

ARTICLE

A Probasin-MerCreMer BAC Allows Inducible Recombination in the Mouse Prostate

Andreas Birbach,^{1,2*} Emilio Casanova,² and Johannes A. Schmid¹

¹Department of Vascular Biology and Thrombosis Research, Medical University of Vienna, Vienna, Austria

²Ludwig Boltzmann Institute for Cancer Research, Vienna, Austria

Received 11 May 2009; Revised 23 July 2009; Accepted 25 July 2009

Summary: Tissue-specific transgene expression in the prostate epithelium has previously been achieved using short prostate-specific promoters, rendering transgenic mouse lines susceptible to integration site-dependent effects. Here we demonstrate the applicability of bacterial artificial chromosome (BAC) technology to transgene expression in the prostate epithelium. We present mouse lines expressing an inducible Cre protein (MerCreMer) under the control of regulatory elements of the probasin gene on a BAC. These mouse lines show high organ specificity, high transgene expression in anterior, dorsal and lateral prostate lobes, no background Cre recombination using a reporter strain and adjustable amounts of Cre-induced recombination upon tamoxifen induction. Together with two recently reported transgenic lines expressing the Cre-ERT2 protein from small prostate-specific promoters, these mouse lines will be useful in research focused on prostate-specific disorders such as benign hyperplasia or cancer. *genesis* 00:1–8, 2009. © 2009 Wiley-Liss, Inc.

Key words: prostate; MerCreMer; bacterial artificial chromosome; transgenic mouse; cancer research

INTRODUCTION

Animal models of prostate-specific disorders are used in basic research to monitor disease progression and in pre-clinical applications to evaluate treatment options. However, current mouse models of, for instance, prostate cancer do not appropriately reflect the human disease (Ahmad *et al.*, 2008). Among points of concern, the following could be solved by improving transgenic mouse technology:

1. Transgenic mouse lines express a transgene or delete an endogenous gene (transgenic/knock-out approach) in a high number of cells in the prostate epithelium. However, human prostate carcinoma likely develops from a few modified cells at different locations, resulting in the multifocal growth of tumors (Miller and Cygan, 1994; Ruijter *et al.*, 1996).

2. Depending on the type of promoter used, transgene expression starts either during mouse development or in the young adult (Cleutjens *et al.*, 1997; Zhang *et al.*, 2000). However, prostate cancer models reflecting a disease of the elderly may benefit from expression onset in older animal.
3. In the human-being, the prostate is a single structure encircling the urethra. This is not reflected in rodents, where four pairs of bilateral lobes called anterior, ventral, lateral and dorsal lobes constitute the prostate (Ahmad *et al.*, 2008). Although some publications refer to one or the other lobe as “most comparable to human prostate”, a consortium has declared that there is currently no data supporting these claims and thus it is still unclear whether one, if any, of the distinct lobes represent a better “model” of the human prostate (Shappell *et al.*, 2004). There are considerable differences in size between the murine prostate lobes, with the anterior lobe being the largest and the lateral prostate the smallest lobe. However, currently used prostate promoters express highest in dorsal or lateral or ventral lobes (Cleutjens *et al.*, 1997; Wu *et al.*, 2001; Zhang *et al.*, 2000). A promoter with high expression in the anterior lobe could significantly facilitate daily work with mouse models, especially for assays for which more starting material is required.

The Cre/loxP system has been widely applied in transgenic mouse research to generate both tissue-specific knockout animals and achieve tissue-specific transgene

Additional Supporting Information may be found in the online version of this article.

* Correspondence to: Dr. Andreas Birbach, Medical University of Vienna, Department of Vascular Biology and Thrombosis Research, Schwarzschanerstrasse 17, A-1090 Vienna, Austria.

E-mail: andreas.birbach@meduniwien.ac.at

Contract grant sponsor: Ludwig Boltzmann Gesellschaft.

Published online in

Wiley InterScience (www.interscience.wiley.com).

DOI: 10.1002/dvg.20558

expression. For the mouse prostate, many mouse lines have been established based on Probasin-Cre mouse lines expressing a constitutive form of Cre recombinase from a small composite promoter (ARR2PB) (Wu *et al.*, 2001). These mouse lines express Cre, starting from young adulthood, in many cells of the different lobes, with rather low expression in the anterior lobe, documenting the concerns mentioned above. Recently, some progress has been made with the generation of two different transgenic lines, one expressing the inducible Cre protein Cre-ERT2 from the ARR2PB promoter in a knock-in approach, the other expressing the same protein from the human prostate-specific antigen (PSA) promoter (Luchman *et al.*, 2008; Ratnacaram *et al.*, 2008).

Here we present a novel approach using a bacterial artificial chromosome (BAC) containing most if not all of the regulatory elements of the highly prostate-specific mouse probasin gene. BAC transgenics have been used to reliably mimic the expression of endogenous genes and be independent of positional effects using small promoters (Giraldo and Montoliu, 2001). In this work, we have used the BAC to express the MerCreMer protein, an inducible Cre protein shown to have very low background activity and high controllability (Verrou *et al.*, 1999; Zhang *et al.*, 1996).

