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Stable Expression of the Alkaline Phosphatase Marker Gene by Neural Cells in Culture and after Transplantation into the CNS Using Cells Derived from a Transgenic Rat

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Multipotent stem cells and more developmentally restricted precursors have previously been isolated from the developing nervous system and their properties analyzed by culture assays *in vitro* and by transplantation *in vivo*. However, the variety of labeling techniques that have been used to identify grafted cells *in vivo* have been unsatisfactory. In this article we describe the characteristics of cells isolated from a transgenic rat in which the marker gene human placental alkaline phosphatase (hPAP) is linked to the ubiquitously active R26 gene promoter. We show that hPAP is readily detected in embryonic neuroepithelial stem cells, neuronal-restricted precursor cells, and glial-restricted precursor cells. Transgene expression is robust and can be detected by both immunocytochemistry and histochemistry. Furthermore, the levels of hPAP on the cell surface are sufficient for live cell labeling and fluorescence-activated cell sorting. Expression of hPAP is stable in isolated cells in culture and in cells transplanted into the spinal cord for at least 1 month. We submit that cells isolated from this transgenic rat will be valuable for studies of neural development and regeneration.

Key Words: **alkaline phosphatase; transgenic rat; transplant; stem cell; neuron; astrocyte; oligodendrocyte; NRP; GRP; O2A.**

INTRODUCTION

Early embryonic development is characterized by a progressive restriction in cellular developmental fate. Multipotent stem cells and more developmentally restricted precursors have been isolated from the developing nervous system and their properties analyzed by culture assays and by transplanting these cells into the CNS. Transplant experiments have convincingly demonstrated that cells survive, integrate, and differentiate *in vivo*, and that their properties inferred from *in vitro* experiments appear to reflect their characteristics *in vivo*. It has however, been difficult to identify cells for prolonged periods after transplantation. Hoechst dye, which intercalates into double-stranded DNA, works well for marking transplanted nondividing cells (28) but is lost rapidly from dividing cells. BrdU can also label dividing cells but is not detectable after more than two or three cell divisions (23). Thymidine labeling has similar problems (21). DiI and DiO also label cell surface proteins but the label is diluted in cells that have divided (5).

Alternative strategies to label dividing cells have been devised. A common strategy has been to use recombinant viruses that express reporter genes, using adenovirus, lentivirus (18), and retrovirus (20). These strategies have allowed investigators to follow cells for longer periods, though labeling efficiencies in general are low. Furthermore, viral-transduced marker genes are turned off after transplantation and expression is not detected for more than 2 to 3 weeks (19, 24). We have observed that expression of green fluorescent protein (GFP), β-gal, and alkaline phosphatase under regulatory control of either the cytomegalovirus (CMV) or the viral LTR is diminished 2 weeks after transplantation, and expression is virtually undetectable by 3–4 weeks (30).

Instead of relying on labeling techniques, it is possible to use xenotransplants and follow the grafted cells through the use of species-specific antigens (1) or to transplant cells from animal strains in which markers differ at a specific allele and therefore can be detected by a specific antibody (14). These strategies have indeed allowed detection of transplanted cells weeks,

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months, or years after transplantation, but they also have disadvantages. Xenotransplants are rejected unless long-term immune suppression is applied (7). Allele-specific markers exist but are few in number and often the specificity of the antibodies is not high.

An option that bypasses many of these problems is the use of transgenic animals in which marker gene expression is driven by a ubiquitous promoter and can be detected not only in the transplanted cells but also in their progeny. Transgenic mice models where β-gal, GFP, and alkaline phosphatase have been expressed using the ROSA26 promoter or other ubiquitous promoters, such as chick β-actin promoter and E-2f, have already been used in transplant paradigms (2, 26). However, the small size of mice makes them inconvenient for some behavioral tests and for certain embryological manipulations. Rats have therefore remained the standard model for such experimental paradigms. It would therefore be useful to have a transgenic rat in which precursor cells were genetically labeled and could be used as a source of marked donor cells in transplant experiments. Furthermore, if the donor rats were inbred, then immune suppression could be omitted for many transplant experiments.

Although it has been difficult to obtain embryonic stem (ES) cells from rats and even from some mouse strains (4, 22), it is possible to obtain transgenic rats by oocyte injections (16, 17). In this article we characterize marker gene expression in the nervous system of a transgenic rat line carrying the human placental alkaline phosphatase transgene (hPAP) under the control of the ROSA26 promoter (11). We show that hPAP driven by a ubiquitous promoter is expressed in multipotent and more restricted precursor cells during development and that this expression can be used to stably mark these cells in culture and after transplantation.

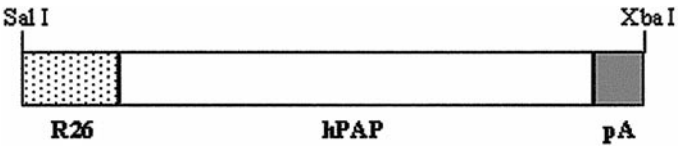


FIG. 1. Generation of the R26-hPAP transgenic rat. Construction of the R26-hPAP transgene (illustrated above) was described in Kisseberth *et al.* (11). It contains the gene regulatory elements from the ROSA26 genomic locus (R26), the coding sequence for human placental alkaline phosphatase (hPAP), and a noncoding element carrying the SV40 polyadenylation signal (pA). This transgene was microinjected in fertilized ova of the F344 strain to generate transgenic rats.

MATERIALS AND METHODS

Substrate Preparation

Poly-L-lysine (pLL, Sigma, St. Louis, MO) was dissolved in distilled water (13.3 μg/mL) and applied to tissue culture plates for 1 h at room temperature. Excess pLL was withdrawn and the plates were rinsed once with water. The plates were then incubated with laminin (15 μg/mL, Gibco BRL, Grand Island, NY) for at least 4 h at 37 or 4°C overnight. The dishes were rinsed once with neuroepithelial stem cell (NEP) medium without growth factors just before plating the cells.

Neuroepithelial Cell Cultures

Transgenic rat embryos were removed at embryonic day 13.5 (E13.5) and placed in a petri dish containing Hank's balanced salt solutions (HBSS). The spinal cord was separated from the surrounding connective tissue using two No. 5 forceps. The meninges were peeled off and the spinal cords were digested with 0.05% trypsin/EDTA solution for 10–15 min at 37°C. The trypsin was neutralized with excess medium and the tubes were spun at 1000 rpm for 5 min. Cells were resuspended in NEP basal medium and gently triturated with a fire-polished pipette to get a single cell population. Cells were plated onto pLL/laminin-coated dishes at a density of 25,000 cells/35 mm dish. For maturation studies the cells were plated at a density of 5000 cells/35-mm dish. All experiments were done with passage 1–3 cells.

