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in Mice and Rats

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The ability to unambiguously mark a cell's genotype is essential for studies in which genetically distinct cell populations must be distinguished from one another *in vivo.* One approach to this challenge has been the creation of transgenic mice expressing a transgene marker that is easily detectable, with no background staining. Multiple transgenic mouse strains bearing constructs with different combinations of promoter elements and coding sequences have been described, each with its own advantages and limitations. In this report we describe the use of an 800-bp promoter fragment isolated from the βgeo integration site in ROSA26 mice to target expression of two marker genes. We demonstrate that the ROSA26 promoter directs ubiquitous expression of human placental alkaline phosphatase and enhanced green fluorescent protein during embryonic and postnatal development in mouse and rat. We further demonstrate the general utility of these transgenes for marking donor cells in transplantation studies. © 1999 Academic Press

Key Words: **chimera; green fluorescent protein (GFP); placental alkaline phosphatase; ROSA26; transgenic mouse; transgenic rat.**

INTRODUCTION

Experiments involving cell or tissue transplantation, whole animal chimeras, and cell lineage analysis each require a method to identify subpopulations of cells *in vivo* that may not be distinguishable by morphological criteria (Rossant and Spence, 1998). For these purposes, an ideal genetic marker should be (1) developmentally neutral, causing no adverse developmental effects; (2) ubiquitously expressed in all cells throughout development; (3) precisely localized to expressing cells; (4) easily and reliably detectable in a variety of tissue preparations (living cells, whole tissue mounts, fixed and paraffin-embedded tissue sections); and (5) compatible with simultaneous visualization of other markers. Previous approaches to cell marking have exploited differences between species in repetitive DNA sequences, which can be visualized using *in situ* hybridization. However, there is evidence from interspecific mouse chimeras that cells from different species mix less frequently than in intraspecific chimeras (Goldwitz, 1986). Additionally, the interspecific approach requires use of immunocompromised or immunotolerant recipients when performing cell or tissue transplants into postnatal animals. A second approach has been to exploit genetic enzyme polymorphisms including tyrosinase, glucose phosphate isomerase (GPI, Chapman *et al.*, 1971; Gearhart and Oster-Granite, 1978), and malic enzyme (NADP-dependent malate dehydrogenase, Gardner *et al.*, 1984) variants (including the null allele), which produce phenotypes that can be identified histochemically, immunohistochemically, or in tissue homogenates. Although useful in certain contexts (e.g., marking coat color or assessing the percentage of contribution by donor genotype to certain tissues in chimeras), polymorphic enzymes generally have not provided markers that can be used easily to assess widespread tissue chimerism at the cellular level.

A third approach has been the creation of transgenic mouse strains expressing a transgene marker that can be detected easily and for which there is no background staining in nontransgenic mice. The bacterial *lacZ* gene (which encodes the protein β-galactosidase) has been used extensively as a reporter gene for *in vivo* promoter analysis

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in transgenic mice, as a label for transfected cell lines, and as a marker in promoter-trapping studies. Multiple lines of transgenic mice also have been created that overexpress *lacZ* under the regulatory control of different promoters. However, each strain has shortcomings. In particular, transgenes are not well expressed in some tissues, and there can be variable staining among individual cells within a tissue or cell line (MacGregor *et al.,* 1987; McBurney *et al.,* 1994). More recently two additional genes have been employed to mark cells. First, the human placental alkaline phosphatase (hPAP) gene has been used as a reporter for studies of gene expression in cell culture systems (Henthorn *et al.,* 1988), for investigating cell lineage *in vivo* using recombinant retroviruses (Fields-Berry *et al.,* 1992; Fekete and Cepko, 1993), and to mark cells in transgenic mice (Deprimo *et al.,* 1996). The hPAP enzyme is heat stable, allowing it to maintain enzymatic activity during paraffin embedding, crucial for studies that require tissue sectioning with outstanding preservation of microscopic tissue morphology. Second, the green fluorescent protein (GFP), derived from the jellyfish *Aequorea victoria* has found increasing use as a reporter gene *in vitro* and *in vivo* (Chiocchetti *et al.,* 1997; Zhou *et al.,* 1997; Ikawa *et al.,* 1998; Hadjantonakis *et al.,* 1998). GFP is a fluorescent protein, thereby requiring no chemical substrate for visualization, and it permits identification of marker gene expression in living cells.

The usefulness of transgenic mice expressing cell markers depends on the gene targeting strategy employed. In some studies, tissue-specific promoters have been used to direct marker gene expression to a specific cell type or stage of development; however, for many studies ubiquitous marker gene expression is required. This is particularly important for cell lineage analyses in chimeras: all cell types derived from a marked genotype should continue to express the marker regardless of differentiation status. Several gene promoters have been identified and employed in transgene construction that exhibit a widespread cell-type expression pattern, including mouse metallothionein (Stevens *et al.,* 1989; Rhim *et al.,* 1994); β-actin from human (Nilsson and Lendahl, 1993), rat (Beddington *et al.,* 1989), and chicken (Sands *et al.,* 1993); ubiquitin (Schorpp *et al.,* 1996); and SV40 early (Takeda and Toyoda, 1991) promoters. However, these often do not reliably or reproducibly target transgene expression to all cells in the body, and the MT promoter requires heavy metal induction for maximal expression. Recently, a ubiquitously expressed gene was identified through use of promoter trap methodology in mouse embryonic stem cells (Friedrich and Soriano, 1991). The line of mice developed from these cells, ROSA βgeo 26 (ROSA26), displayed expression of a *lacZ* reporter gene in all cells throughout embryonic development and in adult tissues. Subsequently, the *lacZ* integration site in the ROSA26 genome was cloned and the promoter region of the locus characterized. An 800-bp DNA fragment located 5′ of the associated transcripts' exon 1, when fused to a *lacZ* reporter gene, targeted β-galactosidase activity to ES cells *in vitro* (Zambrowicz *et al.,* 1997). For the current study, the ROSA26 promoter (R26) was combined with the coding sequence for each of the two marker genes, hPAP and GFP, to determine whether this gene regulatory element could reliably and reproducibly target expression of heterologous coding sequences to all cells in the body. We demonstrate that the R26 promoter directs ubiquitous expression of reporter genes during embryonic and postnatal development in mouse and rat and that cell marking employing this promoter efficiently identifies transgenic cells in transplantation studies.