RESULTS

Generation of the Expression Construct and Transgenesis

We selected the mouse probasin gene based on its highly specific prostate expression in database entries and confirmed by the literature (Greenberg *et al.*, 1994; Su *et al.*, 2002). A BAC containing the full-length gene and more than 190 kb of 5' as well as more than 35 kb of 3' flanking sequence was chosen to be the backbone of the transgenic expression unit (Fig. 1a). We used Red-ET cloning to insert the MerCreMer coding sequence at the translational start of the probasin gene (Casanova *et al.*, 2001; Zhang *et al.*, 1998). The resulting BAC construct was linearized and purified for subsequent microinjection into oocytes. Of more than 40 offsprings, three transgene-positive mice were identified and two of them characterized in more detail.

Expression of the Inducible Cre Protein in Transgenic Mice

Since both wild-type male mice (1 copy) and transgenic male mice (x copies) contain the probasin flanking DNA (X-chromosomally in wild-type mice), copy number was determined using quantitative PCR and an amplicon located in the probasin 5' region (Fig. 1b). One line (line 2) contained 3 ± 1 copies of probasin DNA, i.e. one to three copies of the transgenic BAC, while another line (line 14) contained 10.7 ± 1.7 copies of probasin DNA, i.e., 8–11 transgenic BAC copies. MerCreMer expression in these lines was first monitored via quantitative RT-PCR to determine tissue specificity (Fig.

1c). Good expression of the Cre fusion protein was seen in all four prostate lobes, with lowest expression in ventral prostate. However, high Cre RNA was also detected in the testis. This aberrant expression using a prostate specific promoter could result from nonspecific RNA synthesis detected by cDNA production using random priming. In line with this, a Cre antibody did not detect MerCreMer protein in the testis (Fig. 1e). More importantly, functional studies indicated no recombination in the testis (Fig. 2, Supporting Information Fig. 1). MerCreMer is an inducible Cre protein which translocates to the nucleus upon ligand stimulation of the murine estrogen receptor (Mer) by tamoxifen derivatives. Tamoxifen-inducibility of the fusion protein was demonstrated by staining transgenic prostate sections against Cre after five rounds of intraperitoneal tamoxifen (1 mg) injections into the mice (Fig. 1d). Prostates from tamoxifen-treated mice showed nuclear Cre protein in a subset of epithelial cells, whereas only diffuse cytosolic staining was observed in prostates from vehicle-treated mice.

Cre-Induced Recombination in a Reporter Strain

Next, we aimed to test *in vivo* recombination efficiency of our lines expressing the inducible Cre protein. To this end, the mice were bred with reporter mice which can be induced to express green fluorescent protein (GFP) upon Cre-mediated excision of a Stop cassette containing chloramphenicoltransferase (CAT). Transgene expression in the reporter mice is under the control of the ubiquitous CAGGS promoter and overall Stop cassette excision using a Cre deleter strain has been shown to result in high GFP fluorescence in all tissues tested (Kawamoto *et al.*, 2000).

We activated the MerCreMer protein in mice by five consecutive intraperitoneal tamoxifen injections (1 mg each) on consecutive days, a protocol often used for inducible Cre strains (Metzger and Chambon, 2001). First we analyzed GFP mRNA expression in a panel of tissues including Cre mRNA-positive prostate lobes and testis. GFP was expressed only in prostate lobes, with no background expression in either testis or another control organ (Fig. 2a). Secondly, these data were backed up by FACS analysis of different organs (Fig. 2b). GFP fluorescence was seen in all prostate lobes, with anterior, dorsal and lateral prostate showing a significantly higher number of fluorescent cells than the ventral lobe (Supporting Information Fig. 1). Again, no significant fluorescence was found in the testis (Supporting Information Fig. 1). In line with the copy number of transgenic BACs, line 14 showed a much higher number of fluorescent cells indicating Cre-mediated recombination in more cells than line 2 (Fig. 2b). No fluorescent cells were observed in prostates from vehicle-injected mice, indicating low or no background.

In order to obtain data on the tissue distribution of cells showing Cre-mediated recombination, cryosections from tamoxifen-injected and control mouse prostates were cut. GFP fluorescence could be directly

observed after embedding, with Hoechst 33342 (bis-benzimide) counterstaining indicating the number of cells (nuclei) in the samples. In prostates from tamoxifen-treated line 14 animals, GFP fluorescence was observed in a large number of cells, highlighting the gland structure. In tamoxifen-treated line 2 animals, GFP-positive cells were sparsely distributed over the prostate glands, in line with the FACS data. No GFP fluorescence was observed in animals which had been injected with vehicle alone (Fig. 2c, Supporting Information Fig. 2).

