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To study the tissue specificity of these promoters, we repeated the experiments in kidney epithelial cell line 293, using a promoter-less construct (pBG) as a control for residual activity. In this case, all MCK constructs displayed very weak transcriptional activity, to levels equal to those measured in the promoter-less construct, as reported elsewhere (6). In contrast, the MGP construct displayed significant expression of EGFP, pointing to its low tissue specificity.

In summary, since laser scanning cytometry has become available, diverse applications have been described (1), such as cell-cycle analysis, enzyme kinetics, molecular binding and translocation, and FISH analysis. Here, we show that laser scanning cytometry is a highly sensitive technique for the automated assessment of gene transfer efficiency and promoter activity of EGFP constructs. Thus, laser scanning cytometry has unique features that broaden the utility EGFP as a reporter protein.

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Visualization of Whole-Mount Skeletal Expression Patterns of *LacZ* Reporters Using a Tissue Clearing Protocol

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ABSTRACT

Gene targeting or trapping constructs that utilize the lacZ gene encoding β -galactosidase activity to trap promoter expression have become an increasingly important way to disrupt gene function and monitor gene expression. A number of genes targeted in this way have revealed both ex-

pected and unexpected developmental abnormalities of the skeleton. The use of X-gal staining to monitor gene expression in developing skeletal structures is hampered in these mutants because, during the critical latter stages of mouse embryonic development, visualization is hindered by the opacity of overlying soft tissue. Here, we report the development of a reliable method to clear exogenous tissue in late-stage embryos and neonates that still preserves skeletal X-gal staining patterns. This protocol reveals (i) specific cell staining in localized regions of developing bone and cartilage in two different genetic models and (ii) that the intensity of X-gal staining is consistent with the level of expression of lacZ. We conclude that this protocol accurately reflects both the specificity and intensity of expression and will facilitate the analysis of mouse skeletal development.

INTRODUCTION

The use of skeletal staining protocols allows visualization of developing skeletal structures in late-stage mouse embryos and neonates. These stages of development are particularly important in the study of bone biology because they mark the onset of processes of endochondral and intra-membranous ossification in the embryo. The standard protocol used to study these processes involves the use of Alizarin red and Alcian blue dyes. These preferentially stain bone and cartilage, respectively, which, when followed by treatment with potassium hydroxide to clear exogenous tissue, present a colorful view of the entire developing skeleton against a background of transparent soft tissue (5,6). This technique has been used to monitor not only the progression of skeletal development in normal mice but also the consequences of genetic mutations on these structures (1,7-9,11-13).

Genetic modification of mice by gene targeting and trapping techniques has had a major impact on biological research in revealing the consequences of gene loss. Adaptations of these techniques to include the use of marker genes to report on promoter expression patterns of targeted genes have been particularly useful to the study of spatial and temporal developmental regulation of gene expression. The genetic marker

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most commonly used in this approach has been *lacZ*, whose detection using X-gal stain has proved a reliable and sensitive method to study expression patterns. Recently, *lacZ* promoter trap constructs have been used to elucidate the expression of genes involved in skeletal development (1,3,9,11). The usefulness of these mutants has been hampered in these studies because, in the latter stages of embryonic development and neonates, X-gal staining of skeletal structures is obscured through the overlying skin and musculature. To address this problem, we investigated the potential of using a potassium hydroxide tissue clearing protocol similar to that used normally with Alizarin red and Alcian blue, following staining for *lacZ* expression using the X-gal substrate in whole-mount. We used mice strains carrying *lacZ* reporters in two genes, *Hs2st* and *ESP*, expressed in skeletal structures.

MATERIALS AND METHODS

Genetic Strains of Mice

The generation of mice carrying the *Hs2st*^{TgNst125Nimr} gene trap mutation, which is an insertion of the *lacZ* gene in the Heparan sulfate 2-sulfotransferase gene (*Hs2st*), has been described previously (4). *Hs2st* is expressed differentially during embryogenesis in a variety of tissues including skeletal structures. Mice homozygous for the gene trap (*Hs2st*^{-/-}) die perinatally and exhibit various abnormalities including skeletal defects. *Hs2st* mutant mice were maintained as heterozygotes (*Hs2st*^{+/-}), and *Hs2st*^{-/-} mice were generated by intercross.

Mice carrying a targeted mutation of the embryonic stem cell phosphatase (*ESPTm1PJM*) were generated by insertion of a promoter trap *lacZ* gene by homologous recombination into this locus.

