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A strategy for the analysis of gene expression during neural development

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ABSTRACT **The delineation of cis-acting elements regulating stage-specific gene expression has played a critical role in the definition of the transcriptional circuitry regulating differentiation. In complex tissues such as the central nervous system, differentiation often requires temporally and spatially dynamic epigenetic cues that cannot be reproduced in cell culture. In these cases, identification of critical regulatory sequences and subsequent characterization of cognate transcription factors have been limited by lack of a rapid and efficient assay system for gene expression. We report a methodology that combines particle-bombardment transfection procedures and organotypic slice culture techniques to provide an acute assay system for transcriptional control in the developing central nervous system. Using this system, we demonstrate that cell-specific regulation of the brain lipid-binding protein (BLBP) gene in Bergmann glia, astrocytes, and migrating granule cells is conferred by 1.7 kb of 5′ flanking sequences and that Purkinje cell-specific expression of the calbindin D_{28k} gene in cerebellar cortex can be achieved with 1.1 kb of flanking DNA. Nearly 100% cotransfection of multiple DNAs can be achieved, allowing the design of precisely internally controlled experiments and providing the potential for rapid and efficient genetic analysis of gene function in single cells in a wild-type environment.**

The elucidation of molecular mechanisms that underlie development of the mammalian nervous system has proved to be significantly more difficult than in *Caenorhabditis elegans* or *Drosophila melanogaster* in large part because of the lack of powerful genetic methods for the identification of regulatory molecules that program specific developmental transitions. Two strategies to overcome this limitation are presently in use: (*i*) the isolation and analysis of mammalian homologues to developmental regulators first identified in lower eukaryotes and (*ii*) the direct identification of novel regulatory molecules through molecular and biochemical dissection of signal transduction events that ultimately lead to changes in gene expression in mammals. In both cases, progress in understanding the pathways that control development is limited by the time-consuming and cumbersome methods now available for the analysis of gene expression in complex tissues. Dissection of regulatory sequences that are either potential targets of known transcriptional regulatory proteins or that can serve as affinity matrices for the isolation of novel transcription factors often requires the use of transgenic mice or unwieldy viral vectors. With such methods, analysis of the expression patterns of several different fusion gene constructs in multiple transgenic lines or reconstructed viruses can often take many months or years to complete. In cases where wild-type regulation of transcription can be achieved in cell culture systems, its analysis is accelerated by the use of transient-transfection assays that can be performed in a matter of days. However, gene expression in the developing mammalian central nervous system (CNS) is dependent on regulatory interactions that result from epigenetic events that in turn require specific and complex cell–cell interactions that cannot be reproduced in primary cell culture. In addition, postmitotic neurons are often difficult to transfect. We have developed a method for analyzing regulatory sequences that circumvents these difficulties by introducing fusion gene constructs directly into cultured slices of neonatal mouse cerebellum that maintain the complex cytoarchitecture present in developing tissue. Transfection is accomplished by coating 1-μm-diameter gold beads with plasmid DNA and then shooting them into slices with the PDS-1000 particle delivery system (Bio-Rad). This method is sufficiently rapid, efficient, and accurate to significantly accelerate the routine analysis of gene expression in complex systems.

In this study, we demonstrate the properties of this system and report that 5′ flanking sequences of both the brain lipid-binding protein (BLBP) gene and the calbindin D_{28k}