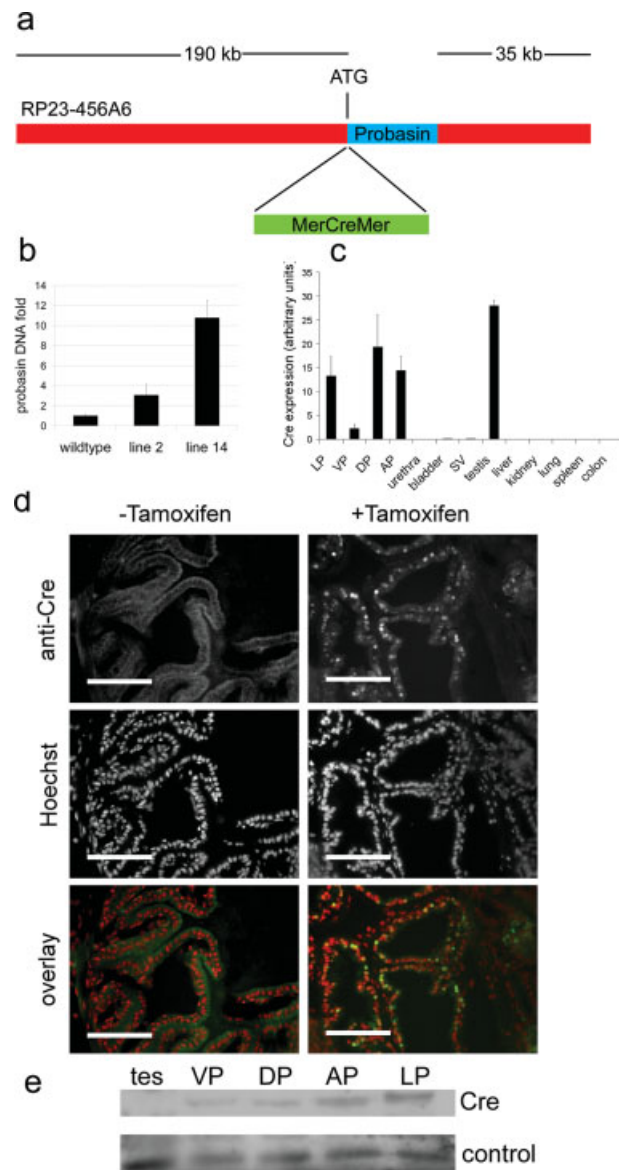
Again, the anterior, dorsal and lateral prostate lobes had higher recombination efficiencies than the ventral lobe, in line with Cre expression levels. In line 14 animals using the aforementioned injection protocol, about 18–25% of ventral prostate luminal epithelial cells were GFP-positive, while the other three lobes had recombination efficiencies between 45 and 60%. Using the same protocol in line 2, GFP positive luminal epithelial cells ranged between 2.5% (ventral lobe) and 20% (lateral lobe) (see quantification in Fig. 4b). Histological observation of the prostate gland following tamoxifen treatment showed normal tissue architecture (Supporting Information Fig. 3).

Recombination Occurs in Luminal Epithelial Cells

In order to determine the cell type specificity of Cre-mediated recombination, we analyzed the tissue section data in more detail. Probasin is a protein secreted by the luminal epithelial cells of the prostate and thus transgenic expression from a probasin expression cassette is expected to yield luminal epithelial expression only. We concentrated on areas with high stromal content on our sections, distinguished from the epithelium by the distinct structure

(glandular epithelium with infoldings indicates epithelium, less organized tissue in between glands indicates stroma). We could not find any expression in stromal cells, although the epithelium was highly fluorescent in our tamoxifen-treated animals (Fig. 3a). In the epithelium, two cell types occur in every prostate gland: Basal epithelial cells can be readily identified by their flat, elongated shape and elongated nuclei stretching along the basement membrane, in contrast to the tall, cuboid-shaped, round nuclei-containing luminal epithelial cells. Using these criteria, we could show that basal cells do not express any GFP in the tamoxifen-treated MerCreMer/reporter animals (Fig. 3a). Identity of basal cells was further confirmed by staining against basal cell-specific cytokeratin 14, which did not show overlay with GFP staining (Fig. 3b). Immunohistochemistry against another basal cell marker, p63, confirmed that elongated nuclei distinct from

FIG. 1. Cloning and expression analysis of a prostate-specific BAC expression unit expressing the inducible MerCreMer protein. **a**, a BAC (RP23-456A6) carrying the exons as well as 5' and 3' flanking regions of the mouse probasin gene was modified by homologous recombination/ET-cloning to insert the MerCreMer cDNA at the translational start (ATG) of the probasin gene. **b**, probasin DNA copy number was determined using quantitative real-time PCR. Line 14 contains several (8–10) copies of the transgenic BAC, while line 2 represents a single or low-copy number integrant ($n = 3$ animals per genotype). **c**, Cre expression in various organs of male transgenic mice. MerCreMer mRNA levels were determined using Cre-specific primers in quantitative real-time PCR. Line 14 animals express Cre in all prostate lobes (AP, anterior prostate; VP, ventral prostate; LP, lateral prostate; DP, dorsal prostate), with mRNA also detectable in testis ($n = 3$). Line 2 animals show a similar pattern with Cre expression levels being ca. 100-fold lower (not shown). **d**, MerCreMer expressing animals translocate Cre to the nucleus upon tamoxifen induction. Line 14 animals received five i.p. injections of 1 mg tamoxifen each on consecutive days and were sacrificed 2 hours after the final tamoxifen boost. Cryosections were stained for Cre and nuclei counterstained with Hoechst 33342. **e**, Western Blot shows staining for MerCreMer protein with anti-Cre antibody in samples of testis and the prostate lobes (upper panel). Lower panel shows loading control (actin). Error bars in quantifications represent standard deviations. Scale bars, 100 μ m.