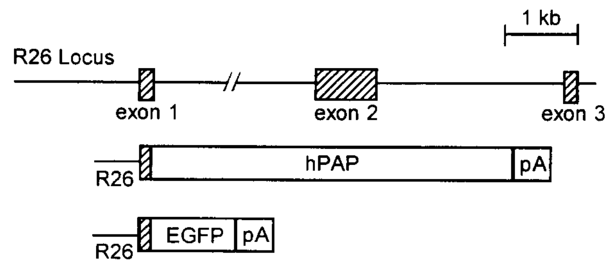

FIG. 1. R26–hPAP and R26–EGFP transgene constructs. A 0.8-kb fragment of 5′ sequence containing the putative regulatory elements of the ROSA βgeo 26 (ROSA26) mouse strain insertion site was fused to the hPAP (middle) and EGFP (bottom) coding sequences.

MATERIALS AND METHODS

Fusion Gene Construction and Production of Transgenic Animals

A Kozak ATG consensus sequence in exon 1 of the ROSA26 (R26) genomic locus was mutagenized to a *Bam*HI site (Zambrowicz *et al.,* 1997). The resulting 0.8-kb *Sal*I–*Bam*HI fragment containing the putative gene regulatory elements of the locus was fused to an hPAP coding sequence to which had been added the *SV40* polyadenylation signal (Fig. 1). The 6.1-kb *Sal*I–*Xba*I fragment containing the R26–hPAP transgene was isolated and microinjected into fertilized FVB/N and C57BL/6 mouse eggs to produce transgenic mice (Brinster *et al.,* 1985). Transgenic Fischer 344 (F344) rats were produced and identified in essentially the same manner.

To construct the R26–enhanced green fluorescent protein (EGFP) transgene, the 0.8-kb *Sal*I–*BamH*I R26 fragment was inserted between the *Sal*I/*BamH*I site of the pEGFP-N1 plasmid (Clontech) containing the mutant EGFP. This GFP has been modified to encode a protein that has a single, red-shifted excitation peak and fluoresces about 35 times more intensely than wild-type GFP when excited at 488 nm. The 1.8-kb *Sal*I/*Afl*I fragment containing the R26–EGFP transgene was isolated and used to produce transgenic mice (Fig. 1).

Genotyping and Enzyme Histochemistry

R26–hPAP transgenic mice were genotyped by enzyme histochemistry using tail blood blotted onto glass slides or filter paper

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and then air dried. Endogenous heat-labile alkaline phosphatases were inactivated by incubation in substrate buffer (0.1 M Tris–HCl, pH 9.5, 0.1 M NaCl, 5mM $MgCl_2$) at 65°C for 30 min. Substrate buffer was then discarded and replaced with fresh buffer containing 0.17 mg/ml of the substrate 5-bromo-4-chloro-3-indolyl phosphate (BCIP). Samples were incubated 8–24 h at 37°C. Genotyping by histochemistry was confirmed in a subset of animals by polymerase chain reaction (PCR) analysis using the primers SVpoly-1f (5′-CTGATGAATGGGAGCAGTGGTGGAATG-3′′ and SVpoly-2r (5′-GCAGACACTCTATGCCTGTGTGGAG-3′), which produced a 360-bp product in transgenic animals. R26–EGFP transgenic animals were identified by PCR using the primers EGFP-1f (5′-TGAACCGCATCGAGCTGAAGGG-3′) and EGFP-2r (5′-TCC-AGCAGGACCATGTGATCGC-3′), which produced a 306-bp product in transgenic animals.

Alkaline Phosphatase Histochemistry of Whole Embryos and Paraffin-Embedded Tissue

To visualize staining of whole embryos, embryos were incubated in substrate buffer for 30 min at 65°C and then incubated with BCIP for 4–24 h. Embryos were postfixed overnight in neutral buffered formalin and stored in 70% ethanol. To visualize microscopic tissue staining, adult tissues or whole embryos were fixed in 4% paraformaldehyde at 4°C for 2–4 h. Fixed tissues were dehydrated through alcohol to Hemo-De (Fisher) and paraffin embedded, and then 5-μm sections were cut and mounted on poly-L-lysine-coated slides. Sections were deparaffinated in Hemo-De, rehydrated, heated at 65°C for 30 min to block endogenous alkaline phosphatase activity, and incubated with BCIP. Some sections were counterstained with nuclear fast red.

Alkaline Phosphatase Solution Assay

Tissues were frozen in liquid nitrogen and stored at −80°C. Frozen tissue was homogenized in 4 ml of TMNC buffer (50 mM Tris, pH 7.5; 5 mM $MgCl_2$; 100 mM NaCl; 4% Chaps) and then centrifuged for 10 min at 1750*g* at 4°C. Supernatants were removed and centrifuged again at 16,000 rpm for 5 min at 4°C. Aliquots of supernatants were stored at −80°C. Stored tissue supernatants were heated to 65°C for 15 min to quench endogenous heat-labile alkaline phosphatase activity. Samples were diluted 1:10–1:100 as necessary with TMNC buffer and 10 μl of each sample was placed in triplicate into 96-well plates. Two-hundred microliters of 1× SEAP reaction buffer (1.0 M diethanolamine, pH 9.8; 0.5 mM $MgCl_2$; 10 mM L-homoarginine) was added to each sample and samples were incubated at 37°C for 10 min. Twenty microliters of 120 mM *p*-nitrophenylphosphate (*p*-NPP) was next added to each well and mixed, and the OD_{405} was read after a 5-min incubation. Blank wells had 20 μl of *p*-NPP added to 120 μl of 1× SEAP (O'Connor and Culp, 1994). Protein content was determined using a procedure based on the method of Bradford (Bio-Rad protein assay). hPAP enzymatic activity was expressed in relative units as OD_{405}/total protein.

