

Temporally and spatially regulated somatic mutagenesis in mice

Frieder Schwenk, Ralf Kühn, Pierre-Olivier Angrand¹, Klaus Rajewsky and A. Francis Stewart^{1,*}

Institute for Genetics, University of Cologne, Weyertal 121, 50931 Cologne, Germany and ¹Gene Expression Program, European Molecular Biology Laboratory, Meyerhofstrasse 1, 69117 Heidelberg, Germany

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ABSTRACT

In mice transgenesis through oocyte injection or DNA recombination in embryonal stem (ES) cells allows mutations to be introduced into the germline. However, the earliest phenotype of the introduced mutation can eclipse later effects. We show in mice that site-specific genomic recombination can be induced in a selected cell type, B lymphocytes, at a chosen time. This precision of somatic mutagenesis was accomplished by limiting expression of a Cre recombinase–estrogen receptor fusion protein to B lymphocytes by use of tissue-specific elements in the promoter of the transgene employed. The expressed fusion protein remained inactive until derepressed by systemic administration of an exogenous ligand for the estrogen receptor, 4-OH-tamoxifen. Upon derepression the Cre recombinase enzyme deleted specific DNA segments, flanked by loxP sites, in B lymphocytes only. The efficiency of recombination in cells expressing the fusion protein could be varied from low levels to >80%, depending on the dose of ligand administered. Our work presents a paradigm applicable to other uses of site-specific recombination in somatic mutagenesis where both temporal and spatial regulation are desired.

INTRODUCTION

Regulated somatic mutagenesis is essential for the analysis of gene function during development and in the mature organism. In mice transgenesis through oocyte injection or DNA recombination in embryonal stem (ES) cells allows mutations to be introduced into the germline. However, the earliest phenotype of the introduced mutation can eclipse later effects. Somatic mutagenesis is essential for studies of gene function during subsequent development.

Recent work on somatic mutagenesis has focused on the use of site-specific recombination (1–3). Two site-specific recombinases (SSRs), Cre recombinase from the *Escherichia coli* phage P1 and FLP recombinase from the 2 μ circle of *Saccharomyces cerevisiae*, have been shown to possess the properties required for genomic manipulations in a wide range of living systems, including mice, plants and flies. Genomic manipulations with SSRs require

expression of an SSR protein and introduction of at least two recombination target sites at chosen positions in the genome. The type of recombination event mediated by the SSR depends on the disposition of the recombination target sites, with deletions, inversions, translocations and integrations being possible (1).

Previously we showed that the ligand-dependent characteristics of steroid hormone receptors can be imposed on FLP recombinase by expressing FLP recombinase–steroid receptor fusion proteins (4). These fusion recombinases are inactive in the absence of a cognate steroid hormone, but respond rapidly to hormone administration. Ligand dependency can be similarly imposed on Cre recombinase (5–7). Thus SSR–steroid receptor fusion proteins present an experimentally convenient way to determine the time of recombination by ligand administration.

Somatic mutagenesis in mice using SSRs requires, for precision, a near complete absence of background recombination, both in cell types other than the target tissue and before the chosen time of induction. To accomplish the first of these two objectives we restricted expression of Cre recombinase to a given cell type by use of a tissue-specific promoter, since previous work in mice has shown effective spatial regulation of recombination based on this strategy (8–11). To accomplish the second, Cre was expressed as a fusion protein with a mutant estrogen receptor ligand binding domain (EBD) that is insensitive to the endogenous hormone β -estradiol but still responsive to the synthetic estrogen antagonist 4-OH-tamoxifen. Thus two levels of regulation, spatial and temporal, were combined in a single transgene (Fig. 1).

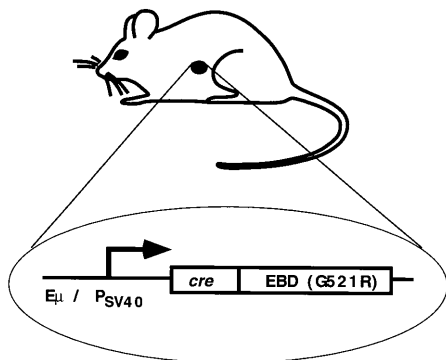
MATERIALS AND METHODS

DNA constructs

To generate the CreED4 coding sequence the stop codon of the Cre reading frame was replaced by the linker peptide VRGS and then fused to amino acid 304 of the human estrogen receptor. The pgkCreED4 vector was derived from the plasmid pgk-neo-bpA through replacement of the *neo* gene by the Cre–EBD coding sequence. To construct the E μ /P_{SV40} Cre–EBD(G521R) expression vector the 2.0 kb *SmaI*–*XhoI* fragment from p44CreEBD-4, which contains Cre fused to the EBD(G521R) coding region (P.-O. Angrand, unpublished results), was ligated into the poly-linker of pE μ hGH (F. Schwenk, unpublished results).