The NEP basal medium used in all experiments was a chemically defined medium modified from that described elsewhere (10, 27). The medium consisted of DMEM-F12 (GibcoBRL, Grand Island, NY) supplemented with additives (3) and bFGF (25 ng/mL, Peprotech, Rocky Hill, NJ) and neurotrophin-3 (NT-3, Upstate Biotechnology, Lake Placid, NY).

Histology

E13.5 embryos were fixed with 4% paraformaldehyde for 12 h. They were then rinsed thoroughly with phosphate-buffered saline (PBS) and immersed sequentially in 2, 10, and 20% sucrose for at least 30 min each or until the embryo sank. The embryos were embedded in OCT and stored at −80°C. Frozen transverse sections (16 μm thickness) were cut with a cryostat (Leica) whose cutting chamber temperature was set at −16°C.

Fluorescence-Activated Cell Sorting (FACS)

Fluorescence-activated cell sorting was carried out as described by Stemple and Anderson (27). Cells were live labeled with 1:500 dilution of a monoclonal antibody against hPAP (Sigma) for 1 h at room temperature and then labeled with FITC-conjugated secondary (1:500, Southern Biotech, Birmingham, AL) for 30 min at room temperature. The cells were trypsinized, washed 3× with PBS, and passed through nylon mesh

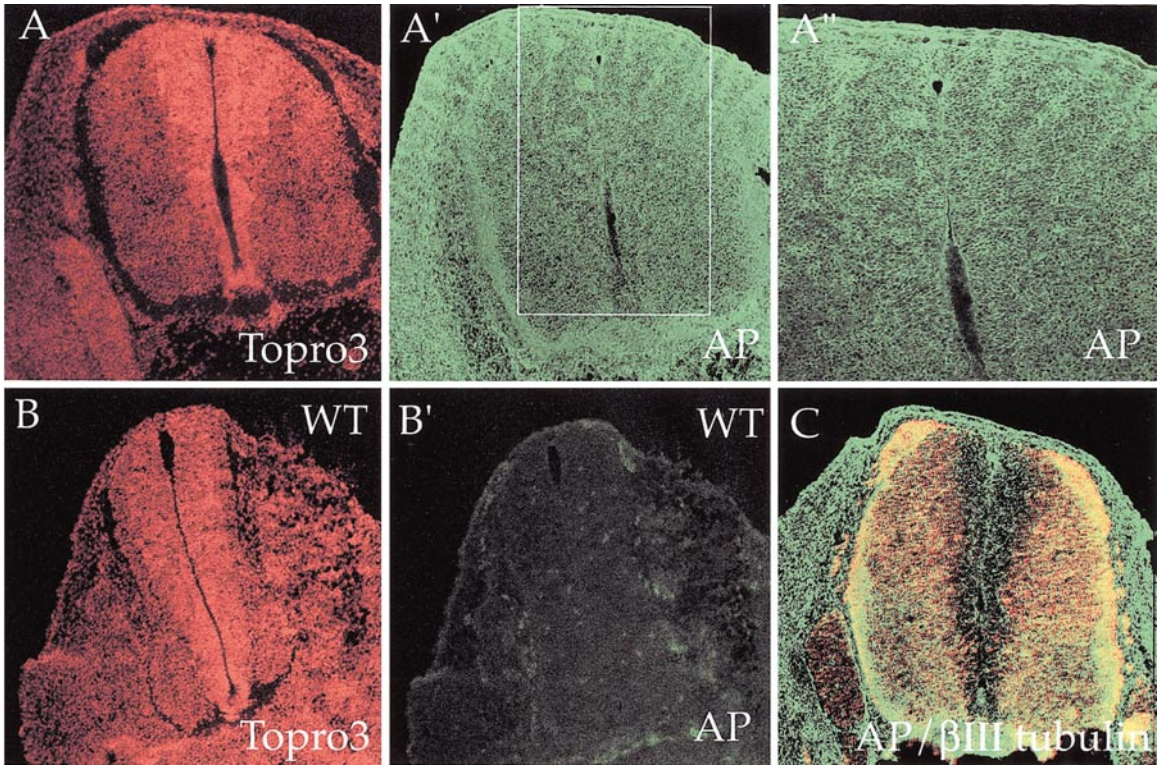


FIG. 2. Confocal images of E13.5 transverse cryosections show expression of hPAP throughout the neural tube. hPAP expression was detected by using an antibody to hPAP on paraformaldehyde fixed sections. (A′) Uniform expression of the alkaline phosphatase (AP) transgene throughout the neural tube. (A) The same section counterstained with topro3, a nuclear stain, to reveal the total number of nuclei present in the field. (A″) Higher magnification of the area enclosed in the box in A′. E13.5 wild-type embryos were used as controls and were processed in parallel for AP immunoreactivity. (B) Total number of nuclei present in the field while B′ shows the absence of AP immunoreactivity in the wild-type animals. (C) A section from a transgenic animal and is double-labeled with hPAP (green) and β-III tubulin (red) to demonstrate transgene expression in undifferentiated neuroepithelial cells and neuronal precursor cells. Original magnification of A″ is at 40× and the others at 20×.

of 20 μm pore diameter to get a single cell suspension. After cell sorting on a FACS cell sorter (Becton-Dickinson, Mountain View, CA), labeled and unlabeled cells were collected in siliconized glass tubes containing NEP basal medium and were plated on pLL/laminin-coated dishes. Cells lost their sorting label within 48–72 h and could subsequently be relabeled for analysis.

Antibody Panning

Antibody panning was carried out as described by Wysocki and Sato (29) with minor modifications. The dissociated cells were plated on an E-NCAM antibody (5A5, Developmental Studies Hybridoma Bank, Iowa City, IA)-coated dish to allow binding of all E-NCAM$^+$ cells to the dish. E-NCAM antibody-coated dishes were prepared by sequentially coating petri dishes with an unlabeled anti-mouse IgM antibody (10 μg/mL, Southern Biotech) overnight, rinsing dishes with DPBS, followed by coating with 5A5 hybridoma supernatant for 1 h at room temperature. Cells were allowed to bind to the plate for 1 h at room temperature. Unbound cells were removed and the plate was rinsed eight times with DPBS. Bound cells were mechanically scraped off and plated on pLL/laminin-coated dishes in 1 mL of NEP medium in mass (20,000 cells/35-mm dish) culture. Growth factors were added every other day. In all cases, an aliquot of cells was analyzed the next day to determine the efficiency of the immunopanning. In general, greater than 90% of the bound cells expressed detectable E-NCAM immunoreactivity. Cell populations that did not were repanned or discarded. Passage 2 immunopanned cells were used for subsequent transplant experiments.