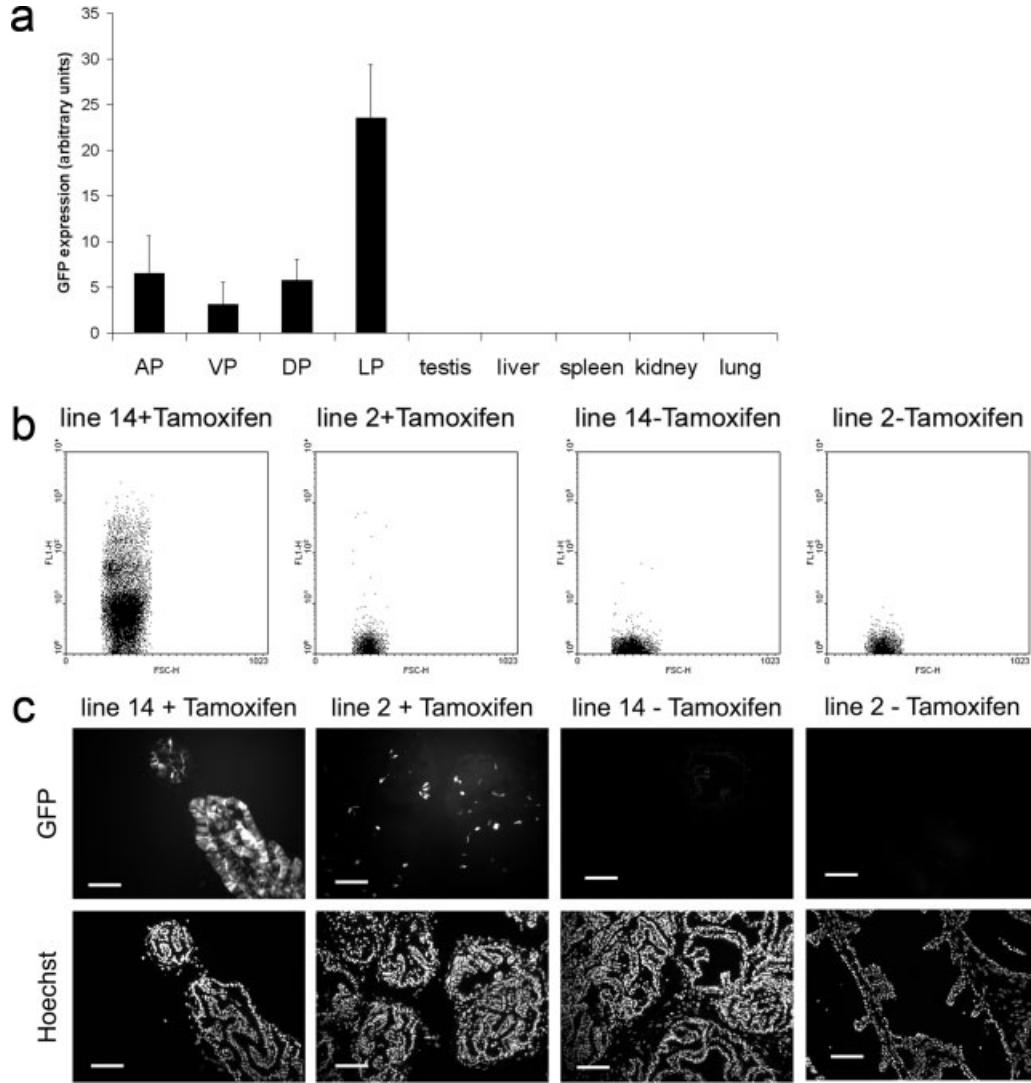


FIG. 2. Functional recombination in ProbasinBAC-MerCreMer mouse lines. **a**, GFP mRNA quantification in PbBAC-MCM line14/CAG-CAT-EGFP double transgenic mice after tamoxifen induction. High expression in lateral prostate is due to high GFP levels in individual cells rather than higher numbers of recombined cells (cf. Fig. 3). **b**, FACS analysis of anterior prostates from double transgenic mice of both line 14 and line 2, tamoxifen-induced and non-induced. GFP fluorescence (FL1 channel) is plotted on the ordinate in logarithmic scale. **c**, cryosections of anterior prostates from double transgenic mice of line 14 and line 2, tamoxifen-induced and noninduced. Scale bars, 100 μ m.

the ones filled with GFP belong to basal cells (Fig. 3c). In total, we counted more than 5,000 cells including 500 stromal and 350 basal cells and never found a single positive stromal or basal cell.

Regulating Recombination Efficiency by Tamoxifen Levels

Having demonstrated that our two lines show a high and low degree of recombination, respectively, we wanted to investigate whether the recombination efficiency could be regulated by tamoxifen levels. To this end, we compared the previously applied protocol (five injections of 1 mg tamoxifen each on consecutive days) to a simple protocol (a single tamoxifen injection of 1

mg) in double transgenic line 14/reporter animals. Analysis of prostate gland fluorescence using cryosections revealed that the single injected animals showed significantly less cells having undergone Cre-mediated recombination than the animals receiving five rounds of tamoxifen injections (Fig. 4a). Quantification of GFP-positive cells in these animals showed that single injected line 14 animals had recombination efficiencies comparable to line 2 animals receiving five rounds of injection, ranging from 0.6% (ventral lobe) to 15% (lateral lobe) (Fig. 4b). On the other hand, we attempted to further enhance recombination efficiency by increasing the tamoxifen dose to 2.5 mg per injection. However, this higher dose resulted in significant toxicity in our mice and is not recommended.