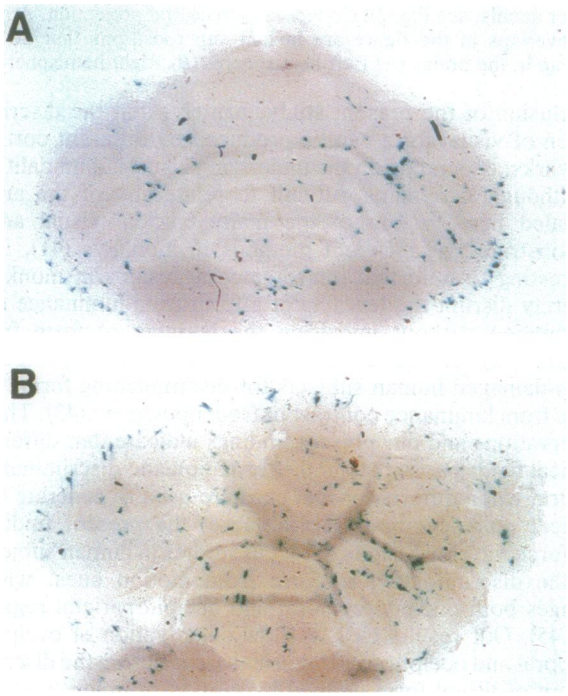

FIG. 1. Coronal slices (400 μm thick) cut from cerebella of mice at postnatal day 10 (*A*) or 9 (*B*), cultured for 24 hr, and then shot with a CMV/*lacZ* construct loaded onto 1-μm-diameter gold particles and incubated for 48 hr before fixation and staining for β-galactosidase activity with X-Gal. Positive cells are blue.

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Abbreviations: BLBP, brain lipid-binding protein; CMV, cytomegalovirus; CNS, central nervous system; X-Gal, 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside.

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gene can confer cell type specific expression in postnatal cerebellar slice preparations.

MATERIALS AND METHODS

Preparation of Organotypic Slices. Coronal slices of 400-μm thickness were cut from mouse cerebella at postnatal day 9 or 10 and cultured by the method of Stoppini *et al.* (1). The slices were placed on 0.4-μm-pore culture plate inserts (Millipore) that sat in the wells of a six-well plate (Falcon). Each well contained 1 ml of culture medium [50% Eagle's minimum essential medium/25% horse serum/25% Hanks' balanced salts solution containing 20 mM Hepes, glucose (5.5 mg/ml), penicillin (100 units/ml) and streptomycin sulfate (100 μg/ml)], just enough for the medium to touch the bottom of the culture-plate insert membrane, while leaving the slice completely exposed to the air. The slices were then incubated at 37°C in a 5% CO_2 atmosphere. In this study, all mice were administered ketamine (44 mg/kg of body weight) prior to dissection. All procedures used were in accordance with institutional guidelines.

Transfection of Slices. After 24 hr in culture, the slices were transfected with a plasmid carrying a gene fusion consisting of the cytomegalovirus (CMV) immediate early promoter/enhancer driving a *lacZ* reporter gene (2). The DNA was loaded onto 1-μm-diameter gold beads by the method of Sanford *et al.* (3). The gold beads were suspended in 70% (vol/vol) ethanol, washed with distilled water, and then suspended in 50% (vol/vol) glycerol. Twenty microliters of beads was then removed and 4 μg of plasmid, 20 μl of 2.5 M $CaCl_2$, and 8 μl of 0.1 M spermidine were added with continuous vortexing. The gold was pelleted by spinning for 2–3 sec in a microcentrifuge and the pellet was washed with 70% and then 100% ethanol. The pellet was finally resuspended by vigorous vortexing and trituration in 20 μl of 100% ethanol until there were no clumps. Six microliters of the suspension was dropped on the center of a macrocarrier that had been wiped with 100% ethanol. The gold beads were shot into slices at 2200 psi (1 psi = 6.89 kPa) by the PDS 1000 He Biolistic particle delivery system (Bio-Rad). The organotypic cultures were returned to the incubator for 48 hr and then fixed and stained for the presence of β-galactosidase. When two plasmid constructs were cotransfected, the procedure was identical, except that 8 μg of a 1:1 mixture of both plasmids was precipitated onto the beads.

Staining of Slices. Slices were fixed for 30 min in 4% paraformaldehyde. They were then washed three times in phosphate-buffered saline (PBS). Slices that were transfected with a β-galactosidase construct were immersed in a solution containing 20 mM $K_3Fe(CN)_6$, 20 mM $K_4Fe(CN)_6$, 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside (X-Gal; Stratagene) at 1 mg/ml, and 2 mM $MgSO_4$ at 37°C for several hours and then washed in PBS and mounted on microscope slides. Slices that were transfected with an alkaline phosphatase construct were incubated overnight with 1% (vol/vol) Triton X-100 in PBS and washed for 10 min in alkaline phosphatase buffer (100 mM NaCl/5 mM $MgCl_2$/100 mM Tris HCl, pH 9.5). They were then left for 5 min in a solution containing levamisol at 250 μg/ml, nitro blue tetrazolium at 250 μg/ml, and 5-bromo-4-chloro-3-indolyl phosphate at 200 μg/ml in alkaline phosphatase buffer.

