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We hope that making available the relevant information on Pachyonychia Congenita will be a means of furthering research to find effective therapies and a cure for PC.
Methods in Experimental Dermatology

Improved detection of lacZ reporter gene expression in transgenic epithelia by immunofluorescence microscopy


Abstract: The bacterial lacZ gene is commonly used as a reporter for the in vivo analysis of gene regulation in transgenic mice. However, several laboratories have reported poor detection of β-galactosidase (the lacZ gene product) using histochemical techniques, particularly in skin. Here we report the difficulties we encountered in assessing lacZ expression in transgenic keratinocytes using classic X-gal histochemical protocols in tissues shown to express the transgene by mRNA in situ hybridization. We found that lacZ reporter gene expression could be reliably detected in frozen tissue sections by immunofluorescence analysis using a β-galactosidase-specific antibody. Moreover, we were able to localize both transgene and endogenous gene products simultaneously using double-label immunofluorescence. Our results suggest that antibody detection of β-galactosidase should be used to verify other assays of lacZ expression, particularly where low expression levels are suspected or patchy expression is observed.

Introduction

The well-characterized bacterial Escherichia coli lacZ gene, which encodes β-galactosidase (β-gal), is commonly used as a reporter of gene expression in transgenic organisms [for review see (1)]. Standard histochemical detection of β-gal activity involves incubation of tissue samples in the presence of a suitable chromogenic substrate such as X-gal (5′bromo-4-chloro-3-indolyl-β-D-galactopyranoside) or Bluo-gal (5′bromo-3-indolyl-β-D-galactoside), which upon cleavage by the enzyme form an insoluble blue-colored indigo precipitate. The Bluo-gal substrate has a lower diffusion rate than X-gal but the latter is preferred for light microscopy analysis because it gives a brighter color reaction (2). Several factors are known to affect the sensitivity of X-gal staining including substrate availability, the level of enzyme activity, temperature and oxidation potential. In addition, the activity of endogenous β-gal must be minimized through the use of neutral-alkaline buffers or inhibitory substrate analogs such as D-galactono-1,4-lactone (1).

In our analysis of transgenic mice expressing the lacZ reporter gene under the control of the mouse keratin 6α (MK6α) promoter (3), we observed weak and patchy staining of the reporter in keratinocytes using standard histochemical X-gal staining techniques. A survey of the literature reveals that others have experienced similar problems with X-gal staining particularly in older mouse embryos (>14.5 days) and in adult tissues (4–9). However, we found that immunofluorescence microscopy analysis, using a specific antibody against β-gal, proved a reliable method for the detection of the reporter in transgenic tissues.

Materials and methods

DNA constructs and production of transgenic mice

The production of transgenic mice containing MK6α-lacZ promoter constructs is described elsewhere (3). Transgenic lines were established by matings with BALB/c mice and the F1 or

Donna Mahony,
Seetha Karunaratne and
Joseph A. Rothnagel
Department of Biochemistry and Molecular Biology and the Institute for Molecular Bioscience, University of Queensland, Brisbane, QLD, Australia

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Joseph A. Rothnagel, Department of Biochemistry and Molecular Biology, University of Queensland, Brisbane, Queensland, 4072, Australia
Tel.: +61-7-3365-4629
Fax: +61-7-3365-4699
e-mail:josephro@biosci.uq.edu.au
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$F_2$ offspring used in all subsequent analyses. Analysis of hair bearing skin was performed in BALB/c animals to avoid autofluorescence by the pigmented hair fibers of C57BL mice.

**Histochemical staining of whole tongue tissue**

Tongue biopsies (4 mm) were taken, washed briefly in phosphate-buffered saline (PBS) and then incubated at 37°C overnight in 100-150 µl of an X-gal staining solution (pH 7.5). This consisted of 100 µg/ml X-gal (suspended in N,N-diethylformamide) in 5 mM potassium ferriyanide, 0.1% NP-40, 0.1 M sodium phosphate pH 7.7 (10).

**Histochemical staining of tongue and skin sections**

Tongue and back skin tissues were dissected and embedded in Tissue Tek II Optimal Cryo Temperature (OCT) compound (Miles, Elkhart, IN, USA) on dry ice. Sections (10 µm) were cut at -20°C and mounted on poly-L-lysine-coated slides and allowed to dry for 30 min at room temperature prior to staining. Initially sections were fixed in 0.25% glutaraldehyde in 0.1 M phosphate buffer (pH 7.5) for 10 min or 2% paraformaldehyde (PFA) and washed three times for 10 min in PBS before placing in X-gal staining solution (see above) at 37°C overnight (10). However, we found that both pre- and postfixation of tissues inhibited β-gal activity and consequently only unfixed sections were used.