Imaging of EGFP Fluorescence

EGFP expression in adult tissues was assayed by imaging fresh whole tissue collected in PBS. Alternatively, tissues were fixed for 30 min in 4% paraformaldehyde at 4°C, embedded in HistoPrep (Fisher), and frozen in liquid nitrogen. Cryostat sections (12 μm) were mounted on aminosilane-coated slides, rehydrated with PBS, and imaged immediately. Unfertilized ova; one-, two-, four-, and eight-cell embryos; and blastocysts were collected from superovulated FVB/N females mated to hemizygous R26–EGFP transgenic or FVB/N nontransgenic males. The same stages were collected from superovulated hemizygous R26–EGFP females mated to FVB/N nontransgenic males. Ova or embryos were placed into 30-μl microdrops of BMOC under paraffin oil. E 12.5 embryos were collected from FVB/N females mated to R26–EGFP males and placed into PBS. Tissues and embryos were imaged with a laser-scanning confocal microscope (a Bio-Rad MRC 1000 scanhead mounted transversely to an inverted Nikon Diaphot 200). For EGFP imaging of whole embryos or tissues, filters were employed to provide excitation at 488 nm with a 15 mM krypton/argon mixed gas air-cooled laser, detecting emission at wavelengths greater than 515 nm. The Bio-Rad MRC-1024 Laser Sharp (version 3.2) software controlled the microscope settings. EGFP also could be detected using an ordinary epifluorescence microscope equipped with a filter set for fluorescein.

Tissue Transplantation

Mice carrying a fusion transgene that targets expression of urokinase-type plasminogen activator (uPA) to hepatocytes develop liver disease (Sandgren *et al.,* 1991). These mice can be used as recipients for transplanted normal hepatocytes, which clonally

FIG. 2. Embryonic and fetal transgene expression. (A–D) Fetal expression of R26–hPAP and R26–EGFP transgenes is ubiquitous and recapitulates the ROSA26 β-galactosidase expression pattern: (A) Whole-mount X-gal staining of ROSA26 +/− fetus (E12.5). (B) Whole-mount BCIP staining of R26–hPAP +/− mouse fetus (E 12.5). (C) Whole-mount fluorescence at 488 nm of R26–EGFP +/− mouse fetus (E 12.5). (D) Whole-mount BCIP staining of R26–hPAP +/− rat fetus (E 15). Insets, nontransgenic fetuses processed identically. Bars, 2 mm. (E–H) hPAP is expressed in all tissues during fetal development: (E) E 7.5, (F) E 9.5, (G) E 12.5 R26–hPAP +/− mouse fetuses and (H) E 14.5, R26–hPAP rat fetuses were fixed, paraffin embedded, sectioned, and then stained with BCIP and counterstained with nuclear fast red. Bars, 250 μm, 500 μm, 1 mm, and 1 mm, respectively. All fetal tissues stained blue, indicating expression of the R26–hPAP transgene. Maternal tissues were unstained. (I–L) Expression of the R26–EGFP transgene in ova and preimplantation embryos: R26–EGFP expression as indicated by fluorescence at 488 nm was observed in all (I) one-cell embryos collected from superovulated +/− R26–EGFP females mated to nontransgenic males, consistent with maternal EGFP expression. (J) At the eight-cell stage, maternal EGFP still could be detected, although fluorescent intensity was reduced at this stage in approximately half of the embryos collected. (K) At the blastocyst stage, some embryos displayed strong fluorescence while others did not fluoresce. (L) Late four-cell embryos derived from superovulated nontransgenic females mated to +/− R26–EGFP males fluoresced, consistent with zygotic expression of the transgene in half the embryos. Asterisks: non-transgenic embryos. Bars, 50 μm.

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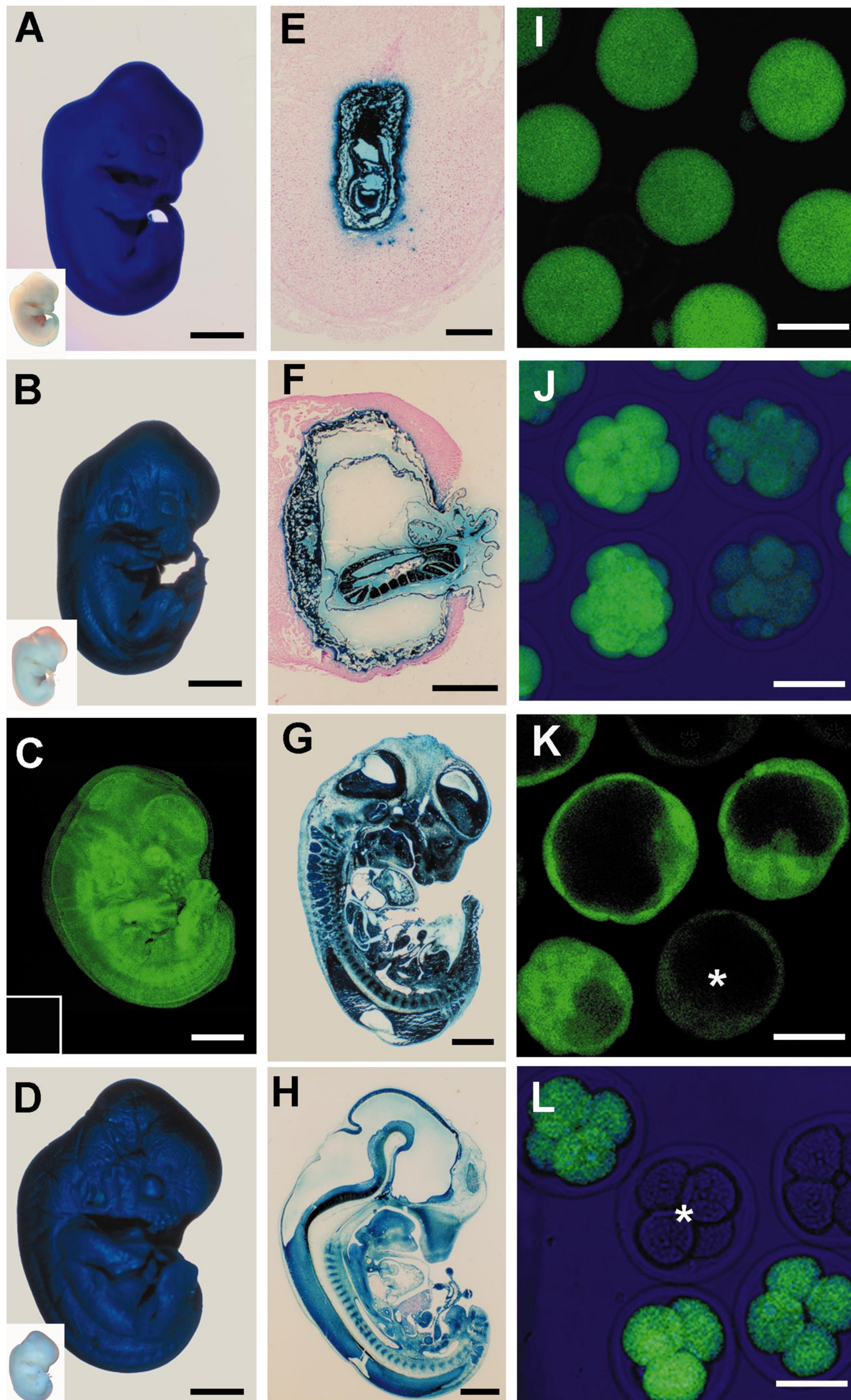