Intraspinal Grafting

NRPs (10,000 cells in 1 μL of basal media) were microinjected into the cervical spinal cord of adult Sprague–Dawley female rats (225–250 g) using a Hamilton syringe containing a glass pipet pulled to 50- to 100-μm inner diameters. The glass tip was advanced slowly into the spinal cord using a micromanipulator; cells were then injected over 1 min and the tip slowly withdrawn. Animals were immunosuppressed with Cyclosporin A injection solution (Sandoz Pharmaceuticals, East Hanover, NJ) administered subcutaneously

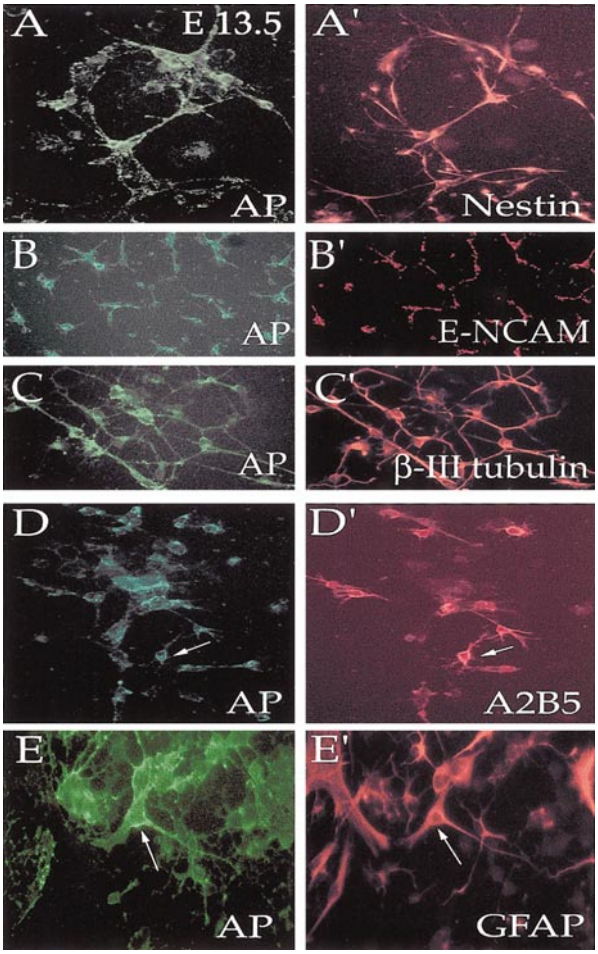


FIG. 3. Expression of the transgene is detected on cells in neuronal, oligodendrocytic and astrocytic lineages in embryonic dissociated spinal cord cultures of transgenic rats. E13.5 cells were isolated from transgenic rat neural tubes and grown in culture. After 2 days in culture, the cells were double-stained for AP (A, green) and nestin (A′, red), demonstrating that most of the AP-expressing cells were nestin-positive. After 3 days the cells were double stained for AP (B, green) and E-NCAM (B′, red) to show that early neuronal cells express AP. The AP expression (C, green) on neuronal precursor cells was further confirmed by double-staining with another early neuronal marker, β-III tubulin (C′, red). The AP expression (D, green) was also seen on small, bipolar A2B5-positive glial precursor cells (D′, red). To confirm the expression of the transgene on astrocytes, the A2B5-positive cells were treated with 10% serum for 5 days and double-stained for AP (E, green) and astrocytic marker GFAP (E′, red). Arrows point to AP+/GFAP+ cells with the typical flat morphology of astrocytes.

at a dose of 1 mg/100 g body weight starting 2 days prior to cell grafting and daily thereafter. Animals were sacrificed at 1 and 4 weeks after transplantation and transcardially perfused with saline followed by 4% paraformaldehyde. The spinal cord segments of interest were dissected out and cryoprotected in 30% sucrose–0.1 M phosphate buffer at 4°C for 2 days. The spinal cord segment was blocked in OCT, frozen, and sectioned parasagittally using a cryostat at 20-μm thickness and sections were collected on gelatin/pLL-coated glass slides.

BrdU Incorporation

To assess the proliferation of hPAP sorted cells, E-NCAM$^+$ cells, and A2B5$^+$ cells, 5-bromo-2′-deoxyuridine (BrdU, 10 μM, Boehringer Mannheim, Mannheim, Germany) was added to the cells for 12 h. The cells were then fixed with 2% paraformaldehyde for 15 min at room temperature, followed by incubation in

