Elimination of background recombination: somatic induction of Cre by combined transcriptional regulation and hormone binding affinity

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ABSTRACT

Somatically inducible Cre lines are used extensively to study gene function. However, a background level of spontaneous recombination due to unregulated expression of Cre is particularly confounding for cancer models in which following the pathogenesis of the disease requires the introduction of sporadic mutations that are monitored over time. In three transgenic mouse lines, two with Cre activity controlled at the transcriptional level (Ahcre, Mx1cre), and one controlled at the protein level (R26creERT), we have identified sporadic recombination at the R26R reporter locus in multiple tissues. Detailed analysis of the intestinal epithelium suggests that recombination can occur both during development and as an ongoing process in adult life. Here we present a new inducible Cre transgenic line, AhcreERT, in which control of Cre activity is regulated at two levels: by transcriptional control of the Ah promoter and by a requirement for Tamoxifen binding. There is no detectable background intestinal recombination in adult AhcreERT mice on the R26R background. Inducible and dose-dependent recombination can be achieved by a single combined treatment with β-napthoflavone and Tamoxifen.

INTRODUCTION

The use of site-specific recombinases temporally and spatially to engineer specific gene mutations in mice has become a widely used research tool to understand gene function in vivo (1,2). The use of tissue-specific promoters to regulate Cre recombinase has allowed the study of genes which are ‘embryonic lethal’ when mutated in the germ line (3). However, even tissue-specific expression involves the introduction of mutations from the earliest stage at which the promoter is active, often before the relevant tissue has fully matured. Both germ-line- and possibly tissue-specific, gene mutations may allow for adaptive responses in which other gene products or pathways modify the response of the tissue over time. A second generation of strategies has focussed on controlling the timing of recombination. Broadly, there are two approaches: (i) to induce transcription of cre and (ii) to regulate the activity of Cre fusion proteins using mutant hormone-binding domains that bind synthetic ligands such as Tamoxifen (4,5). Transcriptional regulation has been reported with the interferon promoter (6) and the tet on/tet off systems in which a chimeric transactivator initiates/represses transcription from a promoter containing the tet operon in the presence of doxycycline (7,8). More recently, we have reported transcriptional regulation of cre using a xenobiotic responsive (CYP 1A1) promoter (9).

Developing these approaches demands a compromise between the level of unwanted background recombination and that induced upon treatment. For example, a common feature of many models is incomplete recombination resulting in tissue mosaicism (10–12). The starting point for the analysis of such tissues is based on in situ methods such as laser capture microscopy and immunohistochemistry (13). Any level of background recombination creates the likelihood that such analyses will include tissue predating Cre induction. This is potentially confounding because cells may over time become functionally adapted to altered patterns of gene expression. Further, in disease models where latency is involved, the respective contribution of pre-existing background and regulated genetic modification is important to define. This applies particularly to cancer models where the aim is to recapitulate the natural history of the disease by introducing a low frequency of sporadic gene mutations at a defined time or location and to follow the development of resultant pathologies.

Having determined that the extent of recombination in the Ahcre line is dose dependent and therefore had the potential to allow induction of sporadic gene mutation, we also identified that there was a low level of spontaneous background recombination. Here we describe the frequency and distribution of that background. For comparative purposes we show that similar levels of background recombination are a feature of both the transcriptionally regulated Mx1cre and the Tamoxifen inducible R26creERT lines and are therefore likely to be a feature of most inducible lines in which there is a single controlling element to regulate Cre. As an alternative we have generated a transgenic line, AhcreERT, in which Cre activity...

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is both transcriptionally regulated by the Ah promoter and dependent on Tamoxifen binding to mediate cytoplasmic–nuclear translocation. We show that background recombination is eliminated in AhcreER T mice and that inducible recombination is obtained in tissues in which the Ah promoter is conditionally active.