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proliferate in the diseased host liver (Rhim *et al.,* 1994). For the present study, to generate transplantation chimeric livers, (FVB/N × C57BL/6)F1 or Swiss nude mice carrying a major urinary protein (MUP)–uPA fusion transgene (Weglarz and Sandgren, submitted) were administered 1×10^5 R26–hPAP-marked hepatocytes or R26–EGFP-marked hepatocytes via splenic injection, after which cells travel via portal circulation to the liver. Donor cells were isolated from FVB/N R26–hPAP or R26–EGFP transgenic mice or from F344 R26-hPAP transgenic rats via two-step EDTA/collagenase perfusion (Klaunig *et al.,* 1981). Recipients were sacrificed between 4 and 25 weeks after transplant, and livers were collected, fixed, and stained or viewed fresh or as cryosections under fluorescent microscopy to identify donor-derived hPAP- or EGFP-marked cells. Chimeric mammary glands were produced by transplantation of 5×10^4 collagenase-isolated mammary epithelial cells from adult R26–hPAP transgenic mice into cleared mammary fat pads of 3-week-old syngeneic FVB/N female recipients (Clifton *et al.,* 1985; Moser *et al.,* 1993). These transplanted cells divided and redifferentiated into morphologically normal mammary epithelium. Fifteen to 18 weeks posttransplantation, reconstituted mammary fat pads were collected, fixed, and stained with BCIP as whole mounts or paraffin sections to identify donor-derived cells.

Immunohistochemistry

Paraformaldehyde-fixed, paraffin-embedded liver sections from MUP–uPA recipient mice transplanted with R26–hPAP rat hepatocytes were stained for 48 h with BCIP as described above. Antigen retrieval was then done by gently boiling slides for 10 min in 0.1 M Tris, pH 9.0. Tissues were cooled, blocked in 0.5% nonfat dried milk, and then incubated with rat-specific bile canalicular monoclonal antibody, Mab 5.4, diluted 1:5 (kindly provided by Dr. Ron Faris), overnight at room temperature. After several PBS rinses, slides were then incubated with biotinylated goat anti-mouse IgG secondary antibody for 30 min at room temperature and then avidin/biotin conjugated to peroxidase (Biogenex), rinsed, exposed to 3,3′-diaminobenzidine (Sigma), and counterstained with nuclear fast red.

RESULTS

Generation of Transgenic Mice and Rats

Transgenic animal lineages are summarized in Table 1. Initial hPAP transgene expression was evaluated by hPAP enzymatic staining of blood collected from founders and offspring in each lineage. The six mouse and two rat R26–hPAP transgenic lineages that were established developed normally into adulthood and were fertile. Sudden deaths occurred in several 804-1 line mice, although no gross or histological lesions were identified. Other individuals from this lineage remained healthy to over 1 year. R26–EGFP transgene expression was evaluated in tail and ear biopsies by fluorescence at 488 nm. All R26–EGFP transgenic lineages developed normally into adulthood and were fertile.

TABLE 1
Transgenic Animal Lineages

Transgene	Species	Strain	No. lineages expressing ubiquitously/No. lineages analyzed[a]
R26–hPAP	Mouse	FVB/N	4/4
R26–hPAP	Mouse	C57BL/6	2/2
R26–hPAP	Rat	F344	2/2[b]
R26–EGFP	Mouse	FVB/N	4/4
R26–EGFP	Mouse	C57BL/6	ND

[a] Transgene expression of R26–hPAP animals was assessed by BCIP staining of tissue sections. R26–EGFP transgene expression was assessed by tissue fluorescence at 488 nm.

[b] Four rat founders were generated, but lineages were established and analyzed only from the two highest-expressing founders.

The R26 Promoter Directs Ubiquitous hPAP Expression during Embryonic Development

Overall, the pattern of R26–hPAP transgene expression recapitulated the β-galactosidase staining pattern observed during embryonic development in the ROSA26 mouse strain (Fig. 2A). R26–hPAP transgene expression was detected with BCIP histochemistry on whole embryos at E 12.5 (Fig. 2B). Whole embryos from each of five examined R26–hPAP transgenic mouse lineages turned uniformly blue when incubated in BCIP, although there were differences in staining intensity among different lineages. There was minimal background staining in nontransgenic embryos (Fig. 2B, inset). Ubiquitous and uniform embryonic transgene expression was confirmed by BCIP histochemical staining of paraformaldehyde-fixed, paraffin-embedded embryos isolated at 7.5, 9.5, and 12.5 days p.c. (Figs. 2E–2G). All transgenic embryonic tissues, and all individual cells within those tissues, stained blue, although there were differences in the intensity of staining between different cell types and, again, between different lineages. R26–hPAP transgenic rat embryos similarly displayed ubiquitous transgene expression based on BCIP staining of whole and sectioned embryos at E 14.5 (Figs. 2D and 2H).