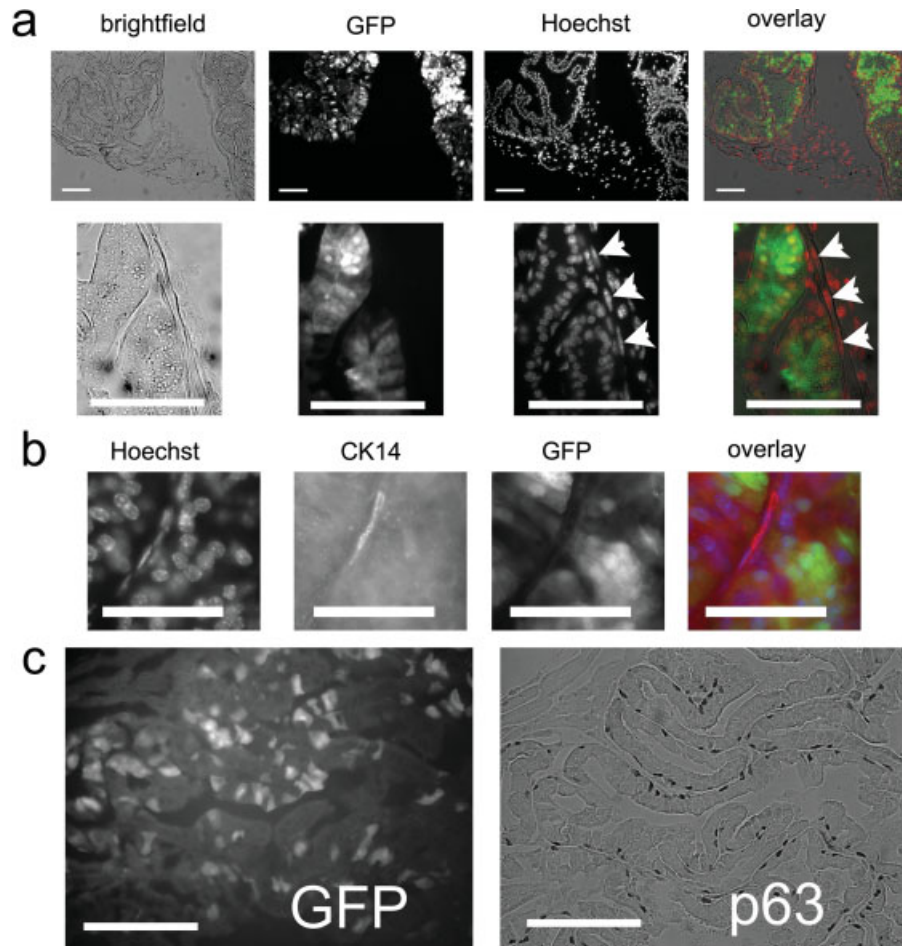


FIG. 3. Recombination is restricted to luminal epithelial cells. **a**, cryosection analysis shows luminal-epithelial specific expression of GFP. Images from double-transgenic line 14 anterior prostates are shown. Upper panel, in regions containing stroma (in the middle between epithelial glands), GFP fluorescence is restricted to prostate glands. Lower panel, within glands, basal cells lining the basal membrane (arrowheads) are negative for GFP. Scale bars, 100 μm . **b**, basal cells in the sections were stained with anti-Cytokeratin 14 antibody. Staining is restricted to regions of flat, elongated cells not overlapping with GFP fluorescence. Overlay: CK14 red, GFP green, Hoechst blue. Scale bars, 50 μm . **c**, Left, section from double transgenic anterior prostate showing GFP fluorescence and digitally enhanced background to visualize tissue organization. Right, DAB staining (black) in the consecutive section (6 μm thickness) showing the corresponding detail. DAB staining is restricted to basal cells at the gland border, not present in GFP positive tall, luminal epithelial cells. Scale bars, 100 μm .

DISCUSSION

The approach using a BAC containing regulatory elements of a prostate-specific gene resulted in high levels of transgene expression in the prostate, making the BAC backbone a valuable resource for other transgenic mouse lines. The high expression in the anterior prostate is in contrast to small promoters currently in use and will be useful for various assays. We had previously attempted to express MerCreMer from a small, prostate-specific promoter in transgenic mice in our lab. Three lines which showed Cre RNA expression in the prostate lobes were bred with reporter mice and Cre activity induced in the same manner described in this work, but no recombination was found (Supporting Information Fig. 4). In contrast to this, all three lines we obtained

from the pronuclear injection of the BAC construct showed recombination when tested with reporter mice (the third line is not shown here, as it was discontinued after initial characterization). This suggests that this BAC construct has potential for the creation of other transgenic animals with prostate-specific expression.

The inducible Cre lines using MerCreMer in this study showed no background recombination and regulatable recombination efficiencies using tamoxifen titration. Recombination efficiency was higher than 50% in some lobes using consecutive tamoxifen injections. On the other hand, very low levels of tamoxifen can induce recombination in only a few cells in the prostate gland, mimicking genetic events in different locations such as in multifocal prostate cancer. Additionally, the inducible Cre lines allow gene deletion or transgene expression by