MATERIALS AND METHODS

Genetically modified animals

Male Mx1 cre (6), Ahcre (9) and R26cre-ERT (5) mice were bred to female cre-activated reporter mice in which the reporters, R26R and R26yfp, were either lacZ (14) or EYFP (15), respectively. Transgenic animals were genotyped by PCR using the following primer combinations. Ahcre: 5'-CCTGACTAGCTGGCATAC-3', 5'-ATTGCCCTGTCTCAT-TAC-3'. Mx1cre: 5'-TTCTGGTTTTGAGCTCCCC-3', 5'-ATTGCCCTGTCTCATAC-3', R26creERT: 5'-TGGCTACACAAAAATTTC-3', 5'-ATTGCCCTGTCTTCACT-3'. R26R and R26yfp reporter strains were genotyped as described previously (14) and double transgenic animals selected for further analysis. Animals were maintained in a specific pathogen-free facility and all animal procedures were carried out under the authority of a UK project licence, the awarding of which includes an ethical review process.

The plasmid pAhcreERT T, containing a fusion gene of cre and mouse Er T [with the G525R mutation (16) in the ligand-binding domain to promote Tamoxifen binding] was made by cloning creERT T into pAhcre (9) by BglII. The AhcreERT expression cassette was released by NotI digestion and transgenic founder animals generated by pronuclear microinjection of CBAXC57BL/6F1 oocytes. Two founder animals were obtained which gave germ-line transmission and both behaved identically in functional assays in crosses to reporter lines (data not shown). One line AhcreERT T was selected for all subsequent experiments and was bred to homozygosity after breeding on a C57BL/6J background for three to four generations.

The cre transgenic animals were crossed to reporter animals. R26creERT T, Mx1cre, Ahcre and AhcreERT reporter mice received no treatment but were killed for determination of background recombination frequencies between 6 and 8 weeks of age. AhcreERT T/R26R mice at 6–8 weeks of age were treated with two intraperitoneal injections given less than 1 h apart of β-naphthoflavone (βNF) dissolved in corn oil (8 mg/ml) followed by Tamoxifen (10 mg/ml) in sunflower oil and killed at the times stated. The dose administered ranged from 8 to 80 mg/kg of βNF and 0.01 to 1 mg of Tamoxifen.

Detection of cre-mediated recombination

For detection of lacZ expression tissues were fixed in ice-cold 2% formaldehyde/0.2% glutaraldehyde in phosphate-buffered saline (PBS) (pH 7.4) for 2–6 h, equilibrated in 20% sucrose/PBS then frozen in liquid nitrogen prior to storage at −80°C. Cryo-sections were cut at 10–15 μm and air dried prior to histochemical detection of β-galactosidase (β-gal) with X-gal substrate (9). Tissues selected for analysis were sectioned at three different levels (with each level being separated by at least 200 μm) and at each level multiple sections were collected onto each slide for X-gal staining. The following tissues were analysed, and were primarily chosen to give good coverage of the principal epithelia: liver, gall bladder, pancreas, fore stomach, glandular stomach, oesophagus, tongue, kidney, bladder, lung and heart. Recombination in small intestine and colon was identified in intestinal wholemounts, prepared luminal side up, as described previously (9). Wholemount preparations were examined using a standard dissecting microscope. For quantification of background recombination in the R26creERT T, Mx1cre and Ahcre lines the entire length of stained wholemounts was scored by cutting it into strips up to 5 cm long, mounting on a microscope slide abluminal side up and examined using a Nikon Optiphot2. Stained crypts were scored (total magnification of 250×) and categorized as being wholly or partially stained and the patch size (number of crypts per patch) also recorded. For the AhcreERT T dose–response experiments a 5 cm length of intestine taken from 25% of the length of the small intestine from the pylorus was scored.

Flow cytometric analysis

Spleens and femurs were transferred immediately into air-buffered DMEM containing 5% FCS. Spleens were disaggregated by passage through a 45 μm cell strainer (Falcon). Bone marrow was removed from femurs by flushing. Cells were recovered by centrifugation, washed with media and resuspended in PBS with 0.5% BSA and 0.05% sodium azide at a final concentration of 2 × 10⁷ cells/ml. Fluorescence (5 × 10⁵ to 5 × 10⁶ cells per sample) was measured with a FACS Calibur flow cytometer (Becton-Dickinson, San Jose, CA) over FL-1 (530 ± 15 nm) and FL-2 (585 ± 21 nm) channels. Scatter plots were analysed using CellQuest software (Becton-Dickinson, San Jose, CA), gating was set using cell preparations from animals not containing the EYFP transgene.