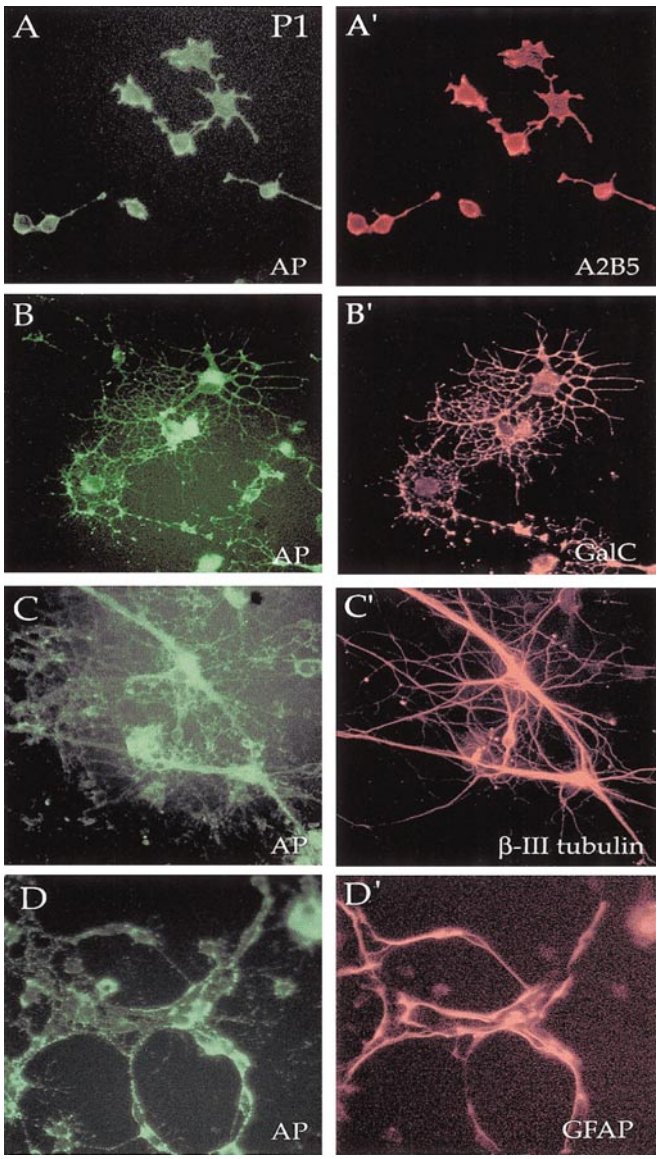


FIG. 4. Transgene expression persists in mature neuronal and glial cells isolated from postnatal day 1 transgenic pups. Cells were isolated from P1 transgenic pups and 3 days later cells expressing the transgene (A, B, C, D) were double-labeled for A2B5 (A′, red) and for oligodendrocyte marker, GalC (B′, red). Transgene expression persisted on postmitotic neuronal cells as shown by β-III tubulin expression (C′, red). (D′, red) Expression was also present in GFAP-positive astrocytes.

95% methanol for 30 min at −20°C. Cells were then washed three times with PBS and 5% goat serum, and permeabilized with 2 N HCl for 10 min. Acid was removed by three washes with PBS and 5% goat serum and the residual HCl was neutralized with sodium borate (0.1 M, Sigma) for 10 min. After rinsing with PBS, cells were incubated with anti mouse-BrdU antibody (1:100, Sigma) for 30 min at room temperature in buffer containing 0.5% Triton X-100. The cells were then incubated with goat anti-mouse IgG1 (1:200, Southern Biotechnology) for 30 min. After three washes with PBS, the cells were observed with a Nikon fluorescence microscope.

Immunocytochemistry and Histochemistry

Staining procedures have been described previously (13). Staining for cell surface markers such as E-NCAM (1:1, DSHB, Iowa City, IA), A2B5 (1:5, ATCC, Manassas, VA), GalC (1:5, kind gift of Dr. Margot Mayer-Proschel), and hPAP (1:500, Sigma) was performed in cultures of living cells. To stain cells with antibodies against internal antigens, cultures were fixed with 2% paraformaldehyde for 15 min at room temperature. Dishes were incubated with the primary antibody such as β-III tubulin (1:500, Sigma), GFAP (1:500, DAKO, Denmark), and hPAP overnight, followed by incubation with an appropriate secondary antibody (1:200, Southern Biotechnology) for 30 min at room temperature. Double labeling experiments were performed by simultaneously incubating cells in appropriate combinations of primary antibodies followed by noncrossreactive secondary antibodies. Negative controls with omission of primary or secondary antibodies were run simultaneously. DAPI histochemistry was performed as described previously (10). DAPI staining was generally done after all other antibody staining had been completed. Labeled cells were viewed using a Nikon fluorescence microscope and photographed using a QED digital camera.

The alkaline phosphatase chromogen reaction on the cultured cells was developed by first fixing the cells with 2% paraformaldehyde for 30 min. The cells were washed with PBS containing 0.1% Tween 20 for 10 min and then washed again with alkaline phosphatase buffer (100 mM Tris, 100 mM NaCl, 50 mM $MgCl_2$, 5 mM levamisole, 0.1% Tween 20) for another 10 min. The cells were incubated in alkaline phosphatase buffer containing NBT (1 mg/mL) and BCIP (0.1 mg/mL) reagent for 15 min. The reaction was developed in the dark. The cells were washed with PBS 5× and fixed lightly with 4% paraformaldhyde and images were obtained using a QED digital camera.

For staining of transgenic rat embryos, cryostat sections were obtained and fixed with paraformaldehyde. Sections were collected on gelatin-coated slides, treated with 0.2% paraformaldehyde, and then washed with PBS 3× for 10 min to remove OCT. The hPAP or β-III tubulin antibody was diluted in blocking buffer containing 0.1% Triton X and incubated overnight at 4°C. The slides were rinsed and incubated with appropriate secondary antibodies for 30 min at 4°C. Slides were rinsed with PBS and mounted with coverslips and images were obtained on a Biorad confocal microscope.

Adult spinal cord tissue sections from the intraspinal grafting experiments were washed three times in PBS, heat-treated at 60°C in PBS for 1 h to inactivate endogenous alkaline phosphatase, briefly washed in alkaline phosphatase buffer (100 mM Tris, 100 mM NaCl, 50 mM $MgCl_2$, pH 9.5), and incubated at room temperature in the dark with 1.0 mg/mL NBT, 0.1 mg/mL BCIP, and 5 mM levamisole (Sigma) in alkaline phosphatase buffer for 1 h to overnight. To facilitate identification of grafted cells *in vivo,* neuronal-restricted precursors (NRPs) were also labeled with the lipophilic fluorescent dye PKH-26 (Sigma) according to standard protocols. Briefly, NRP cells were incubated in 4.0 μM solution of PKH-26 in diluent C for 5 min and washed as directed to remove any unbound dye. PKH-26 labeling was visualized *in vitro* under a rhodamine filter.

RESULTS

Generation of the Transgenic Rat

The R26-hPAP transgenic rat line 7-6, carrying the regulatory element from the ROSA26 genomic locus (31) cloned upstream of the hPAP coding sequence, was generated as described elsewhere (11). The transgene construct is shown in Fig. 1. Briefly, the R26-hPAP element was isolated from plasmid DNA and microinjected into fertilized ova of the F344 rat strain. Ova were transferred into pseudopregnant foster rats, and offspring were screened by PCR for presence of transgene DNA (11). The 7-6 line carrying this transgene was used in the studies described below. It has been assigned the genetic designation: TgN(R26ALPP) 14EPS.