**Quantitative determination of β-galactosidase activity**

Total β-gal activity in tissue extracts was determined using a fluorescence-based assay (Fluoro-Ace Kit, Bio-Rad, Hercules, CA, USA). Protein extracts were obtained from back skin and tongue of $F_2$ progeny by grinding the samples in liquid nitrogen followed by repeated freeze/thaw cycles according to the manufacturer's instructions. Extracts prepared from non-transgenic animals were used to determine control levels. β-gal activity was normalized to total protein content of each extract as determined by a standard colorimetric assay (11).

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**Figure 1.** A comparison of histochemical X-gal staining in tongue and hair follicles. X-gal staining of whole tongue biopsies was efficient and gave a rapid indication of the levels of β-gal expression in different transgenic lines. Note the differences in expression between two different transgenic lines. β-gal expression is localized to the anterior tip of the tongue in a low β-gal expressing line (a, left) but is localized throughout the tongue in a high β-gal expressing line (a, center). Non-transgenic tongue is negative for X-gal staining (a, right). X-gal staining of unixed tongue sections from several different transgenic lines showed a considerably weaker signal than in tongue biopsies. A representative example of a section taken from a high expressing line (as determined by X-gal staining of whole tongue) shows X-gal staining concentrated in the papillae with much lower staining in the interpapillary regions (b). X-gal staining of back skin tissue sections (c) was generally weak with sporadic staining seen in the outer root sheath (ORS) of some hair follicles (arrowhead). DP indicates the dermal papillae of the hair follicle. Scale bar: 500 µm (a); 30 µm (b); 30 µm (c).
Immunofluorescence analysis

For immunofluorescence studies tissue samples were dissected from several transgenic animals from six different founder lines, rinsed in PBS and embedded in OCT on dry ice (12). Sections (8–10 μm) were cut at –20°C and mounted on poly-L-lysine-coated slides and allowed to dry for 30 min at room temperature prior to staining. Excess OCT was removed by three 5-min washes in PBS. Blocking was performed in FBT buffer (5% fetal bovine serum, 1% bovine serum albumin, 0.05% Tween 20, 10 mM Tris-HCl pH 7.5, 100 mM MgCl₂) for 1 h at room temperature. Primary antibodies used include rabbit anti-E. coli βgal (Cortex Biochem, San Leandro, CA, USA; 1:1000 in FBT) and rabbit anti-MK6a (Babco, Richmond, CA, USA; 1:500 in FBT). Following overnight incubation at room temperature sections were washed three times in PBS and the secondary antibodies, goat anti-rabbit Alexa 488 conjugate (Molecular Probes, Eugene, OR, USA; 1:500 dilution) and biotinilated goat anti-guinea pig (Sigma, St Louis, MO, USA; 1:40 dilution) were applied for 1 h and then washed. The biotinilated guinea pig antibody was detected using Texas Red conjugated to streptavidin (Gibco BRL, Gaithersburg, MD, USA; 1:400 dilution). Specimens were mounted in fluorescent mounting medium (Dako, Carpinteria, CA, USA) and imaged with a confocal laser-scanning microscope (Bio-Rad MRC 600).

Results

Successful histochemical analysis of transgenic lacZ expression relies on sufficient penetration of the substrate into the tissue and above threshold levels of β-gal activity (1). We found that histochemical X-gal staining worked very well on whole, unfixed tongue biopsies (Fig. 1a) indicating efficient X-gal penetration in this tissue and adequate enzyme levels. We, therefore, used tongue staining as a rapid, semi-quantitative assay for transgene expression (Fig. 1a). Initial attempts with X-gal staining on 10 μm tongue sections that had been fixed with paraformaldehyde or glutaraldehyde (two commonly used fixatives) were not successful and no staining was detected (data not shown). In contrast, X-gal staining of unfixed frozen tongue sections was readily detectable in high expressing animals (Fig. 1b) but staining was inconsistent in animals with low levels of transgene expression (data not shown). In 10-μm back skin sections X-gal staining was routinely found to be minimal, diffuse, and sporadic irrespective of the level of transgene expression (Fig. 1c). X-gal staining in back skin, when present, was localized to the outer root sheath of hair follicles (Fig. 1c) but did not appear to be confined to a particular region within this structure.

We then performed quantitative enzymatic assays for β-gal activity to determine whether the weak and sporadic staining in back skin was a result of poor transgene expression or due to lack of sensitivity of the X-gal staining technique. These assays revealed significant β-gal activity in back skin and tongue extracts, with levels of enzyme activity 13-fold higher for back skin and sixfold higher for tongue than control extracts (Fig. 2). In addition, Northern analysis, reverse transcription-PCR (RT-PCR), and mRNA in situ hybridization confirmed the presence of MK6a–lacZ transcripts in all K6 expressing epithelia of transgenic animals including tongue and back skin (3) (Fig. 3d). Taken together with the enzymatic assays, these data suggest that the apparently low levels of lacZ expression observed in follicles using X-gal staining is due to a limitation of the technique rather than a lack of expression in transgenic tissues.