The R26 Promoter Directs Ubiquitous EGFP Expression during Embryonic Development

The presence of endogenous heat-stable isoforms of alkaline phosphatase in preimplantation embryos precluded evaluation of R26–hPAP expression (data not shown). We therefore used R26–EGFP mice to assess R26 promoter activity at this stage of development. Nontransgenic FVB/N ova fertilized with sperm from R26–EGFP hemizygous males displayed EGFP fluorescence beginning at the late four-cell stage (Fig. 2L), and expression was maintained at the eight-cell and blastocyst stages. Earlier stages did not fluoresce. Unfertilized ova and fertilized one-cell ova from hemizygous R26–EGFP females displayed uniform fluores-

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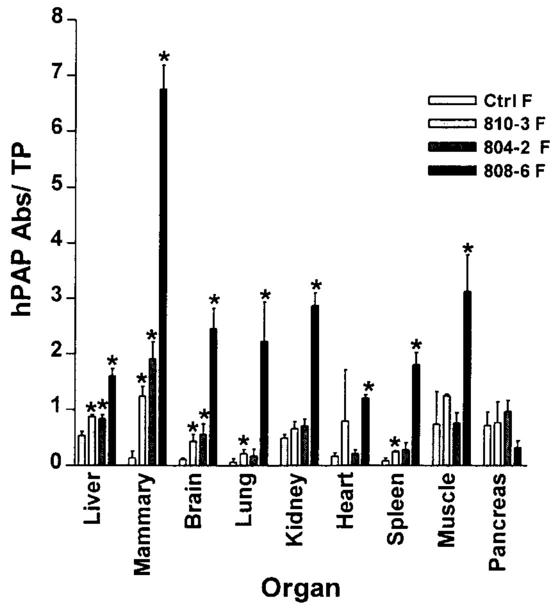

FIG. 3. hPAP solution assay of tissue homogenates isolated from various tissues of three R26–hPAP transgenic mouse lineages and nontransgenic controls. Heat-stable alkaline phosphatase activity was significantly higher (asterisks) in tissue homogenates from transgenic lineages versus controls for most tissues. The 808-6 lineage expressed at a consistently higher level than the other lineages analyzed.

cence (Fig. 2I). Fluorescence persisted in all embryos derived from hemizygous R26–EGFP females mated to nontransgenic males through the four-cell stage (Fig. 2J). At the eight-cell (morula) stage, approximately half of the embryos displayed reduced fluorescence (Fig. 2K), and at the blastocyst stage half of the embryos did not fluoresce at all, consistent with loss of maternally derived EGFP in nontransgenic embryos. Ubiquitous fluorescence also was observed in whole mounts of E 12.5 embryos (Fig. 2C). Eggs and embryos isolated from matings between nontransgenic mice displayed no background fluorescence.

The R26 Promoter Directs Ubiquitous Transgene Expression to Adult Tissues

A p-NPP solution assay was used to measure heat-stable alkaline phosphatase activity present in tissue homogenates from adult transgenic R26–hPAP mice (Fig 3). One transgenic lineage, 808-6, had consistently higher alkaline phosphatase activity in all organs except pancreas compared to nontransgenic controls. hPAP also was highly expressed in the testes of males from this lineage. Tissues from the two other transgenic lineages assayed had consistently lower levels of alkaline phosphatase activity, although activity generally was greater than in nontransgenic tissue homogenates.

To determine relative levels of transgene expression among different cell types within an organ, sectioned adult tissues were stained histochemically with BCIP (Fig. 4). The blue BCIP reaction product was visible in the cytoplasm of all cells. In some cell types, particularly hepatocytes and pregnant mammary epithelium, the reaction product was localized to the cytoplasmic membrane. The relative staining intensity of different organs was compared among four different transgenic mouse lineages (Table 2). In general, BCIP histochemical staining intensity correlated with the p-NPP solution assay results; however, *in situ* BCIP staining clearly demonstrated a visual difference in all lineages between R26–hPAP transgenic mice and nontransgenic controls, even in tissues that showed no significant difference based on the p-NPP solution assay. *In situ* enzymatic staining also permitted determination of relative expression levels in individual cell types within an organ. BCIP histochemical staining of R26–hPAP transgenic rat tissues showed a similar ubiquitous and uniform staining pattern in the two transgenic lineages examined (Fig. 4).

Transgene expression was evaluated in R26–EGFP mice by confocal fluorescent imaging of fresh whole tissue collected from four transgenic lineages. All tissue examined from transgenic mice displayed increased fluorescence relative to nontransgenic controls, and fluorescence was strongest in the 1309-10 lineage. Fixed, frozen sections were examined from the high-expressing 1309-10 lineage (Figs. 4A–4E). All organs examined from this lineage expressed the transgene, exhibiting specific fluorescence at 488 nm. For both R26–hPAP and R26–EGFP mice, the highest-expressing lineages also displayed the highest transgene

TABLE 2

Relative BCIP Staining of FVB/N R26–hPAP Transgenic Mouse Tissues

	810-3	804-2	808-6	804-1
Liver	+/−	3+	3+	1+
Mammary	2+	3+	3+	4+
Brain	3+	4+	4+	4+
Lung	2/3+	4+	4+	4+
Kidney	2/3+	4+	3+	3+
Heart	1+	3+	3/4+	3+
Spleen	1+	3+	3+	3+
Skeletal muscle	1+	2+	2+	2/3+
Pancreas (acinar)	+/−	2+	2+	2+
Pancreas (islet)	+/−	3+	3+	4+
Small intestine	1+	2+	3+	2+
Smooth muscle	3+	4+	4+	4+
Ovary	3+	ND	4+	3+
Testis	2+	3+	4+	4+

Note. Relative staining intensity was determined by visual inspection of paraformaldehyde-fixed, paraffin-embedded sections stained for 24 h with BCIP at 37°C. One adult male and one adult female were analyzed for each lineage. ND, not determined.