RESULTS

For this method to be suitable for analyzing regulatory elements of cell-type-specific genes in the developing CNS, it must be capable of transfecting each cell type with high efficiency. To test whether all cell types could be transfected, a construct consisting of the CMV promoter/enhancer driving the *lacZ* gene was shot into coronal slices cut from mouse cerebella at postnatal day 9. Two examples of the results of this type of experiment are shown in Fig. 1. In both slices

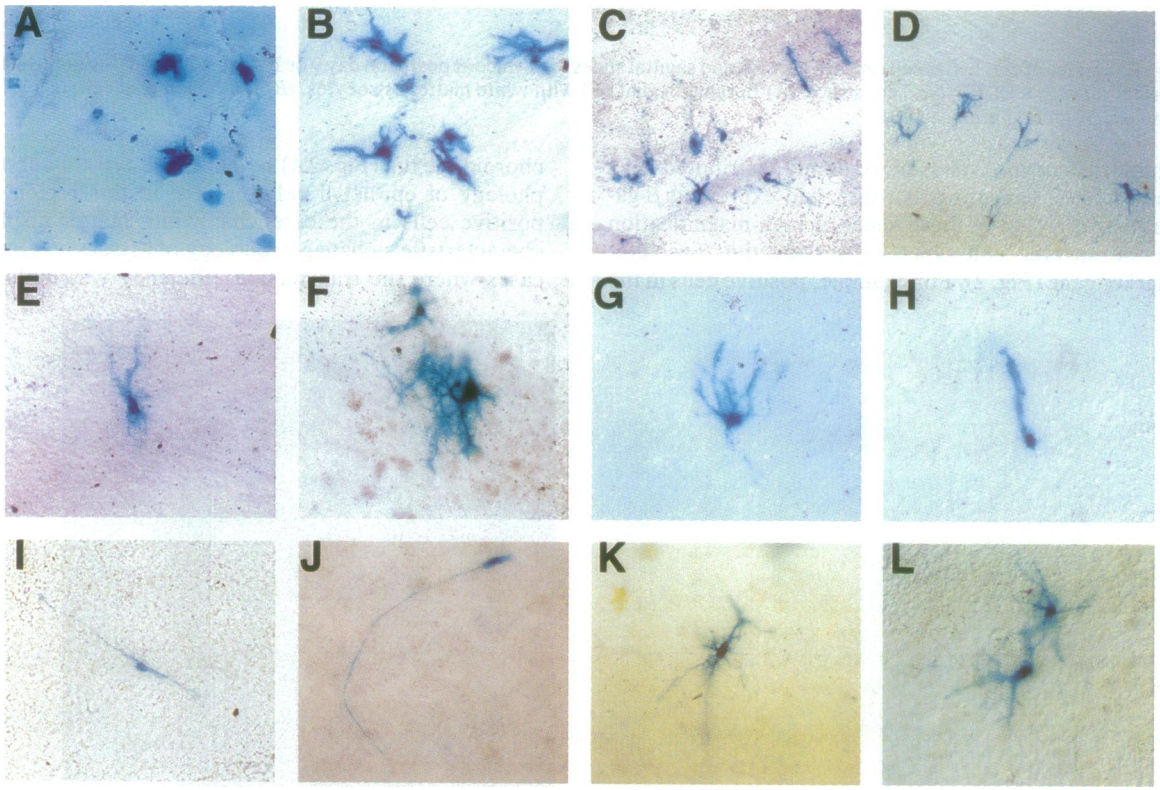

FIG. 2. Examples of cerebellar cell types in coronal and sagittal slices from mice at postnatal day 9 or 10 that exhibited β-galactosidase activity after transfection with a CMV/*lacZ* construct. (*A*) Epithelial cells in the choroid plexus. (*B*) White matter astrocytes. (*C*) Purkinje cells. (*D*) Interneurons. (*E*) Bergmann glial cell. (*F*) Purkinje cell. (*G*) Stellate neuron. (*H*) Granule cell. (*I*) Lugaro cell. (*J–L*) Miscellaneous interneurons.