Expression of Alkaline Phosphatase in Neural Cells

The R26 promoter fragment used to drive hPAP appears to be expressed ubiquitously (25). To determine if transgene expression levels in rat neural precursors could be detected by immunocytochemistry at early developmental stages, we stained sections from transgenic and wild-type embryos from E8.5 onward (Fig. 2 and data not shown). hPAP expression was detectable in the developing neural tube at the earliest time point examined and persisted into adulthood. Figure 2 shows expression at E13.5. Expression was seen in the ventricular zone (Fig. 2A′) as well as in the mantle zone (compare A and C), where more differentiated cells and

restricted precursors are present (15). The antibody used for staining was specific, as no signal was detected in sections from nontransgenic animals that were processed in parallel (B′). Expression was seen in the cortex, retina, cerebellum, and along the entire rostrocaudal axis as well as in the developing peripheral nervous system and in migrating neural crest and in developing placodes (data not shown). Thus, expression of hPAP appears to be ubiquitous in regions of ongoing neurogenesis as well as in regions containing differentiated cells.

To confirm that expression of hPAP was present in neural precursor cells as well as differentiated cells, we dissociated embryonic neural tubes and neonatal brains and examined cells for hPAP expression by immunocytochemistry. The identity of the E13.5-dissociated cells was confirmed by double-labeling with appropriate lineage specific markers. hPAP expression was seen in nestin immunoreactive neuroepithelial stem cells (Figs. 3A and 3A′), E-NCAM immunoreactive neuronal-restricted precursor cells (B and B′), A2B5 immunoreactive glial precursor cells (D and D′), and early born astrocytes (E and E′). In general, the vast majority (>90%) of each cell population expressed hPAP, although the levels of expression varied greatly (see cell sorting below). Expression persisted in culture for at least 6 weeks (the last time point examined) and the level of expression appeared unchanged (data not shown).

To demonstrate expression in oligodendrocyte precursors and maturing oligodendrocytes, we dissociated cells from P1 animals and cultured them under conditions that promote oligodendrocyte and astrocyte differentiation. A2B5-immunoreactive glial precursors (Fig. 4A′) that do not express GalC or O4 (data not shown) coexpress hPAP, as do GalC (B′)- and GFAP (D′)-immunoreactive cells. To confirm that postmitotic neurons continue to express hPAP, we examined the expression of hPAP in β-III tubulin immunoreactive cells harvested at P1. At this age neurogenesis is essentially complete (12), and most β-III tubulin immunoreactive cells are postmitotic. β-III Tubulin-immunoreactive cells coexpress hPAP (Fig. 4C′).

Our results thus show that hPAP expression in the transgenic animal is robust, readily detected in precursor and differentiated cell populations, and persistent throughout the period of neurogenesis and gliogenesis.

Alkaline Phosphatase Expression on the Cell Surface Can Be Detected by Live Cell Labeling

hPAP can be detected in the cytoplasm after fixation and cell permeabilization (see Materials and Methods and Fig. 5A). hPAP, however, may be present on the cell surface and also may be secreted (6, 9). If the R26 promoter drives expression robustly in the nervous system, then sufficient hPAP may be present on the cell surface so that live cell labeling could be used to identify transgenic cells. We compared live cell labeling of dissociated cell cultures with labeling of sister plates of cultured cells after fixation. hPAP labeling can be detected on live cells (Fig. 5A). Labeling is punctate, outlines the cell surface, and is seen in processes that extend from clusters of cells. Staining intensity is less than that seen after fixation, suggesting that a significant amount of the hPAP is present in the cytoplasm (B). Nevertheless, staining is robust and the percentage of unambiguously labeled cells is unchanged (data not shown).

The percentage of cells labeled with antibody was higher (>90%) than with a histochemical reaction (60–80%). This difference is likely due to greater sensitivity of the antibody. Prolonging the histochemical reaction from 2 to 8 h labeled a higher percentage of cells, whereas labeling nonexpressing cells did not show detectable staining even at a fivefold higher concentration (Fig. 5C and data not shown). Thus, hPAP is expressed on the surface of cells from transgenic rats and can be detected by antibody or histochemical staining.

Alkaline Phosphatase Expression Can Be Used to Isolate Cells by Fluorescence-Activated Cell Sorting

The ability to identify live cells that express hPAP allows one to perform coculture experiments and count cells by fluorescence-activated cell sorting, as well as determine numbers of cells that persist after transplantation. Such experiments have been difficult to perform with other labeling methods. To determine if sorted cells behave appropriately, we sorted cells that express the highest levels of hPAP immunoreactivity and plated them in culture to determine survival and ability to divide. Our results are summarized in Fig. 6. Figure 6A shows the profile of immunoreactivity. The intensity of staining varies over 100-fold with only a small fraction of cells failing to demonstrate immunoreactivity when compared to nontransgenic cells (Figs. 6A and 6D).

Two populations of cells were isolated separately: the top 30% of cells expressing hPAP most intensely and the next highest 25% of cells (Figs. 6B and 6C). These populations were plated separately and cell survival and differentiation were examined to confirm that hPAP expression was not biased to a particular cell type and that it did not alter cell division or differentiation. Both fractions of cells showed NEP-, NRP-, and GRP-positive cells (data not shown). hPAP-immunoreactive cells incorporated BrdU (F) and subsets of cells expressed E-NCAM and A2B5 (G and data not shown). Data shown are from the highest (M2) fraction (B) but identical results were obtained with the second (M1) fraction (C) as well. Both E-NCAM- and A2B5-immunoreactive cells incorporated BrdU (G and data not