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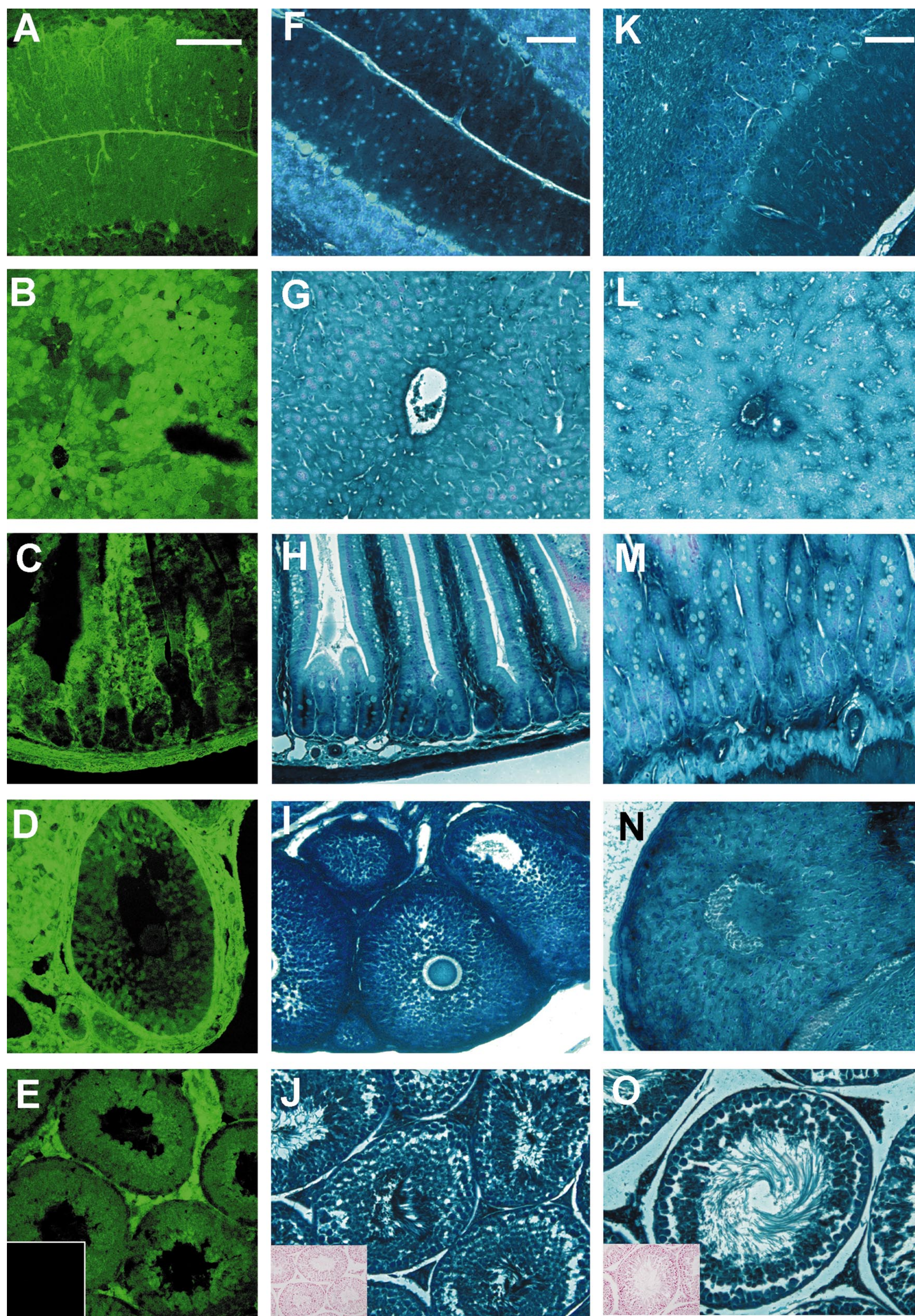

FIG. 4. (A–E) EGFP is expressed in all adult tissues (brain, liver, small intestine, ovary, testis) in R26–EGFP transgenic mice and hPAP is expressed in all adult tissues in R26–hPAP (F–J) mice and (K– O) rats. Tissues from adult R26–EGFP mice were fixed, frozen, sectioned, and imaged at 488 nm. Tissues from adult R26–hPAP mice and rats were fixed, paraffin embedded, sectioned, stained with BCIP, and counterstained with nuclear fast red. Insets, testes from nontransgenic mice or rats processed identically. Bars, 50 μm.

