Characterization of a heat resistant β-glucosidase as a new reporter in cells and mice

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Abstract

Background: Reporter genes are widely used in biology and only a limited number are available. We present a new reporter gene for the localization of mammalian cells and transgenic tissues based on detection of the bglA (SYNbglA) gene of Caldocellum saccharolyticum that encodes a thermophilic β-glucosidase.

Results: SYNbglA was generated by introducing codon substitutions to remove CpG motifs as these are associated with gene silencing in mammalian cells. SYNbglA expression can be localized in situ or detected quantitatively in colorimetric assays and can be co-localized with E. coli β-galactosidase. Further, we have generated a Cre-reporter mouse in which SYNbglA is expressed following recombination to demonstrate the general utility of SYNbglA for in vivo analyses. SYNbglA can be detected in tissue wholemounts and in frozen and wax embedded sections.

Conclusions: SYNbglA will have general applicability to developmental and molecular studies in vitro and in vivo.

Background

A fundamental technique in biological research is the use of reporter genes to track cells or tissues in developmental studies, to quantify or recognize gene expression from defined cis-regulatory elements and to normalise for differential uptake of DNA or delivery vectors in transfection experiments in vitro [1-4]. The most frequently used reporters are Escherichia coli lacZ gene encoding β-galactosidase (βgal), the green fluorescent protein (GFP) of Aequorea victoria and to a lesser degree human placental alkaline phosphatase [3,5,6]. In transgenic studies GFP tends to be the reporter of choice for studies at single cell or intracellular resolution or where viable cells need to be isolated by fluorescence activated flow sorting (FACS). Histochemical detection of lacZ is still widely used at the single cell/tissue level of resolution especially where visualization is in wholemounts of tissues or embryos. Here we present a new reporter protein for cellular and whole organism studies that is validated in vitro and by generating a Cre-reporter mouse in which the reporter is detected in histological sections following induction of Cre recombinase. This new reporter gene, termed SYNbglA, is based on the bglA gene (GenBank: Accession X12575), of the thermophilic bacterium Caldocellum saccharolyticum that encodes a β-glucosidase (βglu) thermostable to 85°C [7].

Results

A mammalian expression construct in which the sub-cloned C. saccharolyticum bglA gene is regulated from the human elongation factor 1α (EF1α) promoter was created to create pEFbglA. For comparison, lacZ was also subcloned to generate pEFlacZ. The EF1α promoter was chosen as, unlike powerful viral promoters such as the cytomegalovirus immediate early promoter (CMV IE1), it does not tend to undergo silencing over time [8]. Following transient transfection cells expressing thermostable βglu could be detected using BCI-glu in fixed cultures with or without heat-treatment (65°C for 20 min; data not shown). Colonies of NIH 3T3 cells transfected with pEFbglA and pEFlacZ were isolated. βglu expression remained detectable after heat treatment unlike βgal which was heat inactivated (Figure 1a-d). To establish if βgal and βglu can be co-localized stable clones separately expressing either reporter were derived and mixed in 1:1 ratio and cultured together for 1-2 days prior to fixation and staining with BCI-glu and Magenta-gal at 37°C. Individual cells in the co-cultures stained with one substrate
only and showed no cross-reactivity demonstrating the potential to visualize these two reporters simultaneously (Figure 1e).

After several passages, cultures derived from bglA+ clones in which all cells stained positive for βglu activity, exhibited large numbers of unstained cells. Such a ‘silencing effect’ has been reported in experiments with the E. coli lacZ gene [9]. Silencing of lacZ is ameliorated by changing its sequence to minimize the number of CpG dinucleotides that are targets for methylation in mammalian cells [9]. The bglA coding sequence contains 109 CpG dinucleotides. Consequently, we undertook to resynthesize bglA such that the nucleotide sequence was depleted for CpG dinucleotides and the codon sequence was biased towards mammalian usage. A nuclear localization signal was also added as a 5’ fusion (Figure 2a). The reporter gene thus generated was termed SYNbglA (GenBank: Accession AY528410).