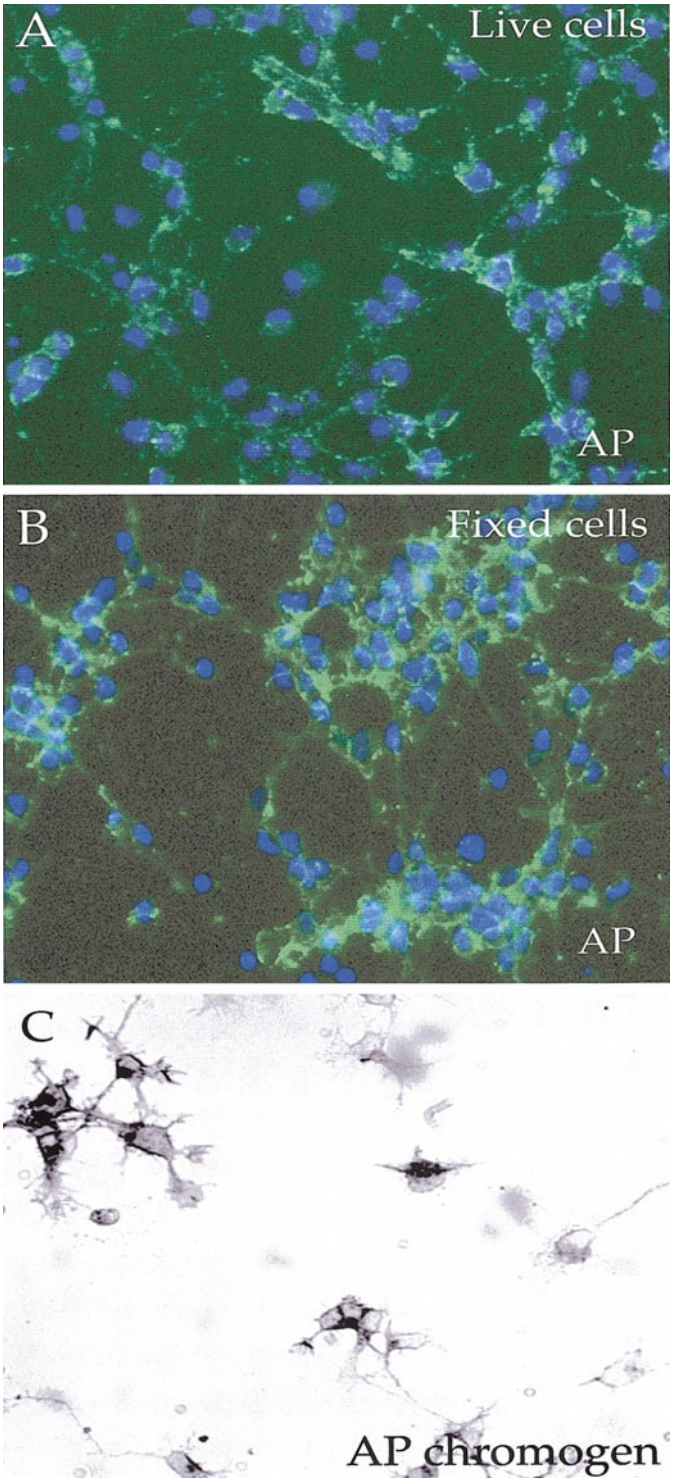


FIG. 5. Alkaline phosphatase expression can be detected by either cell surface or cytoplasmic staining using the antibody or by chromogen reaction. E13.5-dissociated cells were live stained with anti-hPAP antibody (A, green). A sister dish was fixed with 2% paraformaldehyde and stained with anti-hPAP antibody (B, green). DAPI staining (blue) identified all nuclei of cells present in the fields. (C) AP expression can also be detected using a chromogen reaction.

shown), indicating that these precursor cells survive sorting and proliferate and differentiate in culture. Furthermore, these results demonstrated that while the level of AP expression varied, no differences were observed in the ability of cells to proliferate and differentiate as a function of AP expression levels.

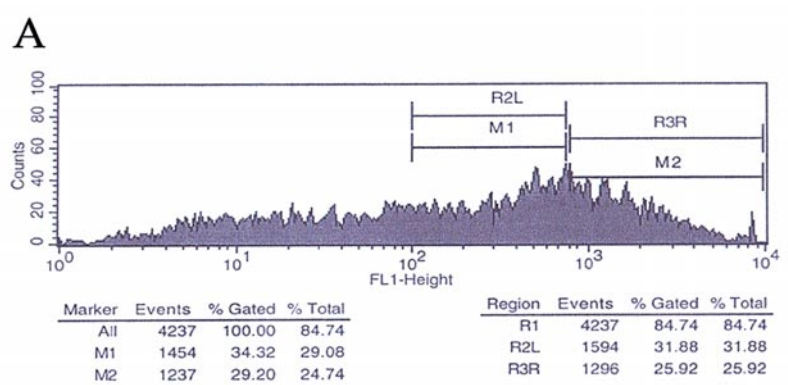
Survival and Transgene Expression of Grafted NRPs in Vivo

An important potential use of this transgenic rat line is to obtain labeled subpopulations of cells whose development can be followed after transplantation. Ideally expression should persist indefinitely and should be seen in processes as well as cell bodies for useful analysis. To determine if cells isolated from the hPAP transgenic rat would be useful in transplant experiments, we isolated neuronal precursors from E13.5 rat caudal neural tubes and transplanted them into unlesioned spinal cords of adult rats. The expression of hPAP was examined at one week and one month after transplantation. Grafted cells were identified by hPAP histochemistry and also by prelabeling with the lipophilic fluorescent dye PKH-26. Grafted cells survived for at least 1 month (Figs. 7C and 7D) and continued to express the hPAP transgene robustly. In some cases, the cell bodies and processes of individual neurons could be visualized (Fig. 7B, inset). Thus, these results demonstrate that cells derived from this transgenic rat continue to express the reporter gene for long periods after grafting and are useful for the detection of transplanted cells *in vivo*.

DISCUSSION

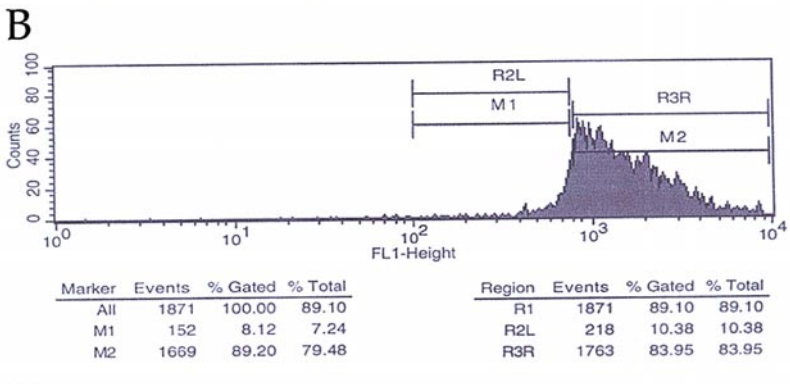
We have characterized cells derived from a transgenic rat in which hPAP is expressed ubiquitously. We show that hPAP can be detected by immunohistochemistry and histochemistry, and that expression is seen throughout development in neurons, astrocytes and oligodendrocytes, as well as in neural precursor cells. Expression can be detected on the cell surface, and live cell labeling can be used to sort cells for transplant and coculture experiments.