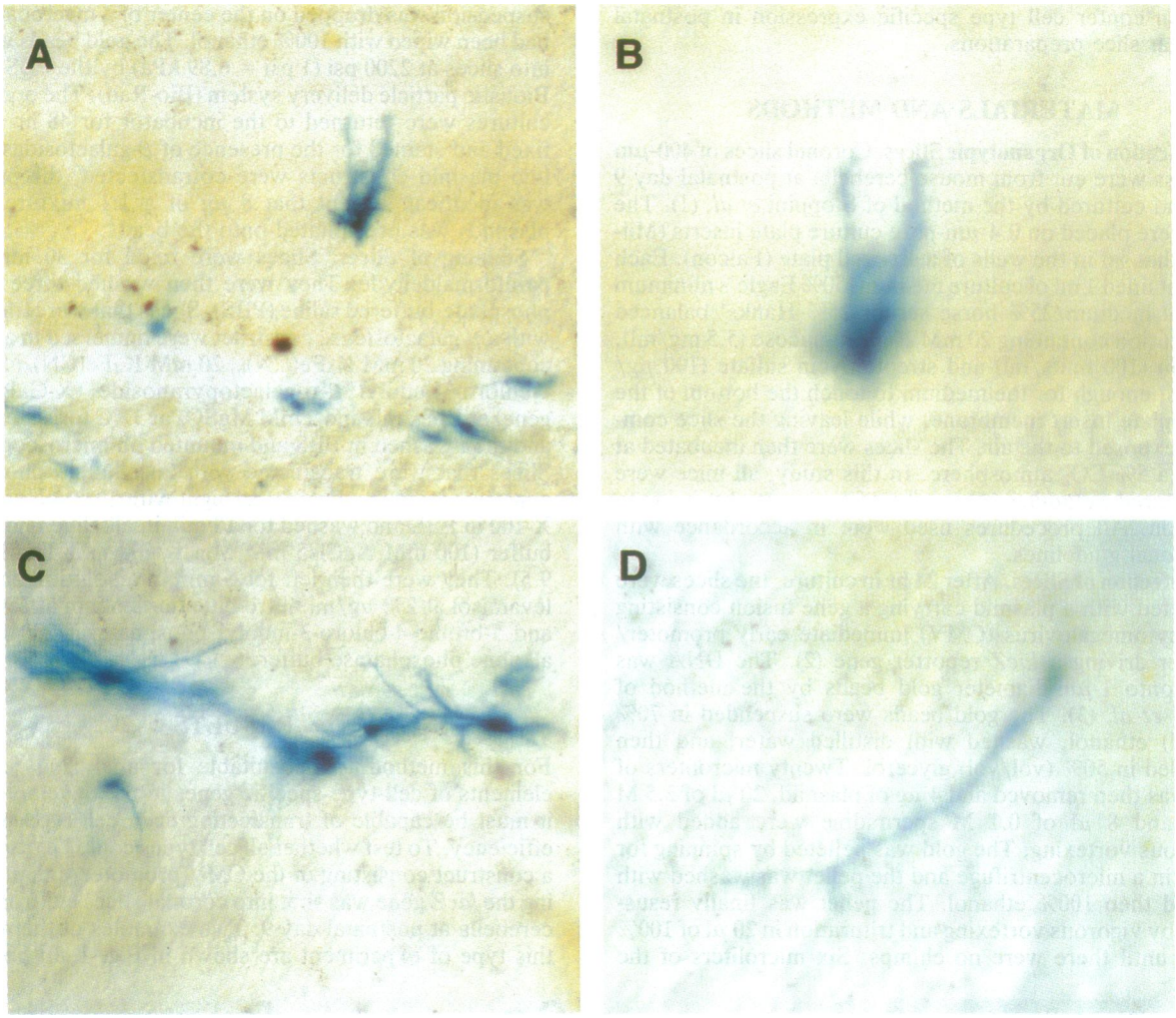

FIG. 3. Examples of cerebellar cell types in coronal and sagittal slices from mice at postnatal day 9 or 10 that exhibited β-galactosidase activity after transfection with a BLBP/*lacZ* construct. (*A*) Bergmann glial cell with white matter astrocytes. (*B*) Bergmann glial cell. (*C*) White-matter astrocytes. (*D*) Migrating granule cell.