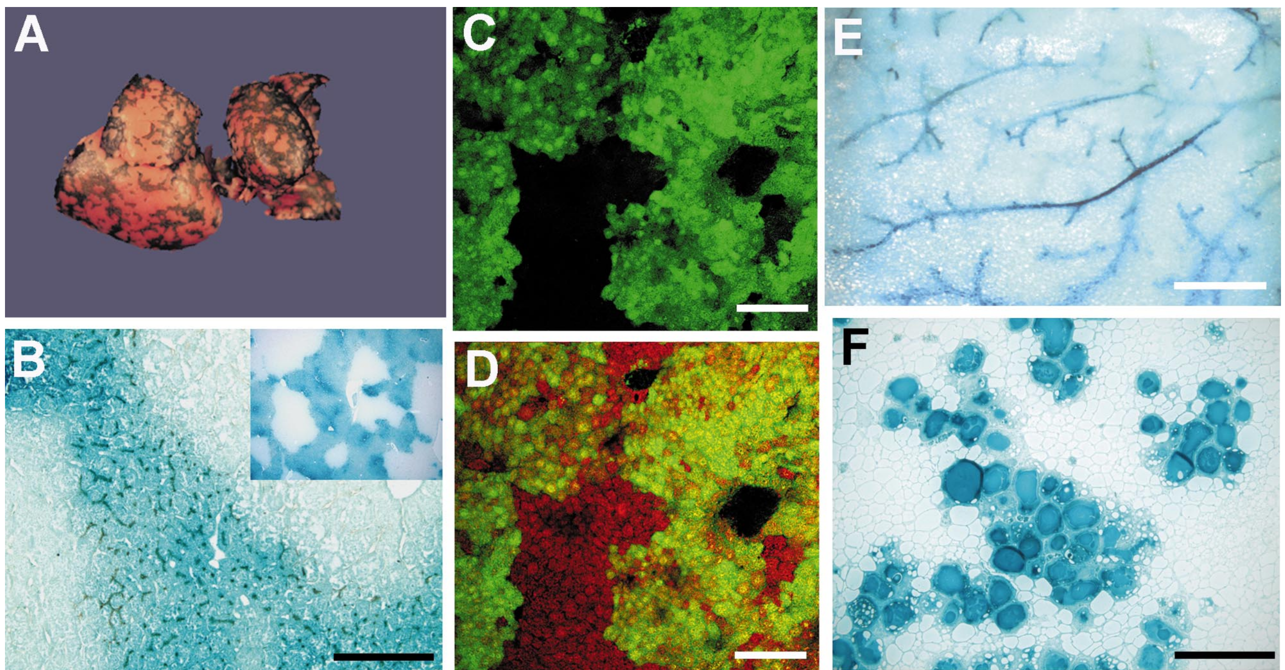

FIG. 5. (A–F) Transplantation of liver and mammary cells bearing marker transgenes. (A) Whole liver BCIP staining of transplanted R26–hPAP-marked rat hepatocytes (line 7-6) growing in immunodeficient recipient diseased mouse liver (14 weeks posttransplant). (B) Transplanted R26–hPAP-marked rat hepatocytes (line 7-6) growing in immunodeficient recipient diseased mouse liver. Blue BCIP staining colocalizes with immunostaining for rat bile canaliculi (25 weeks posttransplant). Paraffin section, BCIP stain. Bar, 125 μm. Inset, low power. (C and D) Transplanted R26–EGFP-marked mouse hepatocytes (green fluorescence) growing in diseased mouse liver (red fluorescence). Fixed, frozen section, propidium iodide-stained, green channel (C); green and red channel (D) (14 weeks posttransplant). Bar, 100 μm. (E) Reconstituted mammary gland (virgin, 15 weeks posttransplant), whole mount of transplanted R26–hPAP (line 804-2) mammary epithelial cells. Bar, 1 mm. (F) Reconstituted mammary gland (day 18 pregnant, 18 weeks posttransplant) from transplanted bitransgenic R26–hPAP/WAP–TGFα mammary epithelial cells. Bar, 250 μm.

copy number as assessed by Southern blotting (data not shown).

R26–hPAP- and R26–EGFP-Marked Transgenic Cells Can Be Identified in Transplantation Chimeric Tissues

Because of the ubiquitous pattern of marker gene expression in R26–hPAP mice and rats and in R26–EGFP mice, cells from these animals should permit (1) monitoring of engraftment of transplanted cells and (2) genotype-specific marking in transplantation and embryonic chimera studies and lineage analyses. To demonstrate this, we employed transgene-marked hepatocytes or mammary epithelial cells in transplantation studies. First, 1×10^5 hepatocytes from R26–hPAP mice or rat or from R26–EGFP mice were transplanted into 3-week-old recipient MUP–uPA transgenic mice that develop transgene-associated liver disease. At 4 or more weeks after transplantation, repopulation of recipient mouse diseased hepatic parenchyma by healthy R26–hPAP-marked mouse hepatocytes (data not shown), R26–hPAP-marked rat hepatocytes (Figs. 5A and 5B), or R26–EGFP-marked mouse hepatocytes (Figs. 5C and 5D) was confirmed by the presence of blue BCIP-stained hepatic foci (Figs. 5A and 5B) or EGFP fluorescent foci (Figs. 5C and 5D). Immunostaining of mouse/rat chimeric liver to detect a rat-specific bile canalicular antigen demonstrated antibody colocalization at the cellular level with the blue BCIP reaction product in transplanted R26–hPAP rat-derived foci (Fig. 5B). Second, 5×10^4 mammary epithelial cells isolated from R26–hPAP transgenic mice were injected into cleared mammary fat pads of 3-week-old syngeneic recipients. At 15 to 18 weeks after transplantation, R26–hPAP-marked cells were identified in BCIP-stained reconstituted mammary gland whole mounts (Fig. 5E). Mammary epithelium stained specifically for hPAP, uniquely identifying cells and structures derived from the donor genotype. The usefulness of the R26–hPAP transgene as a genetic marker was demonstrated further by transplanting bitransgenic R26–hPAP/WAP–TGFα mammary epithelial cells (Fig. 5F). Reconstituted mammary epithelia from these donor cells all stained blue with BCIP in paraffin sections, and, furthermore, displayed phenotypic characteristics of WAP–TGFα mammary epithelium (alveolar hyperplasia and accelerated dif-

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ferentiation during late pregnancy). Importantly, for each of these experimental applications, marked cells were not immunologically rejected by hosts.

DISCUSSION

We have shown that the ROSA26 gene promoter can be used to direct expression of marker transgenes in mice and rats. The ubiquitous and uniform pattern of marker gene expression and the relative ease of marker visualization should make the R26–hPAP and R26–EGFP transgenic lineages useful for experiments that require genetic marking of subpopulations of cells within a larger unmarked cell population. Genetic markers are particularly critical for studying interactions between cells *in vivo* in cases where the respective cell populations (genotypes) cannot be identified by simple morphological criteria or differences in immunohistochemical staining. Genetic markers are especially important for cell lineage analyses. Presently, three reporter genes are used most commonly to mark cells: *lacZ,* hPAP, and GFP. Each has certain advantages and limitations, and these are reviewed below.