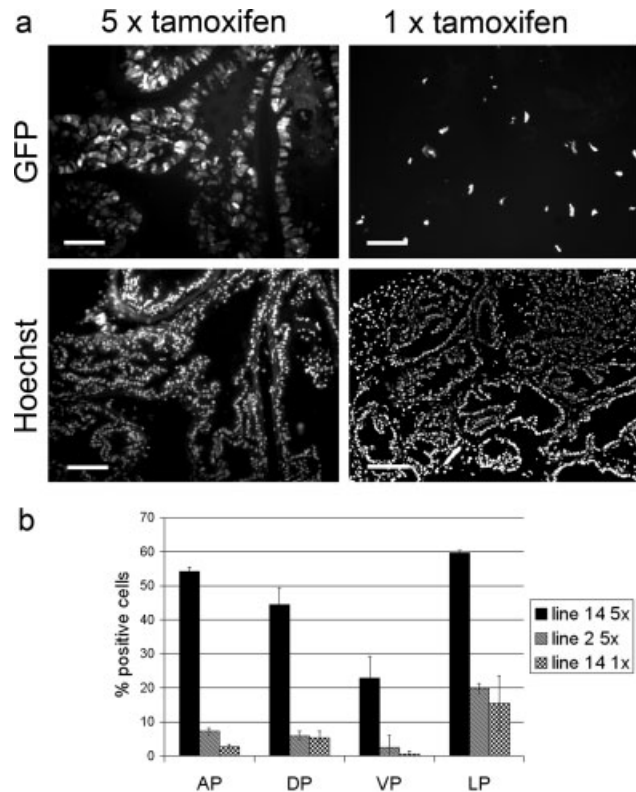


FIG. 4. Recombination efficiency is adjustable. **a**, double transgenic mice P_bBAC-MCM line14/CAG-CAT-EGFP received either five consecutive injections of 1 mg tamoxifen over 5 days or a single 1 mg tamoxifen injection. Cryosections from lateral prostates are shown. **b**, quantification of GFP-positive luminal epithelial cells from double transgenic mice of line 2 or line 14, the latter receiving either five or a single tamoxifen injections. Quantifications from three mice of each group, representing 2,000–3,000 cells of each animal lobe are shown. Error bars represent standard error of mean. Scale bars, 100 μ m.

deletion of a Stop cassette at a certain time defined by the experimenter, rendering it useful for experiments where timelines need to be followed closely or specific age-related phenomena are investigated.

Recently, two other prostate-specific inducible Cre deleter strains have been described in the literature. Both of these use the Cre-ERT2 protein in contrast to our usage of MerCreMer. One line uses the PSA promoter and was described after breeding it to mice harboring a floxed PTEN allele, yielding a model of invasive prostate carcinoma (Ratnacaram *et al.*, 2008). As no breeding with simple reporter mice has been described, it is difficult to judge the recombination efficiency on a cellular level in these mice. Another line uses the small composite ARR2PB promoter, but in a knock-in approach in the endogenous HPRT locus (Luchman *et al.*, 2008). This approach circumvents the problem of integration site-dependent expression levels faced often by the use of small constructs. The authors report complete recombination in the prostate following Cre activity induction by the more active 4-hydroxytamoxifen. In our work, we have used MerCreMer, which had only

been successfully used in two other genetically engineered mouse lines (Sohal *et al.*, 2001; Tannour-Louet *et al.*, 2002). This could indicate that MerCreMer may be less inducible *in vivo* and may need high expression levels to be effective. On the other hand, MerCreMer protein is very tight, leading to no or very low background expression in the absence of ligand stimulation. This is confirmed in our mouse model.

METHODS

BAC Recombination

The BAC clone RP23-456A6 containing the probasin gene and 5' as well as 3' flanking sequences was purchased from the BACPAC resources center (Children's Hospital Oakland Research Institute, Oakland, CA). The 5' and 3' homology regions (HR) were amplified using primer sequences 5'-GAAACATGGGATAGG CACTGGG CATTG-3' (5'HR sense), 5'-CGTGGATCTCC CATCATGT GTGAGCGACTGTCTC-3' (5'HR antisense), 5'-GACGTT AATTAAATCCTCCTGCTCACACTGCAT-3' (3'HR sense), 5'-GATCGCTAGCCCAAGTTCCAGAACAT TCGTTTC-3' (3'HR antisense) into the plasmid pIntron-polyAfrtNotIAmp (Casanova *et al.*, 2001) using the restriction sites within the oligos. The 5'HR antisense oligo already contained the start codon and following nucleotides of MerCreMer instead of the corresponding nucleotides of probasin. MerCreMer was then cloned into the plasmid by overlapping PCR. A linear fragment from the plasmid was used for electroporation of RP23-456A6 containing bacteria and recombination procedures performed following ET cloning (Casanova *et al.*, 2001; Zhang *et al.*, 1998). Recombined BACs were confirmed by Southern blotting, and the selection cassette (Ampicillin) was excised by transient expression of an F₁p recombinase plasmid.

BAC Purification, Microinjection, and Animals

BAC DNA was prepared using NucleoBond BAC 100 kit (Macherey-Nagel). DNA (40 μ g) were linearized by NotI digestion, and purified on a self-made column filled with Sepharose CL-4b (Pharmacia) which had been pre-equilibrated with injection buffer (10 mM Tris/HCl pH7.4, 0.1 mM EDTA, 100 mM NaCl) (Yang *et al.*, 1997). Fractions of 0.5 ml were taken and analyzed using pulsed field gel electrophoresis. Fractions containing only the BAC and no vector backbone were selected for microinjection. Pronuclear microinjection into C57BL/6 oocytes was performed at the University of Veterinary Medicine Vienna. The resulting positive founder animals (ProbasinBAC-MerCreMer line 2 and line 14) were either crossed further with C57BL/6 animals or bred with the reporter line CAG-CAT-EGFP on a C57BL/6 background. CAG-CAT-EGFP animals were a gift from Prof. Jun-ichi Miyazaki (Kawamoto *et al.*, 2000). For determining recombination efficiency in the ARR2PB-MerCreMer mice described in the Supporting Information, animals were bred with Z/EG mice (Novak *et al.*, 2000).