several hundreds or thousands of randomly distributed cells were stained blue, indicating that they had expressed β-galactosidase. Inspection of these slices at high magnification revealed the specific cell types expressing the β-galactosidase marker gene (Fig. 2). For example, positive cells in the choroid plexus (Fig. 2*A*) revealed the typical cuboidal morphology of epithelial cells present in this region, whereas positive cells in the cerebellar white matter displayed the characteristic stellate morphology of astrocytes (Fig. 2*B*). In cases where the transfection efficiency was quite high, in-

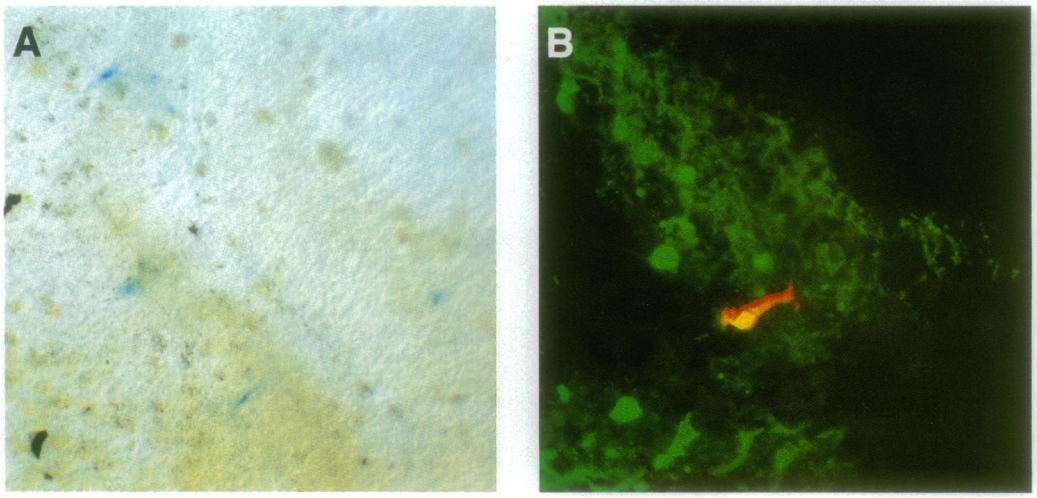

FIG. 4. Parasagittal slices transfected with calbindin promoter construct and assayed for expression of β-galactosidase. (*A*) Positive (blue) cells stained with X-Gal in the Purkinje cell layer. (*B*) Cell colabeled with anti-β-galactosidase antibody (red) and anti-calbindin antibody (green).

spection of the Purkinje cell layer revealed several cells with the typical morphology of Purkinje cells (Fig. 2*C*). In other regions of the slice, mixed populations of cells of diverse morphology were evident (Fig. 2*D*). These data establish that many, perhaps all, of the cerebellar cell types can be studied with this technique and that the position and morphology of each of these cells are quite normal. At this magnification, one can also appreciate the relatively high efficiency of the transfection procedure.

Other transfected cells included Bergmann glia (Fig. 2*E*), Purkinje cells (Fig. 2*F*), stellate neurons (Fig. 2*G*), granule cells (Fig. 2*H*), and Lugaro cells (Fig. 2*I*). In those cases where cell type is not so easily determined (Fig. 2 *J–L*), a counterstain can be employed for unambiguous identification (see below). From these data, we conclude that the CMV promoter is expressed efficiently in most cerebellar cell types, that cells transfected with the Biolistic apparatus are viable and that the efficiency of transfection is sufficient for analysis of transcriptional regulatory sequences.

To demonstrate that this methodology can be employed to study cell-specific transcriptional regulation, the expression patterns of two promoter constructs were examined. The first construct contained a 1.7-kb fragment of the BLBP gene. Detailed analysis of the expression of the BLBP gene in the developing CNS has established that it is expressed in Bergmann glial cells, astrocytes, and migrating granule cells during the first 2 weeks of postnatal cerebellar development (4) and that transgenic mice carrying this construct express β-galactosidase in a pattern that precisely reproduces the expression of the endogenous gene (unpublished data). Transfection of the organotypic slice cultures with the BLBP promoter/β-galactosidase fusion gene resulted in the expected distribution of positive cells (Fig. 3*A*) with typical Bergmann glial (Fig. 3*B*) and astrocytic morphology (Fig. 3*C*), as well as a few scattered cells in which one can clearly identify the characteristic T-shaped axonal morphology of a migrating granule cell (Fig. 3*D*). As a second test of this system, a gene fusion using a 1.1-kb fragment of the calbindin