We, therefore, decided to use an immunological approach because it had been previously reported that antibody detection of β-gal in mammalian cell lines was more sensitive than histochemical techniques (13). In addition, this approach would also permit a direct comparison (by double-label immunofluorescence) of the expression pattern of the transgenes with endogenous MK6a at the cellular level. We first tried a monoclonal β-gal antibody that had previously been used in conjunction
with a peroxidase-labeled secondary antibody (8) but in our hands was not successful because of the high level of non-specific staining in mouse tissues. We then tried several different polyclonal β-gal antibodies from commercial sources and found a rabbit antibody from Cappel-ICN (Costa Mesa, CA, USA), which was initially supplied in solution, gave the best results. However, subsequent batches were only available in a lyophilized form and immunofluorescence staining with the reconstituted antibody always resulted in high levels of non-specific staining. A second rabbit β-gal antibody (Cortex Biochem) was then used and found to work well in immunofluorescence analyses on unfixed frozen tissue sections. Both polyclonal antibodies revealed tissue-specific expression of the MK6α–lacZ transgenes in all K6 expressing tissues examined including back skin (a), tongue (Fig. 3d), footpad and nail bed [data not shown and (3)]. Importantly, immunological detection was more con-

Figure 3. Immunofluorescence analysis reveals lacZ transgene expression in back skin tissue and tongue sections. In normal, unstimulated back skin, β-gal expression (green signal) is detected in the interfollicular epidermis and in hair follicles (a and b). Simultaneous detection of endogenous MK6α (red signal in c; yellow signal in the merged image of a) reveals co-localization of the transgene product with MK6 in the outer root sheath (ORS) cells of the hair follicle. The differential expression of the transgene in the interfollicular epidermis is due to presumptive regulatory elements missing in the K6 transgene [see (3) for details]. In the tongue, the β-gal antibody detects transgene expression throughout the papillary and interpapillary epithelium (d). Anti-β-gal staining was not detected in non-transgenic tissues or in transgenic tissues where the primary antibody was omitted (not shown). Scale bars: 50 μm (a); 330 μm (b and c); 30 μm (d).
sistent than the sporadic and variable expression observed with X-gal staining protocols. Moreover, we were able to determine a difference in the expression pattern of the M6kα-lacZ transgenes compared with that of endogenous K6 in back skin, illustrating the utility of the immunofluorescence approach (Fig. 3a and compare Fig. 3b and c).

Discussion

Several investigators have used the lacZ reporter in transgenic animals to study the inducible aspects of K6 promoter activity because K6 is constitutively expressed in cultured cells (3-6-9). A feature of these studies is the variable and sporadic expression observed for the lacZ transgenes when using standard X-gal staining protocols. A study of the bovine K6β promoter reported weak and patchy transgene expression for constructs containing 4 kb or less of upstream sequences (7) and a similar study of the human K6α promoter reported only sporadic lacZ expression in fewer than 1% of hair follicles for constructs containing 5.2 kb or less of upstream sequences (9). Although these results may reflect the true expression of the bovine and human K6 transgenes in mouse skin or perhaps the sensitivity of these transgenes to position effects at the site of integration, it is also possible that the observed sporadic expression is due to inadequacies of the X-gal staining technique. Moreover, studies on other keratin promoters linked to the lacZ gene have also reported limitations with X-gal staining in transgenic tissues, particularly in keratinocytes (14,15). In instances of low expression, localization of transgenic expression may be further compromised by inactivation of β-gal in keratinocytes giving a false-negative result (14) and in cases of high expression, diffusion of the product away from the initial site of formation may result in a false-positive signal (15).

A number of modifications have been tried to overcome the limitation of X-gal detection of lacZ expression in tissue sections such as embedding tissues in plastic resin and viewing the X-gal stained sections under dark-field illumination (16) or using Bluo-gal as the substrate with visualization of the product under polarized light (4) or fixing the tissues in an ethanol and polyethylene-glycol mixture with subsequent embedding in a low melting-point paraffin in order to preserve β-gal activity (5). Although these methods reportedly increase detection of β-gal activity, they are also time consuming involving multiple dehydration and rehydration steps of the tissue prior to staining and in some protocols further processing after staining. By comparison, immunofluorescence analysis is relatively less demanding and can be used with antibodies against endogenous proteins to aid in the localization of transgene expression. Therefore, this rapid and sensitive technique should be used to confirm X-gal staining in keratinocytes, especially when variegated or mosaic expression is observed or where low expression levels are suspected.

Finally, although lacZ is currently the most popular reporter gene used in transgenic studies, green fluorescent protein (GFP) and its derivatives may become the system of choice in the future. GFP has the advantages of innate fluorescence allowing the expedient co-localization of the reporter with endogenous proteins and the unique ability for following the expression of transgenic fusion proteins in live cells.

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