Copy Number and mRNA Expression Analysis

For quantification of BAC copy number, mouse tail biopsy DNA from F1 generation mice was purified by Phenol/Choloroform extraction and 50 ng were used as template. Probasin 5' flanking DNA was amplified using primers 5'-CAGAACCAGGGCACTCACAGTAG-3' and 5'-GGAACCCAAACAGCACCTCCTATTGTC-3'. DNA template amount was controlled for by amplification of Rosa26 DNA with primers 5'-CCAGATGACTACCTATCC TC-3' and 5'-GAGCTGCAGTGGAGTAGGCG-3'.

RNA quantification was done following RNA purification using the RNeasy Mini Kit (Qiagen). cDNA was prepared using the RevertAid H Minus First Strand cDNA synthesis kit (Fermentas). For quantification of MerCreMer expression, Cre was amplified from cDNA using primers 5'-GTACTGACGGTGGGAGAATGT-3' and 5'-ATTCGGATCATCAGCTACACC-3'. For quantification of GFP expression, primers 5'-GTTTCATCTGCACCACCGG CAAGCTG-3' and 5'-CGAGCTGCACGCTGCCGTCCTC-3' were used. Then 18S rRNA amplified by primers 5'-A CCGCGTTCTATTTTGTGG-3' and 5'-CGCCGGTCCAA GAATTTCA-3' was used as normalization reference.

Cre Recombinase Induction in Animals

Male animals (ProbasinBAC-MerCreMer or Probasin-BAC-MerCreMer/CAG-CAT-EGFP) were injected at 9-11 weeks postnatally. Five injections of 1 mg each, separated by 24 hours each were given intraperitoneally. In some experiments (see Fig. 3), a single injection of 1 mg was used. Tamoxifen was dissolved in sunflower seed oil (Fluka) at 10 mg/ml, using 0.1 ml per injection. For vehicle control, animals were injected with sunflower seed oil only.

Fluorescence Activated Cell Sorting (FACS)

Animals were sacrificed 4-6 days after the last injection to allow for high GFP expression levels. Prostate lobes and control organs were separated under a dissection microscope and grinded manually, followed by filtering through a 70- μ m nylon filter (Cell Strainer; Becton Dickinson). FACS analysis was done using a Becton Dickinson FACScan with 488 nm laser excitation and a 530/30 nm bandpass filter. Intact cells were gated for using forward/side scatter gates.

Cryosectioning and Embedding

Animals were sacrificed 4-6 days after the last injection to allow for high GFP expression. Prostate lobes were separated under a dissection microscope and fixed for 2 hours at 4°C in neutral phosphate-buffered 4% paraformaldehyde. Organs were then transferred to 20% sucrose in phosphate-buffered saline (PBS), stored overnight at 4°C, and shock-frozen in isopentane/liquid nitrogen. Samples were stored at -80°C until cutting. Before cutting, samples were equilibrated to -20°C and embedded in Tissue-Tek (Sakura Finetek). Eight-micrometer sections were cut using a Microm HM500 OM Cryostat and mounted onto SuperFrost slides (Thermo Scientific).

Slides were dried for 2 hours at room temperature, washed several times in PBS, and Hoechst 33342 (bis-benzimide; Sigma) was included in the last but one wash at 100 ng/ml to counterstain nuclei. Samples were embedded in PBS/glycerol (1:7), coverslipped, and sealed with nailpolish.

Immunohistochemistry

For Cre staining, animals were sacrificed 2 hours after the last injection to maximize MerCreMer nuclear translocation. Fixation and cutting were done as described above. For staining, samples on slides were fixed/denatured/permeabilized for 6 minutes in precooled (-20°C) acetone at room temperature, blocked with 5% goat serum in PBS +0.1% Tween 20, and incubated with polyclonal anti-Cre antibody (Kellendonk *et al.*, 1999) diluted 1:1,000. Secondary antibody was either Alexa488 or Alexa555-coupled anti rabbit antibody (Molecular Probes), diluted 1:2000. Counterstaining and mounting was done as described in the previous section. For cytokeratin 14 staining, anti-keratin 14 monoclonal antibody LL001 (a gift from Prof. Birgit Lane, University of Dundee, Scotland) (Purkis *et al.*, 1990) at 1:5 dilution was used, followed by secondary antibody phycoerythrin-anti-mouse (Sigma P9670) at 1:40. Monoclonal anti-p63 antibody (clone 4A4, Sigma P3737, dilution 1:100) for anti-p63 staining was used following antigen retrieval with citrate buffer (BioGenex) which destroyed GFP fluorescence. Diaminobenzidine (DAB) staining was done after secondary antibody treatment (polyvalent secondary antibody, ID Labs) using DAB tablets (Sigma D4293).

ACKNOWLEDGMENTS

The authors thank Thomas Kolbe for oocyte injection, Prof. Jun-ichi Miyazaki for the gift of CAG-CAT-EGFP mice, Prof. Birgit Lane for the LL001 antibody, and Bernd R. Binder for critically reading the manuscript.