The embryonic stem cell phosphatase (*ESP*) gene, also known as osteotesticular phosphatase, is known to be highly expressed in osteoblasts and a limited number of other tissue types (10).

All mice were maintained in the animal facility at the Centre for Genome Research, Edinburgh, and all procedures were carried out in accordance with the United Kingdom Home Office regulations.

X-Gal and Alizarin Red/Alcian Blue Staining Procedures

X-gal staining was performed using an adaptation of a previously described procedure (2). Cadavers were immersed for 5 min in 70% ethanol to loosen skin. An incision was made into the abdomen to aid in the removal of skin under a stereo microscope using fine forceps. We took care not to dismember the car-

cass; thus, small amounts of skin on the paws were left intact. The embryo/neonate was then carefully eviscerated using fine forceps, and all internal organs were removed from the abdominal and thoracic cavities. Embryo/neonate carcasses were then washed briefly in PBS (Sigma, St. Louis, MO, USA) and placed in fixative overnight at 4°C. The fixative was prepared as 0.2% glutaraldehyde, 2 mM MgCl₂, 5 mM EGTA, 0.1 M sodium phosphate buffer, pH 7.3. After fixation, the embryos were washed briefly in PBS and added to the X-gal stain. X-gal stain was prepared by diluting solution A 50-fold with solution B. Solution A consists of 50 mg/mL X-gal in N,N-dimethyl formamide (Sigma) stored in the dark at -20°C. Solution B is 5 mM K₃Fe(CN)₆ and 6 mM K₄Fe(CN)₆ in 0.1 M sodium phosphate buffer, pH 7.3. After overnight staining, the embryo/neonate carcasses were

washed briefly in PBS and fixative before embryo clearing. No X-gal staining was ever observed in non-transgenic mice (see Figure 3B).

Alizarin red/Alcian blue stains were performed with minor modification of the previously reported method (6). The cadavers were skinned and eviscerated as described above and placed into 100% ethanol overnight at room temperature, followed by overnight incubation in acetone at room temperature. From stock solutions of 0.1% Alizarin red in 85% ethanol and 0.3% Alcian blue in 70% ethanol, two staining solutions were prepared. Solution 1 consisted of a 1:20 dilution of 0.1% Alizarin red in 85% ethanol/5% acetic acid, and solution 2 consisted of a 1:20 dilution of both 0.1% Alizarin red and 0.3% Alcian blue in 85% ethanol/5% acetic acid. The carcasses were washed in distilled water and placed in solution 1 for four

days at room temperature, followed by staining in solution 2 for two days.

Embryo Clearing Procedure

The X-gal-stained fetus/neonate carcasses were washed with PBS and incubated in 1% trypsin (from 1:250 porcine pancreas trypsin powder; Sigma) in PBS at 37°C overnight to digest connective tissue. They were then carefully transferred into 20% and 30% glycerol, respectively, for 3 h each at room temperature. Carcasses were then moved into 40% glycerol with 1% KOH and incubated overnight at 37°C. This step resulted in most of the X-gal stain eluting from exogenous soft tissue and the initial clearing of the opaque soft tissue. The skeletal preparations were then transferred into 60% glycerol for three days to allow further tissue clearing, revealing the X-gal-stained skeletons

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against the now translucent soft tissue. For storage, skeletal preparations were first moved into 80% glycerol overnight and then into 100% glycerol.

Alizarin red/Alcian blue-stained embryos were cleared and stored using an identical protocol as described above for the X-gal-stained embryos.

RESULTS AND DISCUSSION

Tissue Clearing Reveals Localized Skeletal X-Gal Staining Pattern Against a Transparent Background of Soft Tissue

To determine the skeletal expression patterns of *Hs2st* and *ESP*, mutant embryos between gestation and birth were stained on day 20 to examine *lacZ* expression. Poor visualization of skeletal staining was encountered because of the opacity of the overlying soft tissue and the presence of *lacZ* expression in some of these tissues. Protocols using benzyl-benzoate/benzyl alcohol have been used successfully to clear early embryos to visualize the underlying X-gal staining (9) or in the extremities of neonates (3). However, we found this protocol not sufficiently stringent to clear whole-mount late-stage fetuses, neonates, or older animals (data not shown). These stages of development are very important in the study of skeletal development. Therefore, we assessed whether X-gal staining of skeletal structures could be visualized by the clearing of soft tissue in a manner akin to that used in the standard protocol for visualization of these structures by Alizarin red/Alcian blue. This would allow the immediate visualization of total expression patterns in the skeletal system in three dimensions, as opposed to laborious reconstruction of serial sections. *Hs2st*^{+/-} and *Hs2st*^{-/-} mice from a heterozygous intercross were sacrificed at day 20 of gestation, and the pups were stained with X-gal as described. Figure 1 shows the staining observed after clearing in a *Hs2st*^{+/-} embryo. These mice are known to express *lacZ* in skeletal structures, and homozygous mutant mice have skeletal deficits underlining the importance of this gene in skeletal development (4). The tissue clearing of the *Hs2st*^{+/-} em-