SYNbglA was subcloned to generate pCMV-SYNbglA that was cotransfected with pSV2neo into 293 cells and G418 resistant clones obtained. The CMV promoter was selected as a more robust test of the resistance of SYNbglA to silencing. Four clones were expanded and all expressed the altered βglu (SYNβglu) strongly and homogeneously as evaluated by BCI-glu staining. The clones were serially passaged (cultures approaching confluence were split 1:4 to 1:8 every 2 or 3 days) and continuously evaluated for expression by staining with BCI-glu. No reduction in the proportion of cells stained or in their intensity was observed up to passage 40, the highest analysed (Figure 2b). We conclude that SYNbglA is not prone to the gene-silencing phenomenon observed with bglA and reported with unmodified lacZ [9,10].

βgal expression can be quantified by detecting cleavage of O-nitro-phenyl-galactoside conjugated sugars in colorimetric assays. We confirmed that SYNβglu expression is quantifiable with ONP-glucopyranoside (not shown). This method was then used to gain insight into the functional stability of SYNβglu compared to βgal. Both proteins were expressed inducibly in stably derived clones of 293EcR cells (engineered to express a heterodimeric transcriptional transactivator that only binds to the DNA binding domain present in an inducible promoter in the presence of Pronesterone A, an ecdysone analogue) [11]. The rate at which reporter enzyme activity decayed following withdrawal of Pronesterone A was determined by harvesting treated cells daily for 6 days. Following the cessation of induced transcription, the rate of decay for SYNβglu is similar to that of βgal, with a 50% reduction in activity observed at 2.65 days and 2.33 days, respectively, suggesting that they have similar stabilities within cells (Figure 3).

In contemporary studies of gene function in vivo, genes are not only deleted or overexpressed constitutively but conditionally mutagenized using site-specific recombi-
nases such as Cre [12,13]. Reporter lines for recombinase activity are necessary to determine the timing, spatial regulation and tissue specificity of recombinase expression [14]. Consequently, we decided to generate an alternative reporter line for assessing Cre activity by targeting the ubiquitously expressed murine ROSA26 locus with SYNbglA placed downstream of a floxed STOP cassette to create a line named R26(\textit{SYNbglA})R (Figure 4). Frozen sections and intestinal wholemounts from adult R26(\textit{SYNbglA})R and non transgenic animals were analysed for βglu activity using BCI-glu. Tissues from R26(\textit{SYNbglA})R and non transgenic animals showed identical staining patterns with all (including liver, oesophagus, bladder, pancreas, muscle and heart) except small intestine showing no staining due to background enzymatic activity. In the small intestine, in both cases, the positive staining was associated with the epithelial brushborder and could be prevented by incubation at 65°C for 20 min prior to incubation in BCI-glu (Figure 5a, b). These results indicate that there is no background expression from the targeted allele and that detectable endogenous βglu is only present in the small intestine.

In order to functionally test the R26(\textit{SYNbglA})R line it was mated to two different Cre expressing lines, PGKcre\textsuperscript{em} and Ahcre. PGKcre\textsuperscript{em} is expressed from a maternally inherited transgene that is expressed during the diploid phase of oogenesis resulting in complete recombination at loxP flanked cassettes even where PGKcre\textsuperscript{em} is not inherited [15]. Male R26(\textit{SYNbglA})R mice were crossed with PGKcre\textsuperscript{em} females and the offspring analysed around the time of weaning. Tissues were excised, fixed and stained as wholemounted tissues in BCI-glu for 48 h. Tis-
sues from age-matched mice not crossed to PGKcrem mice were also prepared. In all tissues of mice obtained from the R26(SYNbgLA)/R/PGKcrem intercross, including heart, thymus, spleen, pancreas, kidney, skeletal muscle, liver, stomach and brain, there was intense staining with BCI-glu, where there was none in the control tissue. This demonstrates that SYNβglu expression is sustained in diverse tissue types following cre-mediated activation (Figure 5c-i).