RESULTS

Background recombination in adult R26creERT T/Mx1cre/Ahcre animals

For all three cre lines the background level of spontaneous recombination has been described previously but using different methods (5,6,9). We utilized the constitutive reporter gene expression obtained from the recombinant R26R locus to undertake an assessment of spontaneous recombination events in cre-R26R double transgenic animals by X-gal staining of adult tissues.

As described previously, the R26creERT T showed most recombination in proximal small intestine (Figure 1A) which was also frequently observed, with numerous blue recombinant cells in each section, in liver, gall bladder, kidney, pancreas (Figure 1B) and glandular stomach (Figure 1C) and to a lesser extent in fore stomach, oesophagus, lung (Figure 1D), heart and bladder. Very little recombination was observed in tongue or colon. The Mx1cre line shows extensive recombination in liver (Figure 1E), kidney and pancreas in which many recombinant cells were observed in each tissue section. Other tissues showing a lower level of recombination, with only occasional blue cells, were small intestine, tongue (Figure 1F), skin, bladder, oesophagus, fore stomach and heart. Very little recombination was observed in colon and glandular stomach with some sections showing no recombinants and only the occasional recombinant
cell in others. In Ahcre animals there was extensive recombination in the cardiac muscle and kidneys of untreated mice (Figure 1G and H). There was a significant level of background recombination in bladder and to a lesser extent oesophagus and liver (Figure 1I). Recombination in glandular stomach, small intestine (Figure 1J), colon, tongue and pancreas was only occasionally observed.

Background recombination in spleen and bone marrow

Background recombination in spleen and bone marrow was measured in cells isolated from R26yfp mice crossed onto three cre lines (Figure 1K–O). The most extensive recombination was observed in Mx1cre [3.19 ± 0.89 and 4.79 ± 0.83 (mean ± SE), n = 6 for bone marrow and spleen, respectively]. There was a much lower level of recombination in R26creER<sup>1</sup> mice (0.26 ± 0.23 and 0.16 ± 0.11, n = 7) and effectively none in Ahcre animals (0.0025 ± 0.0025, 0.07 ± 0.07, n = 4). The Ah promoter is not active in haematopoietic cells, with no cre expression on induction.

Measurement of intestinal recombination

To determine the nature and extent of background recombination in the intestinal epithelium, wholemounts of X-gal-stained small intestine from adult Mx1cre-, Ahcre- and R26creER<sup>1</sup>-R26R mice were prepared. In these preparations a clear spatial pattern of recombination was evident with a proximal-distal...
gradient of epithelial staining for the $R26cre^{ERT}$ and $Mx1cre$ lines. This effect was less evident in the $Ahcre$ line due to large clusters of recombinant areas of epithelium which were variable in position and size from mouse to mouse. Staining in all lines was present as patches of blue recombinant epithelium that was contained in intestinal crypts. The size of recombinant patches was variable: large patches of contiguous crypts were observed as well as individual crypts that were partly or wholly occupied by recombinant epithelium. Other tissue components in wholemounts showed frequent positive β-galactosidase reactivity. These include blood vessels, longitudinal and circular muscle, and a cell type present on the outer surface of the gut (most frequent in the $Ahcre$ line) which we tentatively identify as mesothelial cells of the peritoneal lining (Figure 1A).