bryo was found to reveal specific X-gal staining in skeletal tissue against a translucent background of soft tissue. This staining was found to be predominately limited to cartilage throughout the mouse. Strong staining was restricted to areas of cartilage in all long bones from the epiphyseal growth plate to the articulated surface of the joint, while ossified tissue, such as trabecular and cortical bone, remained essentially unstained. In the thoracic, lumbar, and sacral regions of the spine, the cartilaginous region of the vertebrae stain strongly with X-gal, while staining in the adjacent ossified regions of the vertebrae is undetectable. In the caudal region of the spine, the immature cartilaginous vertebrae stain strongly. Other tissues that stained strongly with X-gal include the sternal region of the rib, the perichondrium around the cartilage of the nasal capsule, the cartilaginous tympanic bulla, the patella, and the primordial cartilage of the hands and feet. Moderate staining was observed in the medial border of the scapula, while weak staining was observed in the mandible of the jaw. Major ossification areas such as calvaria, vertebral ribs, and ventral tubercle are essentially negative. These observations suggest that it

is possible to clear exogenous tissue to reveal the underlying X-gal staining of skeletal structures and that *Hs2st* gene-expression in late embryonic life is predominately restricted to chondrocyte lineages. This observation of lineage-restricted expression has a direct impact on the interpretation of the skeletal abnormalities observed in *Hs2st* homozygous mutants that will be discussed in a later publication. The graded staining patterns from strong to weak staining suggest that this method is sensitive and allows the identification of areas with even low levels of gene expression. To determine whether the expression pattern in whole-mount staining was comparable to that observed using the conventional technique of X-gal staining of frozen serial sections, neonatal head preparations were made, sectioned, and stained using standard methodology. In whole-mount preparations, we had observed that there is strong staining in the perichondrium surrounding the nasal cartilage (Figure 1 and Figure 2A). This staining pattern was also observed in the stained histological sections, with intense X-gal staining surrounding the nasal cartilage (Figure 2B). X-gal staining in whole-mount preparations was

Figure 1. Tissue clearing reveals underlying X-gal staining against a background of translucent soft tissue in a whole-mount *Hs2st*^{+/-} embryo at day 20 post coitum. Blue staining represents the areas of X-gal deposition that mark the expression of *lacZ* from the *Hs2st* promoter, which is highly expressed in cartilage tissue. TV, thoracic vertebrae; LV, lumbar vertebrae; SV, sacral vertebrae; CV, caudal vertebrae; TB, tympanic bulla; NC, nasal capsule cartilage; PC, primordial cartilage of the paws; C, calvarium.

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undetectable in the calvarium of *Hs2st*^{+/-} mice (Figure 1), and this was also confirmed using X-gal stained sections (note the unstained in the calvarium in Figure 2C compared to the intense staining of the adjacent brain tissue). Where comparable, whole-mount and stained sections confirmed the fidelity of the X-gal staining patterns (data not shown).

X-Gal Staining Pattern Is Different in Two Genetic Models

To prove that the X-gal skeletal staining protocol accurately reflects gene expression patterns, a second strain of mice was investigated. These mice carry a heterozygous mutation in *ESP* (*ESP*^{+/-}), a gene with known expression in osteoblasts (10). We pre-