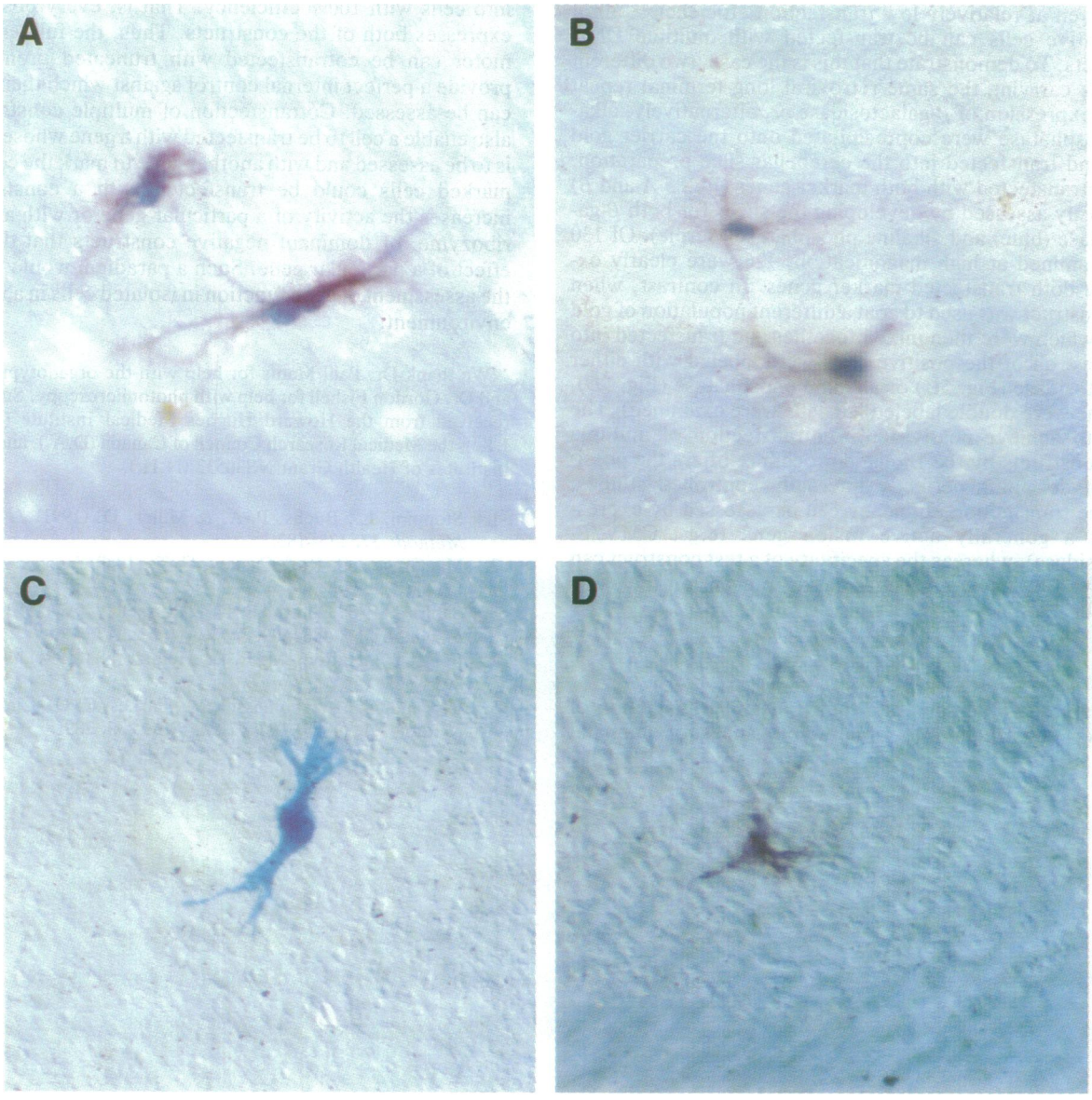

FIG. 5. Cells from coronal slices shot with individual beads loaded with two constructs and cells that were shot with a mixture of beads that were loaded with one or the other construct. (*A* and *B*) Cells that exhibited β-galactosidase activity (blue stain) and alkaline phosphatase activity (purple stain) after being shot with beads coloaded with a retroviral promoter driving a β-galactosidase reporter gene and the same promoter driving an alkaline phosphatase reporter. (*C* and *D*) Cells that exhibited activity of only one reporter gene after being shot with a mixture of beads that were loaded with one construct or the other, but not both.