The *Escherichia coli lacZ* gene, encoding the enzyme β-galactosidase (β-gal), has been used extensively as an *in vivo* reporter. β-Gal activity is easily detected in whole mounts and fresh and frozen sections using a simple histochemical assay that produces a blue reaction product. Stained tissues can be postfixed, paraffin embedded, and sectioned, or they can be processed for electron microscopy. There is minimal endogenous galactosidase activity. The ROSA26 mouse, derived from an ES cell line carrying a promoter-trap *lacZ* construct, expresses the cytoplasmic form of β-gal in all tissues and has been used as a source of marked cells for the production of chimeric embryos and adult tissues (Chen and Behringer, 1995; Merritt *et al.,* 1997; Gould and Dove, 1997). Tam and colleagues (Tam and Tan, 1992) also have reported ubiquitous expression of an X-linked HMG–CoA–*lacZ* transgene construct. The *lacZ* reporter gene and transgene constructs employing this reporter have displayed several limitations. (1) There can be variable and unpredictable expression when *lacZ* is placed under the regulatory control of different promoters (Beddington *et al.,* 1989; McBurney *et al.,* 1994). (2) *LacZ* expression can be developmentally down-regulated, suggested by several reports of decreasing expression of *lacZ* transgenes in later versus earlier developmental stages (Beddington *et al.*, 1989; Cui *et al.,* 1994). This down-regulation may be mediated by changes in *lacZ* methylation. (3) Variable cell-to-cell expression of *lacZ* transgenes has been reported in tissue culture and *in vivo* (MacGregor *et al.,* 1987; Beddington *et al.,* 1989), also possibly mediated by differential methylation. (4) β-Gal is heat labile and does not maintain enzymatic activity after paraffin embedding, preventing *in situ* enzymatic staining of fixed, paraffin-embedded sections with optimal cellular morphology. The latter problem can be partially circumvented for cells expressing high levels of β-gal protein, in which the protein can be detected by immunohistochemistry. (5) Finally, although *lacZ*-expressing tissues can be stained first and then paraffin embedded and sectioned, substrate penetration into tissue is limited and substrate staining intensity is reduced during routine tissue processing. We have determined that substituting chloroform- or limonine-based solvents for xylene during tissue processing can minimize the latter problem (data not shown).

hPAP also has been used as a reporter gene *in vivo* (Fields-Berry *et al.,* 1992; Fekete and Cepko, 1993; Deprimo *et al.,* 1996). As for β-gal, enzymatic activity of hPAP can be demonstrated in tissues by incubation with an appropriate substrate, typically BCIP (Table 2). One line of transgenic mice carrying hPAP under regulatory control of the human β-actin promoter has been reported to display hPAP expression in multiple tissues examined from both embryos and adults (Deprimo *et al.,* 1996). Disadvantages of hPAP as a marker include (1) endogenous heat-stable alkaline phosphatase activity in early embryos, and, as for *lacZ,* (2) limited penetration of tissue by substrate. However, as shown in this report, because hPAP is heat stable, fixed and paraffin-embedded tissue sections can be incubated directly with substrate. In this context, substrate penetration into tissue is not a limiting factor and excellent tissue morphology can be preserved.

Most recently, green fluorescent protein, derived from the jellyfish *A. victoria,* has been employed as a reporter gene *in vitro* and *in vivo.* GFP absorbs blue light and emits green light without the need for any cofactor or substrate. GFP and related mutant forms such as EGFP that have been modified to enhance or shift the GFP fluorescence signal have been used as reporter genes in transgenic mice. When placed under regulatory control of different promoters (e.g., human hemopexin, mouse β-1 integrin, human GFAP) GFP expression has been directed to several different cell types (Chiocchetti *et al.,* 1997; Zhou *et al.,* 1997), and ubiquitous EGFP expression was reported in transgenic mice carrying EGFP under regulatory control of a chicken β-actin promoter and cytomegalovirus enhancer (Ikawa *et al.,* 1998). Nagy and colleagues (Hadjantonakis *et al.,* 1998) have established a mouse embryonic stem cell line carrying this construct that also directs ubiquitous expression in mice generated from these cells. Because there are no mammalian homologs to GFP, there is no specific fluorescence from endogenous GFPs in mammalian cells (although there is variable nonspecific autofluorescence). The unique advantage of GFP versus other reporters is that it can be visualized in living cells, both *in situ* and in isolated cells prepared for cell sorting. It is also quite stable, maintaining fluorescence activity after incubation at 65°C and fixation in 4% paraformaldehyde (Cubitt *et al.,* 1995), although unless fixed it remains highly soluble in alcohols and aqueous solutions.

The two transgene constructs described in this report, R26–hPAP and R26–EGFP, each provide many of the characteristics of an ideal genetic marker. Specifically,

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expression of these constructs appears to be developmentally neutral, they produce a ubiquitous and uniform pattern of marker gene expression throughout development that can be localized at the cellular level, and they provide simplicity and flexibility of visualization techniques as well as compatibility with other tissue procedures. Expression of the R26–hPAP transgene could not be confirmed by histochemical staining in preimplantation embryos due to the presence of endogenous heat-stable alkaline phosphatase activity at these early developmental stages. However, R26-driven zygotic expression of EGFP was detected as early as the late four-cell stage, indicating that the R26–EGFP transgenic lineage should be particularly useful for marking genotypes in preimplantation embryos. The transgene expression pattern observed in our animals is largely consistent with the staining pattern observed in ROSA βgeo 26 mice (Friedrich and Soriano, 1991; Zambrowicz *et al.,* 1997), which already have proven useful for genotype-specific marking of transplanted tissue (Zambrowicz *et al.,* 1997) and embryonic chimeras (Chen *et al.,* 1995; Merritt *et al.,* 1997; Gould and Dove, 1997). It is intriguing that 0.8 kb of the regulatory DNA from the ROSA26-associated gene is sufficient to direct efficient, high-level expression of multiple heterologous coding sequences to all cell types in the body and in a manner that is related to transgene copy number. This promoter should be useful in the construction of other transgenes for which ubiquitous transgene expression is desired. Interestingly, the endogenous R26 locus appears to be a favorable site for efficient homologous recombination in embryonic stem cells (Soriano, 1999). Thus, mouse strains for which germline-competent embryonic stem cells exist can be specifically engineered for ubiquitous expression of desired coding sequences by (1) targeting the endogenous R26 locus in ES cells and then (2) generating mice from targeted cells. The transgenic approach described in this report should remain particularly important in other mouse strains and in the rat and other species for which transgenesis remains the only practical way to manipulate the genome. Finally, we note that R26–hPAP and R26–EGFP transgenic lineages have been created in commonly used genetic backgrounds, FVB/N and C57BL/6 mice and Fischer 344 rats. These transgenic animals should expand the range of questions that can be asked using chimeric tissues.

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