Wholemounts were scored for both the total number of crypts containing recombinant epithelium and the size of recombinant patches (number of crypts per patch) (Figure 2A). The mean frequency of recombinant crypts per small intestine for each line was: $11559 \pm 701$ SE, $R26cre^{ERT}$; $1108 \pm 60$ SE $Ahcre$ and $287 \pm 71$ SE $Mx1cre$. Taking the total number of crypts per small intestine to be $10^6$, this equates to approximately 12, 1.1 and 0.3% of the total epithelium for the $R26cre^{ERT}$, $Ahcre$ and $Mx1cre$ lines respectively. However, the proportion of single (wholly or partly) stained crypts as a percentage of total stained crypts was much lower in the $Ahcre$ line (3.2% ± 0.35, mean ± SE) than in the $R26cre^{ERT}$ (59.7% ± 14.4, mean ± SE) and $Mx1cre$ (33.1% ± 4.26, mean ± SE) lines. The corollary of this observation must be that a larger proportion of recombination in $Ahcre$ mice is present in multi-crypt patches and indeed the median patch size was 6 for the $Ahcre$ line compared to 2.8 and 4.5 for the $R26cre^{ERT}$ and $Mx1cre$ lines, respectively. The proximal–distal pattern of staining did not apply to large patches of recombinant crypts which appeared to be randomly distributed along the intestine (Figure 2B–D).

**Analysis of $Ahcre^{ERT}$ mice**

Solid tissues of untreated $Ahcre^{ERT}$/R26R mice were screened for background recombination by X-gal staining of frozen sections, as described above. In cardiac muscle, kidney, liver, gall bladder, small intestine, pancreas, glandular stomach and oesophagus there was no positive staining for β-galactosidase. In bladder, two positive cells in total were identified in numerous serial sections from two different mice. Intestinal wholemounts ($n = 3$) stained with X-gal scored along their entire length showed no β-galactosidase expression in either the intestinal epithelium or in non-epithelial components of the gut. Flow sorted bone marrow and spleen populations also showed no increase in the frequency of positive cells compared to cells from mice lacking the EYFP reporter (data not shown).

Adult $Ahcre^{ERT}$/R26R mice were treated with 80 mg/kg βNF and 1 mg of Tamoxifen and left for 14 days to 6 months post-induction prior to screening for recombination. Animals tolerated the combined treatment very well and displayed no evidence of toxicity either immediately following treatment or in the longer term. After 14 days, extensive sporadic X-gal positive recombinant cells could be observed in the small intestine, liver and bladder (Figure 3A–C), and pancreas...
Less frequent recombinants were observed in glandular stomach, oesophagus (Figure 3D and E) and gall bladder and fore stomach (data not shown). By 6 months recombinant cells were observed to persist in most of these tissues, including gall bladder and pancreas (Figure 3F and G) although in bladder they were much reduced in frequency and were absent in oesophagus and fore stomach.

To determine the relationship between inducer dose and recombination, cohorts of AhcreERT mice were treated with varying doses of βNF and Tamoxifen and recombination frequency counted in intestinal wholemounts (Figure 4) at 6 weeks post-induction. By varying the dose of βNF between 8 and 80 mg/kg and of Tamoxifen between 0.01 and 1 mg, the extent of recombination can be varied over a 1000-fold range: from $5 \times 10^{-6}$ to $5 \times 10^{-2}$. This dose responsiveness arises from the combined treatment with both inducing agents as no recombination was obtained after treatment with 1 mg Tamoxifen only and after a single dose of 80 mg/kg βNF a low rate of combination ($2.8 \pm 1.8 \times 10^{-6}$, mean ± S.E., $n = 5$) was detected.

**DISCUSSION**

There are two types of analyses where the presence of background recombination could confound the interpretation of somatically engineered gene mutations. First, expression arrays generated from microdissected tissues may identify transcriptional changes within functionally adapted mutant cells which predate induction. This is likely where the level of induced recombination is low and the background high. The second example is in cancer studies, where the objective is to model the aetiology and stages of disease progression. Such models require the introduction of sporadic mutations at a defined time to allow the developing pathology to be monitored. There are relatively few methods for introducing defined mutations in this way. They include (i) viral delivery or regulated expression of recombinases and (ii) the introduction of inactivating but unstable genetic elements which allow expression of altered genes following spontaneous mutagenesis or homologous recombination (5,17,18). All methods suffer from some drawbacks either in terms of leaky background, lack of temporal control or, in the case of viruses, immunological/titre problems [see review by Jonkers and Berns (19)].