Expression of hPAP was seen throughout the rostrocaudal axis of the CNS, consistent with reports using this promoter in mice (25). Expression was robust and reliable and consistent from generation to generation. Expression in heterozygotes was sufficient for detection and we were able to use heterozygotes for our transplant studies (unpublished results). Cells in all the major germinal zones examined expressed hPAP including the retina, cerebellum, subgranular zone of the hippocampus, and the subventricular zone that contributes neurons to the olfactory bulb. A detailed analysis of expression in placodal and neural crest derivatives was not performed but expression was de-



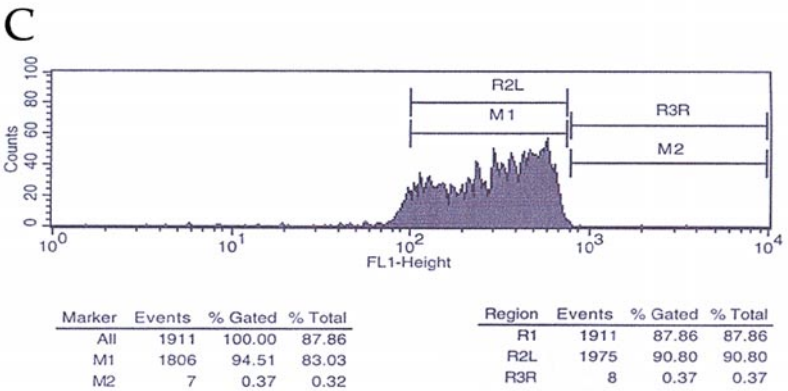
Marker	Events	% Gated	% Total
All	4237	100.00	84.74
M1	1454	34.32	29.08
M2	1237	29.20	24.74

Region	Events	% Gated	% Total
R1	4237	84.74	84.74
R2L	1594	31.88	31.88
R3R	1296	25.92	25.92



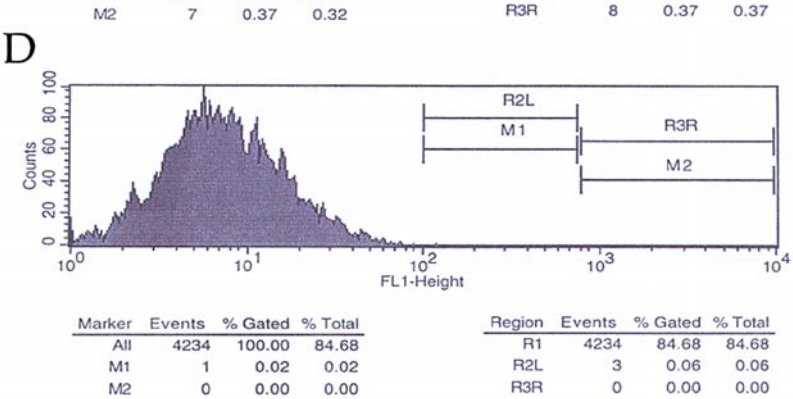
Marker	Events	% Gated	% Total
All	1871	100.00	89.10
M1	152	8.12	7.24
M2	1669	89.20	79.48

Region	Events	% Gated	% Total
R1	1871	89.10	89.10
R2L	218	10.38	10.38
R3R	1763	83.95	83.95



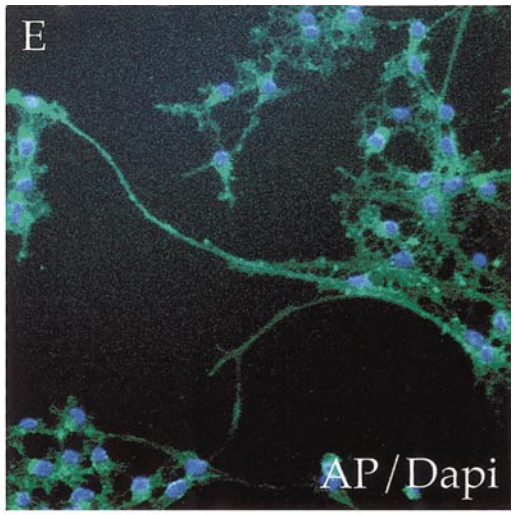
Marker	Events	% Gated	% Total
All	1911	100.00	87.86
M1	1806	94.51	83.03
M2	7	0.37	0.32

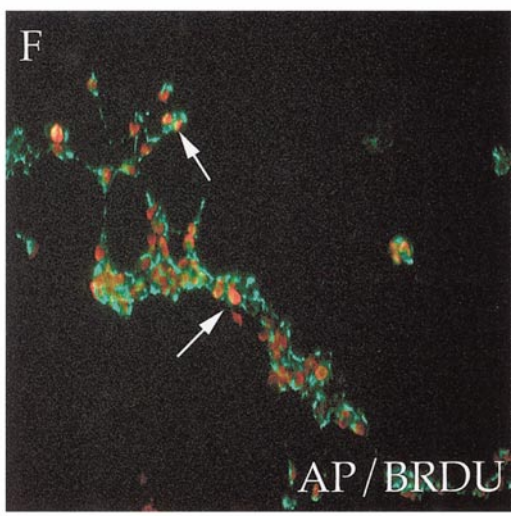
Region	Events	% Gated	% Total
R1	1911	87.86	87.86
R2L	1975	90.80	90.80
R3R	8	0.37	0.37



Marker	Events	% Gated	% Total
All	4234	100.00	84.68
M1	1	0.02	0.02
M2	0	0.00	0.00

Region	Events	% Gated	% Total
R1	4234	84.68	84.68
R2L	3	0.06	0.06
R3R	0	0.00	0.00





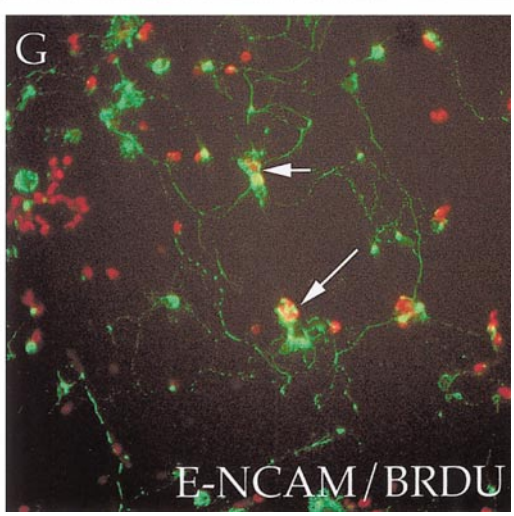


FIG. 6. Fluorescence-activated sorting can be used to isolate AP-labeled cells. E13.5-dissociated spinal cord cells of the transgenic rat were cultured for 15 days and then live-labeled for alkaline phosphatase. (A) The total number of fluorescently labeled cells of varying intensities. M2 represents the brightest 30% of the cells. These cells were collected, resorted and profile is shown in B. M1 in A represents the next brightest 25% of the labeled cells. These cells were also re-sorted as shown in C. (D) Control in which cells incubated with just the FITC conjugated secondary antibody to determine background fluorescence. Note that all the cells are present at the left-hand corner of the plot. The sorted cells were grown for 3 days in culture and stained for alkaline phosphatase (E, green). The cells showed a robust expression of AP and virtually all the cells were positive as shown by DAPI staining of the nuclei (E, blue). A sister dish was processed for BrdU incorporation (F, red) and demonstrated that most AP+ cells (F, green) were dividing. To confirm that the sorted cells can generate neural precursor cell populations, the cells were stained for E-NCAM/BrdU (G, green and red, respectively) and A2B5/BrdU (data not shown).