dicted that we would observe staining in the ossified bone structures and other skeletal elements in these mutants. Figure 3, C and D, shows a comparison of rib cage X-gal stains of these mice compared to the rib of a non-transgenic mouse stained with Alizarin red/Alcian blue (Figure 3A) and a non-transgenic mouse stained with X-gal as a negative control (Figure 3B). Mammalian ribs consist of both ossified bone and cartilaginous tissue. In Figure 3A, these areas are clearly identified as the ossified Alizarin red-stained vertebral rib and the cartilaginous Alcian blue-stained sternal rib. Figure 3C shows the localization of X-gal staining in the *Hs2st*^{+/-} mice as being restricted to the cartilage of the sternal region of the rib (see Figure 3A; compare the X-gal-stained region to the Alcian blue-stained region). Figure 3D shows that, in *ESP*^{+/-} mice, staining spans both the ossified vertebral and cartilaginous sternal regions of the rib. This confirms that *ESP* is expressed in osteoblast lineages and cartilage and would suggest that the X-gal staining observed using this protocol was faithful to the expected gene expression pattern. In addition, we observed many other differences between *Hs2st* and *ESP* mutant mice that confirmed the specificity of the X-gal staining (data not shown). These observations demonstrate that this X-gal staining protocol can distinguish between chondrocyte and osteoblast gene expression patterns and that the X-gal staining is in line with expected *lacZ* expression patterns. To determine an age limit for the use of this technique, *ESP*^{+/-} mice up to three weeks old were X-gal stained and cleared using the protocol described. By three weeks of age, the clearing of exogenous tissue had reduced efficiency and required several weeks in glycerol/KOH to give moderate clearing. Even at this age, the visualization of the X-gal staining of ribs (Figure 3E) and other skeletal structures was possible, showing that this technique is useful with mice up to this age.

X-Gal Staining Is Enhanced in Homozygous Versus Heterozygous Mice

The intensity of X-gal staining is associated with the expression levels of

Figure 2. Skeletal X-gal staining patterns are consistent between whole-mount preparations and sections. (A) Frontal view of the head of *Hs2st*^{+/-} day 20 post-coitum mouse showing staining in the perichondrium of the nasal cartilage and undetectable staining of the calvarium. (B) Stained section of neonatal *Hs2st*^{+/-} mouse showing intense staining in the perichondrium of the nasal cartilage. (C) Stained section of neonatal *Hs2st*^{+/-} mouse showing the absence of staining in the calvarium. Br, brain; Ca, calvarium; NC, nasal capsule cartilage; PER, perichondrium of the nasal cartilage; S, surface of the skin.

Figure 3. Areas of X-gal staining of skeletal structures differ when *lacZ* is expressed from two different promoters. (A) Newborn wild-type mouse rib stained using a standard Alizarin red/Alcian blue skeletal stain. (B) Wild-type day 20 post-coitum mouse stained with X-gal and cleared showing the absence of any background staining. (C) X-gal staining of rib from day 20 post-coitum *Hs2st* mouse showing the restriction of expression to the cartilaginous regions of the rib. (D) X-gal staining of rib from newborn *ESP*^{+/-} mouse showing expression in both the ossified bone and cartilaginous regions of the rib. (E) Rib of X-gal stained and cleared three-week-old *ESP*^{+/-} mutant mouse; note that the clearing of tissue is reduced, but staining is still clearly visible in both the ossified bone and cartilage of the rib. SR, sacral rib; VR, vertebral rib; VC, cartilaginous region of the vertebrae.

Figure 4. X-gal staining is enhanced in *Hs2st*^{-/-} compared to *Hs2st*^{+/+} mice. (A) Hind limb staining in *Hs2st*^{+/+} mice. (B) Hind limb staining in *Hs2st*^{-/-} mice. Fe, femur; T, tibia; F, fibia; C, cartilage of the knee joint; P, patella; GP, epiphyseal growth plate; ASJ, articulated surface of joint.

the *lacZ* gene. Therefore, we predicted that this should be evident when homozygous animals were compared to the heterozygotes because heterozygous littermates possess two copies of the *lacZ* gene. *Hs2st*^{-/-} mutant animals were identified by their skeletal abnormalities and used in the X-gal staining and tissue clearing protocol with the heterozygous littermates that were processed simultaneously. Figure 4 shows a comparison of the intensity of X-gal staining of the knee-joint cartilage of these animals. The developmental abnormality of the bone and cartilage structure of the *Hs2st*^{-/-} mutant is clearly visible, including foreshortening and structural abnormalities of the ossified bone and slight thickening and abnormality of the cartilage of the joint. In addition, we could clearly see that the levels of X-gal staining were enhanced in cartilaginous regions of the homozygous mice (compare the intensity of color in Figure 4, A and B). Although it is not possible to easily quantify the levels of intensity of X-gal staining between heterozygous and homozygous animals, the changes in intensity show that the intensity of X-gal staining to some extent reflects the magnitude of *lacZ* expression.

CONCLUSION

We have developed a reliable method to visualize *lacZ* gene expression in whole-mount skeletal structures by coupling an X-gal staining protocol

with a tissue clearing protocol. We have demonstrated that this protocol can reveal the spatial and temporal expression patterns and the magnitude of gene expression in late-stage embryos, neonates, and older mice. We conclude that this protocol is a fast, reliable, accurate, and sensitive method for the visualization of gene expression patterns in skeletal structures.

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