Ahcre is a mouse line in which Cre recombinase is conditionally expressed from the rat cytochrome P450 1A1 promoter in several gastrointestinal tissues, following treatment with the inducing agent β-napthoflavone [16]. The Ahcre line was originally validated with the R26R reporter line, in which lacZ is expressed from the ROSA26 locus following cre-mediated recombination, allowing the known pattern of recombination to be compared with that obtained in Ahcre/R26(SYNbgLA)R mice. Thus, Ahcre/R26(SYNbgLA)R were either left untreated or treated with five daily intraperitoneal injections of 80 mg/kg β-napthoflavone to activate transcription of cre and mediate excision of the stop cassette allowing expression of SYNbgLA (Figure 5j, k). There was very a very low level of background recombination in the target tissues of untreated adult animals (Figure 5j). After induction, there was near complete recombination with extensive expression of SYNβglu that could be detected with BCI-glu in small intestinal wholemounts (Figure 5k). In the colon of induced Ahcre/R26(SYNbgLA)R mice there was extensive recombination that was maximal proximally and became increasingly mosaic towards the anus (Figure 5l-o). In both untreated and treated mice the pattern of recombination was identical to that observed previously in Ahcre/R26R mice although overall the extent of recombination as determined by expression of SYNbgLA seems greater in more distal regions of the intestine [16].

Increasingly transgenic experiments require the simultaneous application of two or more reporter genes. We wanted to establish if SYNβglu can be co-localized with βgal in tissues. In order to achieve this we chose to analyse the intestinal epithelium and exploit the known clonality of intestinal crypts [17]. Ahcre and reporter strains R26R and R26(SYNbgLA)R were intercrossed to generate animals carrying all three modifications (Ahcre/R26R/R26(SYNbgLA)R mice). These were injected with a single dose of β-napthoflavone to induce Cre submaximally such that mosaic patterns of recombination resulted. The intestines were then analysed for expression of both βgal and SYNβglu after 12 weeks, a time by which the process of crypt monoclonal conversion is largely complete [18]. Intestinal wholemounts or cryostat sections were stained first for βgal (6 h, 37°C) and then, after heat-treatment, for SYNβglu as described in the Methods section using BCI-gal and Mag-glu, respectively. Individual and clusters of stained crypts could be clearly identified and could be related to either reporter expressed alone or occasionally both together (Figure 6a-e).

In order to determine if the introduced SYNβglu could be detected in sections processed for histology in paraffin wax blocks, different fixatives and protocols were tested. Liver, pancreas, bladder and small intestinal samples from Ahcre/R26(SYNbgLA)R mice induced with β-napthoflavone 1-6 weeks previously were fixed in various fixatives for 1-6 h, processed through an ascending series of ethanol, xylene and into paraffin wax at 65°C for embedding. Five micrometer sections were cut, dewaxed in xylene and rehydrated before incubation with BCI-glu.
at 37°C. Clear nuclear localized histochemical product was found in patterns identical to that observed with cryostat sections as described above (Figure 7a and 7b). The main determinant of staining intensity was the fixation protocol with the best results achieved after 1 h fixation with 2% formalin/0.2% glutaraldehyde.

In order to test whether or not SYNβglu can be localized with other markers, we performed immunohistochemistry specific for intestinal cell types in tissue sections already stained for BCI-glu (either for 2 days at 37°C or overnight at 65°C). Villus enterocytes were easily identified in such sections on the basis of their positive staining for villin as were intestinal goblet cells on the basis of staining for muc2 (Figure 7c and 7d).

