Bipolar cells and Müller glial cells were also labeled (data not shown). Moreover, labeled cells were also observed in the pigment epithelium (data not shown). Over 90% of labeled cells were rod photoreceptors. This was expected as these cells are the most abundant retinal cell type and the major cell type that was being produced at the time of injection (27). The number of labeled photoreceptors in a cluster was typically between 2 and 10, with the larger clones found predominantly in the peripheral, as opposed to the central, retina. Interestingly, the cells of a clone always had a strictly radial distribution with their outer segments clustered together and their cell bodies at varying depths in the outer nuclear layer (Fig. 3 *E* and *F*).

Background staining of mock-injected or uninjected retinas was only observed when the tissue was grossly overstained (18 hr as opposed to 2 hr), when staining appeared in the ganglion cell layer.

DISCUSSION

We have constructed a vector containing the bacterial β-gal gene, and here we show that this vector is capable of infection and expression of β-gal and resistance to G418 in a variety of neural cell types. We also show stable, high-level, *in vitro* expression of β-gal as indicated by enzyme assay, ELISA, and immunoblotting data. We have applied this β-gal virus to the study of neural lineage *in vivo* and in culture and have been able to mark cells in both cases.

In the retina, we have shown that it is possible to introduce a retroviral vector *in vivo* into the vicinity of dividing neural precursors in such a way that a small proportion of these cells becomes infected. Whole mount staining and sectioning allowed us to identify and analyze the marked clones. All of the major cell types being generated at the site of injection at this time—i.e., rod photoreceptors, Müller glia cells, bipolar cells, and pigment epithelial cells—were successfully infected. Ganglion cells, horizontal cells, and cone photoreceptors, which become postmitotic before birth, were not labeled. All retinal cell types are initially generated from the same mitotic zone and, after their final divisions, migrate inward to form