LITERATURE CITED

- Ahmad I, Sansom OJ, Leung HY. 2008. Advances in mouse models of prostate cancer. *Expert Rev Mol Med* 10:e16.
- Casanova E, Fehsenfeld S, Mantamadiotis T, Lemberger T, Greiner E, Stewart AF, Schutz G. 2001. A CamKII α iCre BAC allows brain-specific gene inactivation. *Genesis* 31:37-42.
- Cleutjens KB, van der Korput HA, Ehren-van Eekelen CC, Sikes RA, Fasciana C, Chung LW, Trapman J. 1997. A 6-kb promoter fragment mimics in transgenic mice the prostate-specific and androgen-regulated expression of the endogenous prostate-specific antigen gene in humans. *Mol Endocrinol* 11:1256-1265.
- Giraldo P, Montoliu L. 2001. Size matters: Use of YACs, BACs and PACs in transgenic animals. *Transgenic Res* 10:83-103.
- Greenberg NM, DeMayo FJ, Sheppard PC, Barrios R, Lebovitz R, Finegold M, Angelopoulou R, Dodd JG, Duckworth ML, Rosen JM. 1994. The rat probasin gene promoter directs hormonally and developmentally regulated expression of a heterologous gene specifically to the prostate in transgenic mice. *Mol Endocrinol* 8:230-239.
- Kawamoto S, Niwa H, Tashiro F, Sano S, Kondoh G, Takeda J, Tabayashi K, Miyazaki J. 2000. A novel reporter mouse strain that expresses

- enhanced green fluorescent protein upon Cre-mediated recombination. *FEBS Lett* 470:263-268.
- Kellendonk C, Tronche F, Casanova E, Anlag K, Opherk C, Schutz G. 1999. Inducible site-specific recombination in the brain. *J Mol Biol* 285:175-182.
- Luchman HA, Friedman HC, Villemaire ML, Peterson AC, Jirik FR. 2008. Temporally controlled prostate epithelium-specific gene alterations. *Genesis* 46:229-234.
- Metzger D, Chambon P. 2001. Site- and time-specific gene targeting in the mouse. *Methods* 24:71-80.
- Miller GJ, Cygan JM. 1994. Morphology of prostate cancer: The effects of multifocality on histological grade, tumor volume and capsular penetration. *J Urol* 152:1709-1713.
- Novak A, Guo C, Yang W, Nagy A, Lobe CG. 2000. Z/EG, a double reporter mouse line that expresses enhanced green fluorescent protein upon Cre-mediated excision. *Genesis* 28:147-155.
- Purkis PE, Steel JB, Mackenzie IC, Nathrath WB, Leigh IM, Lane EB. 1990. Antibody markers of basal cells in complex epithelia. *J Cell Sci* 97:39-50.
- Ratnacaram CK, Teletin M, Jiang M, Meng X, Chambon P, Metzger D. 2008. Temporally controlled ablation of PTEN in adult mouse prostate epithelium generates a model of invasive prostatic adenocarcinoma. *Proc Natl Acad Sci USA* 105:2521-2526.
- Ruijter ET, van de Kaa CA, Schalken JA, Debruyne FM, Ruiters DJ. 1996. Histological grade heterogeneity in multifocal prostate cancer. Biological and clinical implications. *J Pathol* 180:295-299.
- Shappell SB, Thomas GV, Roberts RL, Herbert R, Ittmann MM, Rubin MA, Humphrey PA, Sundberg JP, Rozenfurt N, Barrios R, Ward JM, Cardiff RD. 2004. Prostate pathology of genetically engineered mice: Definitions and classification. The consensus report from the Bar Harbor meeting of the Mouse Models of Human Cancer Consortium Prostate Pathology Committee. *Cancer Res* 64:2270-2305.
- Sohal DS, Nghiem M, Crackower MA, Witt SA, Kimball TR, Tymitz KM, Penninger JM, Molkentin JD. 2001. Temporally regulated and tissue-specific gene manipulations in the adult and embryonic heart using a tamoxifen-inducible Cre protein. *Circ Res* 89:20-25.
- Su AI, Cooke MP, Ching KA, Hakak Y, Walker JR, Wiltshire T, Orth AP, Vega RG, Sapinoso LM, Moqrich A, Patapoutian A, Hampton GM, Schultz PG, Hogenesch JB. 2002. Large-scale analysis of the human and mouse transcriptomes. *Proc Natl Acad Sci USA* 99:4465-4470.
- Tannour-Louet M, Porteu A, Vaulont S, Kahn A, Vasseur-Cognet M. 2002. A tamoxifen-inducible chimeric Cre recombinase specifically effective in the fetal and adult mouse liver. *Hepatology* 35:1072-1081.
- Verrou C, Zhang Y, Zurn C, Schamel WW, Reth M. 1999. Comparison of the tamoxifen regulated chimeric Cre recombinases MerCreMer and CreMer. *Biol Chem* 380:1435-1438.
- Wu X, Wu J, Huang J, Powell WC, Zhang J, Matusik RJ, Sangiorgi FO, Maxson RE, Sucov HM, Roy-Burman P. 2001. Generation of a prostate epithelial cell-specific Cre transgenic mouse model for tissue-specific gene ablation. *Mech Dev* 101:61-69.
- Yang XW, Model P, Heintz N. 1997. Homologous recombination based modification in *Escherichia coli* and germline transmission in transgenic mice of a bacterial artificial chromosome. *Nat Biotechnol* 15:859-865.
- Zhang J, Thomas TZ, Kasper S, Matusik RJ. 2000. A small composite probasin promoter confers high levels of prostate-specific gene expression through regulation by androgens and glucocorticoids in vitro and in vivo. *Endocrinology* 141:4698-4710.
- Zhang Y, Buchholz F, Muirers JP, Stewart AF. 1998. A new logic for DNA engineering using recombination in *Escherichia coli*. *Nat Genet* 20:123-128.
- Zhang Y, Riesterer C, Ayrall AM, Sablitzky F, Littlewood TD, Reth M. 1996. Inducible site-directed recombination in mouse embryonic stem cells. *Nucleic Acids Res* 24:543-548.