promoter was employed. Calbindin was chosen because its expression in the cerebellum is known to be specific to Purkinje cells (5), although no regulatory sequences for Purkinje cell-specific calbindin transcription have been reported. After transfection of the construct into the slices, positive (blue) cells were found almost exclusively in the Purkinje cell layer (Fig. 4*A*). As expected from the small number of Purkinje cells relative to other cerebellar cell types, many fewer cells were evident in each slice using this gene than had previously been observed with either the CMV or the BLBP promoter. To confirm the Purkinje cell-specific expression pattern, 20-μm-thick sections of transfected slices were immunostained for β-galactosidase (red) and calbindin (green). Although some misexpression occurred (most notably in the choroid plexus), expression of β-galactosidase in the cerebellar cortex was largely restricted to cells that were immunopositive for calbindin polypeptide (Fig. 4*B*).

Another property of the Biolistic transfection procedure that may prove extremely useful derives from the fact that the carrier gold beads physically carry the DNA into the cell. Thus, even at relatively low transfection efficiencies, all of the positive cells can be transfected with multiple DNA constructs. To demonstrate that this is the case, two different plasmids carrying the same retroviral long terminal repeat driving expression of β-galactosidase or, alternatively, alkaline phosphatase were coprecipitated onto the carrier gold beads and transfected into the cerebellar slice preparation. Cells cotransfected with both marker genes (Fig. 5 *A* and *B*) were easily assessed by developing the slices for both β-galactosidase (blue) and alkaline phosphatase (purple). Of 130 cells examined at high magnification, 126 were clearly expressing both transfected marker genes. In contrast, when each construct was used to coat a different population of gold beads, which were then mixed together and transfected into the slices, all of the positive cells were labeled with either β-galactosidase (Fig. 5*C*) or alkaline phosphatase (Fig. 5*D*), and none were double labeled (28 cells were examined). The ability to achieve nearly 100% cotransfection of multiple DNA constructs by the Biolistic procedure offers the possibility of designing perfectly internally controlled studies, since the transfection efficiency can be assessed by expression of a generally active fusion gene (e.g., CMV/β-galactosidase), whereas the specificity of a test construct can be assayed by using a second marker present on the same carrier beads.

DISCUSSION

In this study we demonstrate that transient transfection of promoter constructs into organotypic slices of neonatal mouse cerebellum provides an extremely fast and efficient way of studying the regulation of gene expression during development. This technique makes it possible to transfect all the known cell types in neonatal mouse cerebellum with relatively high efficiency, and promoters reproduce the same patterns of gene expression as *in vivo*. Consequently, the regulatory activity of a particular promoter construct can be assayed within 3 days. The same assay using current methods would require that a series of transgenic mice be made, taking many months of work and thousands of dollars. Using the Biolistic methodology we have identified 5′ flanking regions controlling transcription of two genes that are expressed in specific cell types in postnatal cerebellum. A 1.7-kb region of 5′ flanking DNA of the BLBP gene was sufficient to restrict expression to astrocytes, Bergmann glia, and migrating granule cells. In addition, 1.1 kb of flanking DNA of the calbindin gene was found to restrict expression to Purkinje cells.

Dissection of the regulatory regions of promoters is facilitated because more than one construct can be transfected into cells with 100% efficiency. That is, every positive cell expresses both of the constructs. Thus, the full-length promoter can be cotransfected with truncated promoters to provide a perfect internal control against which their activity can be assessed. Cotransfection of multiple constructs will also enable a cell to be transfected with a gene whose function is to be assessed and with another gene to mark the cell. Thus, marked cells could be transfected with a construct that increases the activity of a particular gene or with antisense, ribozyme, or dominant negative constructs that delete the effect of a particular gene. Such a paradigm would allow for the assessment of gene function in isolated cells in a wild-type environment.

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