Discussion

The βglu encoded by SYNβglA is an easily detected and stable protein that seems an ideal cell marking reporter molecule. It has potential application to cell and molecular studies where gene expression has to be localized. Its potential applications in vivo include studies during development and in the adult where gene expression from defined promoter elements have to be detected or for the fate mapping of tissues or individual cells, for example in clonal studies [19,20].

Like βgal expression levels of SYNβglu can be quantified using colorimetric substrates. Such assays are routinely used for normalizing for vector uptake in transfection experiments with the stability of βgal making it suitable for this purpose. However, such colorimetric assays are relatively insensitive and, as has been pointed out previously for βgal, chemiluminescent substrates can greatly increase sensitivity [3]. Appropriate chemiluminescent substrates are available for the detection of SYNβglu but, to date, we have not attempted to apply them. However, the ultimate limit on sensitivity for βgal is the presence of background enzymatic activity from endogenous, mammalian β-galactosidases and it is likely that the ability to destroy such background by heat inactivation will mean that SYNβglu has an enhanced sensitivity over that of βgal [21].

SYNβglu may have advantages over E. coli βgal for cellular localization in some experimental settings. The thermostability of SYNβglu allows it to be visualized in high-resolution sections from paraffin wax embedded tissues. The size of the coding sequence for SYNβglu is 1.2 kb compared to 3.1 kb for lacZ. This smaller size is advantageous for viral delivery vectors where cloning of large inserts is problematic.

The similarity of SYNβglu and βgal both in terms of processing requirements for visualization and the relative stabilities of the two proteins, together with the observation that they can be co-localized, suggests that they may be used in tandem in compound genetically modified transgenic animals where gene expression changes are being effected. The compatibility of SYNβglu and βgal for dual detection may become especially significant as transgenic analyses become more elaborate. For example, a stem cell marker gene (Lgr5) has been validated in mice by localizing stem cells using an enhanced yellow fluorescent protein targeted to the Lgr-5 locus along with a Tamoxifen activated Cre recombinase [22]. Recombination mediated by the latter is recognized in crosses to R26R mice as clones of cells expressing βgal. A recent study of neuronal function combines use of GFP and human alkaline phosphatase to allow simultaneous detection of cell bodies, neurites and presynaptic sites and envisages the potential to also detect βgal in crosses to mutant strains [23]. In this regard, SYNβglu will add to the limited platform of available reporters. The R26R(SYNbglA)R mice described here will further permit detailed analysis of the pattern of Cre activity in mouse models in which recombination is restricted to specific cell types and tissues and to different stages of development.
Conclusions
SYNβglu is an easily detected reporter protein that has a variety of applications in vitro and in vivo where cell tracking, accurate localisation and high sensitivity is required. SYNβglu may offer some advantages to the E. coli βgal but the ability to use these two reporter systems together suggests that they will complement each other and will be used in tandem. The Cre-reporter animals described here demonstrate the applicability of SYNβglu to transgenic tissues and in the analysis of Cre mediated recombination.

Methods
Plasmid cloning
The bglA sequence was initially PCR amplified from pNZ1065 (gift of Dr D Love) and subcloned into pCR3 (Invitrogen) using primers (5’ ttccatggGGATCCtaagttcccagaaaagttttggttgg 3’ and 5’ ttAGATCTgtgcctacagaatttcctttaatactg 3’) designed to introduce a consensus translation start sequence (bold) and flanking restriction sites (caps) for subsequent cloning [7,24]. This bglA fragment was subcloned into pEF1alpha [25] to generate an expression construct (pEfbglA) containing the human type 1α elongation factor promoter and SV40 polyadenylation sequence. In order to allow a comparison, the equivalent lacZ construct (pEFlacZ) was also made by conventional subcloning (cloning details available on request). The SYNbglA and lacZ cassettes were also subcloned into pIND (Invitrogen, CA, USA) containing five ecdysone response elements upstream of a Drosophila minimal promoter. Additional cloning details are available upon request.