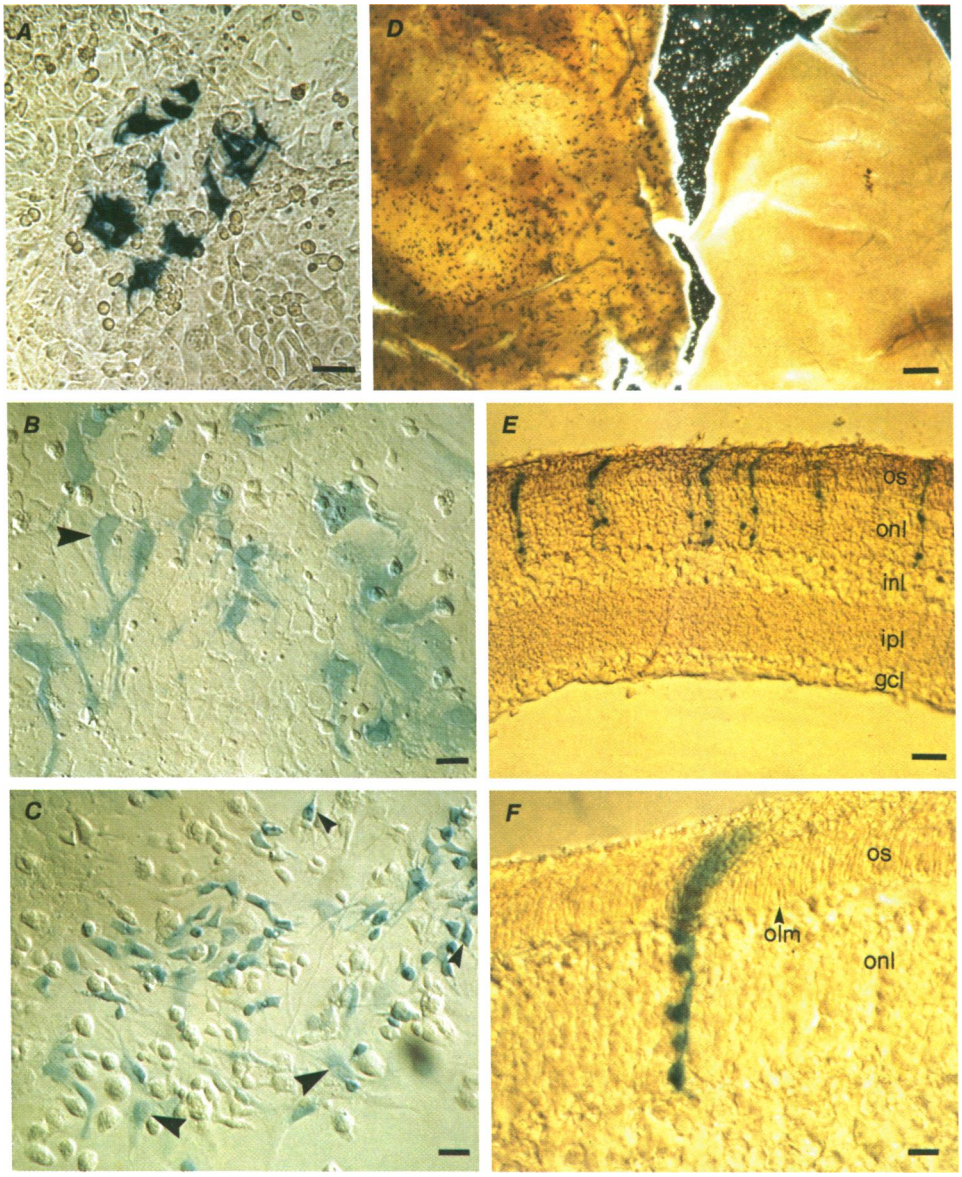


FIG. 3. Infection of cells *in vitro* and *in vivo* with BAG virus. (*A*) D17 cells were infected with BAG virus and stained 2 days later with X-Gal. Infected cells are blue and are surrounded by uninfected cells. (*B*) Portion of a BAG-infected clone in a culture of E15 rat cerebral cortex stained with X-Gal after 7 days *in vitro*. Most of the labeled cells are "fibroblastic" in morphology, but four cells have a different morphology (arrowhead) reminiscent of radial glial cells. (*C*) Portion of a BAG-infected clone in a parallel culture to that shown in *B*. Process-bearing cells are indicated by small arrowheads and flat fibroblastic cells, similar in morphology to those shown in *B*, are indicated by large arrowheads. (*D*) X-Gal-stained whole mounts of retinas from injected rat pups. The retina on the left was injected with BAG virus and the one on the right was injected with a control virus (SVX) that did not contain the β-gal gene. Injections were made between the pigment epithelium and the neural retina on the day of birth. Adult retinas were harvested and processed for X-Gal histochemistry. Each of the blue spots seen in the BAG-infected retina represents one or several labeled cells, as seen in 20-μm frozen cross sections in *E* and *F*. (*E*) Clusters of rod photoreceptors with cell bodies distributed radially within the outer nuclear layer are visible. Each cluster presumably represents a clone of infected cells. os, Outer segments; onl, outer nuclear layer; inl, inner nuclear layer; ipl, inner plexiform layer; gcl, ganglion cell layer. (*F*) Isolated clone near the periphery of the same retina. Six rod photoreceptors are labeled (one is out of the plane of focus). The cell processes are clearly stained, as are the outer segments. olm, Outer limiting membrane. (*A–C*, bars = 50 μm; *D*, bar = 250 μm; *E*, bar = 25 μm; *F*, bar = 10 μm.)

the multilayered retinal structure (27, 30). The observed radial distribution of rod cell bodies in X-Gal-labeled clones is consistent with this radial migration and suggests that little lateral cell movement occurs in the postnatal retina. The larger number of cells in peripheral clones agrees with previous observations that mitosis in the periphery continues for 2–3 days longer than in the central retina (30). These results indicate that the infected cells participate normally in development with respect to their gross appearance and fate. It remains to be shown that β-gal is innocuous in all cells at all developmental stages.