The cause of unregulated background recombination presumably arises from either endogenous substrates or ones found in diet. The proximal to distal gradient of recombination in the small intestinal epithelium for the lines studied here may argue that different contaminants of the diet are capable of inducing Cre activity in each case. These dietary elements or their metabolites may be transported to other tissues. However, the proximal–distal effect is associated with a
small patch size of the recombinant epithelium. Large patches appear to be randomly placed along the intestine, suggesting that another contributing factor to the extent of background recombination is the expansion of recombinant cells that arise during embryonic development. For each of the three lines there is a different proportion of contribution from such embryonic recombinants. It is most striking for the Ahcre line where it contributes 97% of the total background. Indeed this is probably an underestimation as the spatially restricted clusters of epithelial recombinants include individual crypts which appear associated with adjacent larger patches. In many other tissues the recombinant epithelium often also appears to contain large contiguous patches of recombinant cells. This is particularly evident when comparing with the pattern observed in AhcreERTT animals (compare Figures 1 and 3).

Both the ROSA26 and Mx1 promoters have a wide range of inducible tissue expression (6,14,20) while that of the Ahcre line is selectively directed to the epithelia of the gastrointestinal tract (9). Thus only in the Ahcre line can the tissue specificity of inducible and background recombination be compared. Clearly, background recombination is greater in some (heart, kidney), but not all (spleen, bone marrow) of those tissues that are not transcriptionally inducible for the Ah promoter in the adult. In fact, the extent of background recombination in the principal adult target tissues, liver and small intestine, is much lower than in heart and kidney. This may suggest that the Ah promoter is promiscuously active in some tissues during development but later becomes more tightly regulated. This interpretation is also consistent with the predominance of large patches of background recombination in the small intestine.

The AhcreERTT line allows induction of Cre-mediated recombination in the absence of any apparent background. The binary control of cre activity, at both transcriptional and protein levels, must provide this tight control. The additional Tamoxifen requirement presumably ensures that there is no unregulated recombination during the promiscuous phase for Ah promoter activity described above, while the extensive intestinal recombination observed in the R26creERTT mice is avoided due to the stringent regulation of the Ah promoter in the adult. In all target tissues the pattern of recombination is mosaic and apparently sporadic with individual cells (liver) or amplified clones (small intestine) scattered through the tissue. The frequency of intestinal recombination obtained in AhcreERTT mice following a single dose of 80 mg/kg BNF and 1 mg of Tamoxifen is around 6-fold lower than that observed in Ahcre mice (9) following a single dose of 80 mg/kg BNF. We ascribe this lower degree of responsiveness largely to a reduced efficiency of the CreERT fusion protein compared to the native recombinase.

The concept of regulating Cre activity with combined transcriptional and protein controls has been described recently in an in vitro study employing a single agent, a progesterone analogue, that binds a transactivor encoded at one transgene to induce expression of a progesterone-responsive Cre fusion protein at another (21). In contrast, the system validated in vivo in this report employs two independent inducing agents acting at a single transgene.

The optimal frequency with which a desired mutation is introduced will vary with application. Higher frequencies are needed for microdissection, but in the case of low-level sporadic events a rate lower than $10^{-5}$ would seem impracticable in terms of detecting mutant cells or small clones in situ (e.g. in small intestine this would give about 10 mutant crypts per gut distributed along a ~36 cm length) but might suffice if lesions are allowed to develop to macroscopic size. Equally, levels higher than $10^{-2}$–$10^{-3}$ might reduce the certainty of lesions occurring independently in a wild-type background. Again in small intestine a recombination rate of $10^{-5}$ would induce $10^5$ affected crypts per small intestine with a high probability of neighbouring lesions colliding as they reach macroscopic size. The dose-dependent induction of recombination in the AhcreERTT line demonstrates the utility of combining control at the transcriptional and protein level to regulate Cre activity in the absence of detectable background. This simple strategy, which could be applied to other transcriptionally inducible promoters, such as that for interferon, will have an application in developing cancer models which recapitulate the sporadic nature of the disease process.

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**REFERENCES**