In order to generate the final gene targeting construct, conventional cloning was performed to make a cassette comprising loxP-PGKneo.pA-loxP SYNbglA.pA which was subcloned into pROS-MCS-13 [26] containing the two arms of ROSA26 locus homology. Additional cloning details are available upon request.

Cell lines
Mouse NIH 3T3 and human 293-EcR cells were obtained from the American Type Culture Collection and Invitrogen, respectively, and were maintained in standard tissue culture media (Dulbecco’s Modified Eagle’s Medium) containing 10% fetal calf serum. Cells were transfected using the Stratagene MBS kit. Where cotransfection was required for the purpose of antibiotic selection the test plasmid was cotransfected (10:1 ratio) with pSV2neo (Clontech, CA, USA) followed by G418 selection at 1 mg/mL.

Synthesis of SYNbglA
SYNbglA was chemically synthesized by Bionexus using their proprietorial method (Bionexus Inc, CA, USA http://www.bionexus.net/)

Gene targeting
Embryonic stem cell manipulation procedures were performed by the Gene Targeting Service, Babraham Institute (Cambridge, UK). E14 129Ola ES clones were selected with G418 and colonies initially screened by polymerase chain reaction using primers anchored 5’ to the shorter targeting arm (R26TOPV: 5’ ggtaggggggtacgcattagatagagagagcc 3’) and at the introduced splice acceptor (R26SAmut: gtctcacaagggagagtgtg) which amplified a unique 4kb band. Two clones were selected following further screening by Southern blotting using a probe located 5’ to the targeting vector as described previously [26]. These clones, C8 and C10, were microinjected into blastocysts and the resultant chimeras used to establish R26(SYNbglA)R mice. Both clones were evaluated for expression of reporter following induction of Cre in double transgenic Ahcre/R26(SYNbglA)R mice and were found to behave identically.

Enzyme histochemistry
Cellular localization for lacZ in tissue culture plates was performed by routine methods [27] that were modified for detection of bglA expression as needed. Essentially, plates were washed in phosphate buffered saline (PBS) before fixation in 2% formaldehyde/0.1% glutaraldehyde in PBS. After washing with PBS plates were incubated with 0.04% conjugated sugar substrates [5-bromo-4-chloro 3-indolyl-β-D-galactopyranoside (BCI-gal, No. MB1001), 5-bromo-6-chloro-3-indolyl-β-D-galactopyranoside (Magenta-gal, No. M1141), 5-bromo-4-chloro 3-indolyl-β-D-glucopyranoside (BCI-glu, No. MB1002), all from Melford Laboratories, Suffolk, UK] or 5-bromo-4-chloro 3-indolyl-β-D-glucopyranoside (Magenta-glu, No. 31059, Glycosynth, Cheshire, UK) containing potassium ferrocyanide (4 mM), potassium ferricyanide (2 mM) and magnesium chloride (1 mM) in PBS at the temperatures stated.

Protein stability determination
The colorimetric assay for βgal and βglu was carried out using a commercially available kit (No. E2000; Promega, WI, USA) as per the manufacturers instructions, except that the substrate containing solutions [o-nitrophenyl-β-D-galactopyranoside (ONP-gal), Sigma No. N1127, o-nitrophenyl-β-D-glucopyranoside (ONP-glu), Sigma No. N8016] at a concentration of 1.33 mg/mL were prepared independently. The absorbance of the cleaved substrates at 420 nm was determined on a Tecan SpectraFluor Plus plate reader.