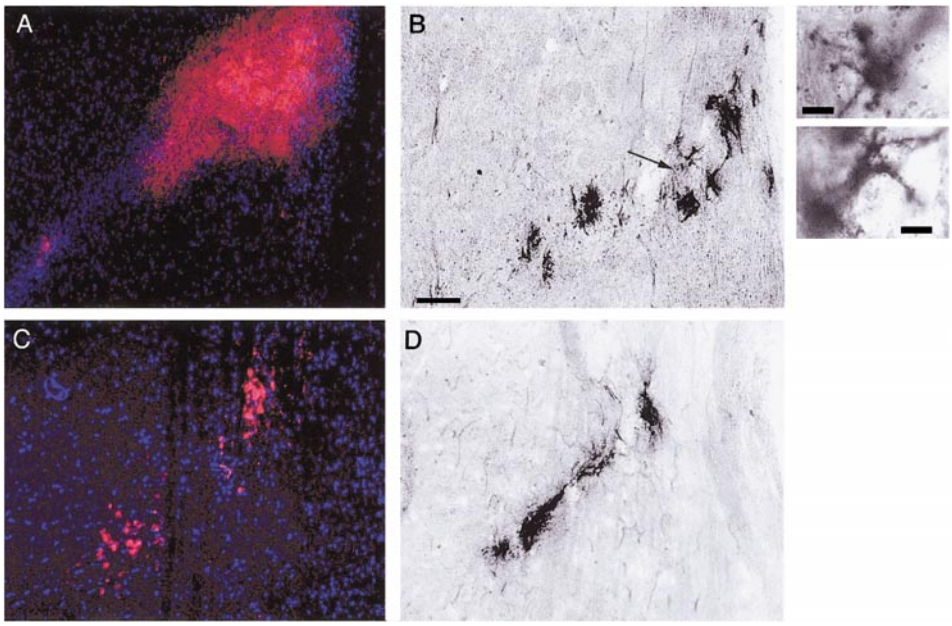


FIG. 7. Survival and transgene expression after grafting neuronal-restricted precursor (NRP) cells derived from a transgenic rat. NRPs purified by immunopanning for E-NCAM from transgenic rat embryos were labeled *in vitro* with the lipophilic fluorescent dye PKH-26 and grafted into the adult spinal cord. At 1 and 4 weeks, animals were sacrificed and the spinal cord sectioned parasagitally. hPAP transgene expression was assessed by AP histochemistry. (A and C) The presence of the grafted cells shown by PKH-26 labeling (red) at 1 and 4 weeks, respectively. DAPI (blue) identifies all nuclei. (B and D) Adjacent tissue sections, demonstrating that hPAP transgene expression persists for at least 1 month (D). The cell indicated by the arrow in B is shown as a high-power inset at two focal planes to visualize the morphology. Note that AP histochemistry easily identified neural processes. Scale bar in B is 100 μm and applies to A–D. Scale bars in the high-power insets are 10 μm.

tected in Schwann cells, sympathetic, enteric, and dorsal root ganglia, as well in placodal derived ganglia. While we cannot rule out absence of expression in specific subsets of cells, our data nevertheless indicate that all major neural precursor cell populations from this transgenic rat express robust levels of hPAP and that this expression can be used to follow the development of these cells in culture or after transplantation.

In each neural region expression was seen in most cells, although the levels of expression varied. Quantification of levels of expression on the cell surface indicated a 100-fold difference between cells but expression was maintained for at least 6 weeks in culture with no apparent changes in expression levels. This difference in hPAP expression could not be attributed to cell-type-specific levels of expression but rather to heterogeneity in each population of cells or in our ability to detect hPAP. We tested this by sorting E-NCAM immunoreactive neural precursors and demonstrating a 100-fold difference in detectable hPAP, yet both high and intermediate-expressing populations contained neural and glial precursors. Both high and intermediate AP-expressing populations were able to proliferate and differentiate in culture. The results also show that the highest AP-expressing cells can be easily selected for using FACS and thus may be helpful in the detection of grafted cells after transplantation.

Our results showing that cell surface expression can be used to detect expressing cells in mixed populations extend the potential uses of this transgenic animal. Clonal culture experiments can be performed by culturing labeled cells with unlabeled cells, thus bypassing the difficulty of growing cells at low density. Transplanted cells can be quantified by FACS, and live cell labeling can be used to follow the behavior of cells in slice cultures. These experiments, which are possible using transgenic mouse cells, have been difficult to perform with rat cells because of lack of donor cells with a readily detectable marker.

The availability of a transgenic rat in an inbred strain F344 now allows experiments to be performed without immune suppression, making this transgene a valuable resource for transplantation studies. We have also demonstrated that cells derived from this transgenic rat can be grafted into other rat strains with immune suppression. Further, our results show hPAP expression in most (90%) of the cells. This efficiency is greater than with most transfection or retroviral infection techniques (5–40%) making this animal an improved source of labeled cells. Of additional importance was our observation that cellular expression of the transgene was stable both in culture and after transplantation. Robust expression was seen after 6 weeks *in vitro* and 4 weeks *in vivo,* and levels of expression were sufficient for the processes were well outlined and readily traced. This is in contrast to our experience with retroviral expression using similar promoters. In these experiments (30), expression was reduced signif-

icantly after 2 weeks and was virtually undetectable after 3 weeks. Several mechanisms for this downregulation have been suggested (8). The stability of expression indicates that cells from this transgenic rat line can be used for long-term reconstitution studies and thus provide a valuable resource for the transplant community.

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