The cerebral cortex culture experiments suggest that we labeled progenitor cells that have not been previously described. Although the culture experiments are currently limited by our inability to characterize the X-Gal-stained cells immunohistochemically, clones that contain morphologically distinctive cell types, such as process-bearing cells together with flat cells, were reproducibly observed. Raff *et al.* (29) have proposed that there is a progenitor cell (O2A) that gives rise to type 2 astrocytes and to oligodendrocytes in the optic nerve. Type 1 flat astrocytes are thought to have a separate lineage (31). Neurons do not develop in the optic nerve. If these separate lineages existed in the cortex, we would expect to see at least two distinct types of clones in our cultures. One type would be made up of flat fibroblastic type 1 astrocytes. The other would be made up of process-bearing

cells—i.e., cells with small cell bodies and long thin processes. Type 2 astrocytes, oligodendrocytes, and the O2A progenitor itself all fall into this latter morphological class. We do see clones that fall into the flat, fibroblastic morphological class (type 1 astrocytes). However, the majority of our clones contain process-bearing cells and flat cells, indicating that a single progenitor has given rise to both types. Cells with such developmental potential do not seem to exist in the optic nerve. The most likely interpretations of our results are either that the cortex is different from the optic nerve in the potential of progenitors found there or that we have succeeded in finding a less committed cell. A third less likely possibility is that our culture conditions allow a greater range of expression of potential phenotypes.

The data presented here demonstrate the utility of retrovirus vectors for the introduction of genes into neural cell precursors. *In vitro* and *in vivo* experiments demonstrate successful infection and subsequent expression from the viral LTR. In addition, *in vitro* experiments have shown that expression can occur from the SV40 promoter within a retroviral vector. The efficiencies that were observed here should enable studies on gene expression and function. The level of expression of β-gal in photoreceptors, as judged by the intensity of X-Gal staining, was among the highest observed in any cell type with which we have worked. Moreover, we could distinguish cells infected with a virus that induces the synthesis of less β-gal (by a factor of 100) than BAG (unpublished observations). This probably means that highly efficient expression will not be necessary for detection in the lineage mapping experiments. After mapping a particular tissue, the function of genes that may play a role in development of the nervous system can be assessed by cloning them into the β-gal vector in place of the *neo* gene. The infection can be monitored by examination of the tissue with X-Gal staining. The behavior of the labeled cells can then be compared to the behavior of cells labeled with BAG. Differences in clone size, morphology, or migration patterns may be interpretable with respect to the activity of the test gene.

The ability to derive lineage relationships from the patterns of X-Gal staining requires that clones be recognizable as such. We have relied on low density of infection giving a few, discrete clones and on the reproducibility of the results. There is currently no feasible method of demonstrating biochemically that any clone is indeed the result of infection of a single precursor cell. The virus does provide a genetic mark of clonality as each provirus integrates into a unique site in the host genome, but the verification of this requires either a Southern blot or the cloning of the fragment that contains the provirus. Either of these approaches requires isolation of at least 10^4 infected cells. Even if such a procedure were feasible, proving clonality for any individual case does not prove the general case.

Interpretation of lineage relationships is limited to cells that express the β-gal marker: unlabeled neighboring cells cannot be assumed to be unrelated, as they may simply fail to express the viral gene products. We have observed that mosaic expression of β-gal occasionally can occur even in clones of infected fibroblasts carried *in vitro*. Other cell types may not express the gene at all. It has been reported that Mo-MuLV infection and subsequent expression from the viral LTR in embryonic and early postnatal mouse brain occur fairly efficiently (32). However, immunohistochemical analysis of the infected cells' identity has not been reported. The method presented here will serve to clarify this issue.

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