The commercially available ecdysone inducibility system was obtained including 293Ecr cells that stably express the VgRXR receptor (a heterodimer of ecdysone receptor and retinoid X receptor) (Invitrogen, Scotland, UK). In 293 EcR cells the heterodimer binds to an ecdysone response element in pIND in the presence of Pron...
testosterone A. Clones of 293EcR cells were obtained by selection with G418 following transfection with plNDSYnbglA or plNDlacZ that contain a neo selection cassette. Clones were screened for conditional expression of βglu and βgal, respectively, in the presence of Pronestosterone A (5 μM). For each reporter one clone was plated out at low density in 2.5 cm² replicated tissue culture wells which were incubated in media containing Pronestosterone A (5 μM) for 24 h. Triplicate wells for each clone were harvested in lysis buffer (Promega, No. 397A) at 24 h intervals after PBS wash (x3). Cells harvested at the end of Pronestosterone A treatment were designated day 0. For each lysate total protein concentration was determined using a Pierce BCA kit (PerBio, No. 23227) and the volumes analysed in the enzyme assay were normalized for total protein content.

Treatment of animals
Homozygous Ahcre mice were crossed with R26(SYNβglA)R animals and offspring carrying both transgenes selected for subsequent experiments. Ahcre mice were genotyped as described and R26(SYNβglA)R mice by polymerase chain reaction using a primer combination (5’ cagaaagtgagcaggttgcc 3’, 5’ gggatacagaagaccaatgcaga3’; 5’ tctctcaacgcgagctgtg 3’) giving a 440 bp and 350 bp for the wild type and targeted R26 loci, respectively [16]. For induction of the Ah promoter, mice received interperitoneal injections of 80 mg/kg β-napthoflavone (βNF; Sigma) dissolved in corn oil (8 mg/mL) at the frequencies stated and controls received either no treatment or corn oil only.

Tissues and immunohistochemistry
Whole tissues for BCI-glu staining were dissected from 3-week-old mice and were sliced to present a cut facet for histochemistry. Tissues were fixed in 4% paraformaldehyde for 2 h, washed in PBS and incubated in BCI-glu at 50°C for 48 h. Intestinal wholenumbs from Ahcre/R26(SYNβglA)R animals were prepared lumenal side up as described previously [16] except that they were fixed in ice cold 2% formaldehyde/0.2% glutaraldehyde in PBS (pH7.4) for 1 h prior to overnight incubation in BCI-glu (as described above) substrate at room temperature. For frozen sections small pieces of intestine were snap frozen in liquid nitrogen and cryostat sectioned. Slides were air dried, fixed for 5 minutes in ice cold 2% formaldehyde/0.2% glutaraldehyde in PBS (pH7.4) for 10 min, before transferral to BCI-glu. For heat inactivation sections were incubated in PBS at 65°C for 10-20 min prior to incubation in BCI-glu. Tissues processed for histology were immersed in the fixatives for the length of time stated and processed into paraffin wax blocks using a Citadel tissue processor (ThemoShandon, Cheshire, UK) with freshly prepared dehydrating ethanol (x1 70%, 30 min; x1 90%, 30 min; x3 100%, 30 min each), xylene (x3, 20 min each) and wax (x2, 30 min each). Sections were cut at 3-5 μm, dewaxed and rehydrated into PBS prior to immersion into BCI-glu, prepared as above, and incubated at 37°C (48 h) or 65°C (overnight).

Abbreviations
BCI-glu: 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside; BCI-glu: 5-bromo-4-chloro-3-indolyl-β-D-glucopyranoside; βgal: β-galactosidase; βglu: β-glucosidase; BNF: β-naphthoflavone; G418: green fluorescent protein; Magenta-gal: 5-bromo-6-chloro-3-indolyl-β-D-galactopyranoside, Magenta-glu: 5-bromo-6-chloro-3-indolyl-β-D-glucopyranoside; PBS: phosphate buffered saline; SYNβglu: synthesized β-glucosidase

Authors’ contributions
SMC performed the experiments shown in Figures 1 and 2. KJ performed the experiments resulting in Figure 3. SAC performed the mouse genotyping. LAH performed the histochemistry and histology. RK performed the data analysis and participated in the experimental design and manuscript preparation. DJW conceived the study and participated in its design, coordination and drafting of the manuscript. All authors have read and approved the manuscript.

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