*To whom correspondence should be addressed. Tel: +49 6221 387 562; Fax: +49 6221 387 518; Email: stewart@embl-heidelberg.de

1. Restrict SSR/LBD fusion protein expression to selected cell type



2. Activate recombinase activity with ligand

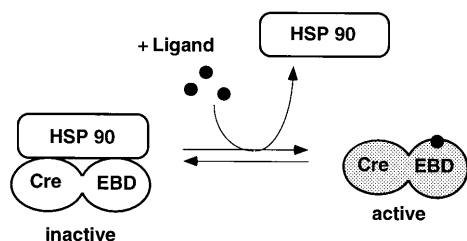


Figure 1. Principle of combinatorial temporal and spatial regulation of site-specific recombination in mice. (1) Expression of a site-specific recombinase–nuclear receptor ligand binding domain (SSR–LBD) fusion protein is directed to a chosen cell type by the properties of the *cis* regulatory elements of the transgene. In this case the immunoglobulin heavy chain enhancer (E_{μ}) is combined with the SV40 early minimal promoter to direct transgene expression exclusively to B lymphocytes. (2) The expressed protein is a fusion of Cre recombinase and the estrogen binding domain (EBD) of the human estrogen receptor mutated at amino acid 521 (G521R). The Cre–EBD(G521R) protein is inactive because the EBD interacts with the ubiquitous Hsp90 complex. The fusion protein is released from the inactive state upon ligand binding by the LBD, allowing recombination between two loxP sites present in the genome. Hsp, heat shock protein.

Transgenic cells

The SV40-transformed murine embryonic fibroblast line MEF5/5 (M. Aguet, unpublished results) was transfected with the vector p12neo (H. Gu, unpublished results), which contains an HSVtk promoter-driven *neo* gene flanked by two loxP sites and, on one side, DNA from the yeast *leu2* gene. A single copy integrant, identified by Southern blotting, was further transfected with a hygromycin resistance vector together with the Cre–EBD(G521R) expression vector pgkCreED4. In all of the four stable transfectants analyzed (containing 3–10 copies of pgkCreED4) Cre activity was induced upon administration of anti-hormone, although to different extents. Clone 4-4 was most efficient and was used for further analysis. Cre activity was measured by Southern blotting of *Xba*I-digested genomic DNA using the adjacent *leu2* probe. Cre-mediated deletion reduces the band detected by the *leu2* probe by 1.2 kb.

Transgenic mice

Transgenic mice were produced by pronuclear injection of the purified DNA fragment into fertilized (C57Bl/6 × CBA) F₂ eggs using standard techniques. Founder mice were screened for Cre–EBD(G521R) expression using the anti-Cre mAB 7.23 (12). Of 14 transgenic mice generated, two showed detectable expression of Cre–EBD(G521R) in B cells.

Ligand administration

Aliquots of 50 mg 4-OH-tamoxifen (Sigma H-6278) were dispersed in 1.5 ml sunflower seed oil with a sonicator. Aliquots were shock frozen and stored at -80°C . After thawing the samples were heated for 5 min at 60°C and injected i.p. through a 26 gauge needle. On gross inspection of the organs of mice treated with 4-OH-tamoxifen (five injections of 8 mg) no abnormalities compared with non-treated controls were found. In bone marrow, spleen and thymus of 4-OH-tamoxifen-treated mice the populations of immature and mature B and T cells appeared normal by flow cytometric analysis.

RESULTS

Ligand-dependant recombination in a fibroblast cell line

The Cre enzyme was expressed as a fusion protein with a mutant human estrogen receptor ligand binding domain, EBD(G521R). The equivalent Gly→Arg mutation in the mouse estrogen receptor (G525R) renders this EBD resistant to activation by the natural estrogen receptor agonist β -estradiol, while allowing activation by the synthetic antagonist 4-OH-tamoxifen (13,14). Cre–EBD(G521R) fusion proteins were first tested in a stable cell assay to evaluate regulatory properties and further characterize the response of the G521R mutation to other estrogens. A fibroblast cell line containing a single copy of a neomycin resistance gene flanked by the loxP target sites for Cre recombinase ('floxed') was transfected with Cre–EBD(G521R) constructs (Fig. 2A) and stable clones isolated. Constitutive expression of Cre–EBD(G521R) did not result in detectable recombination when the cells were grown for up to 4 days in medium alone (Fig. 2B and C). As expected from the known properties of the G525R mutation, addition of high concentrations of β -estradiol resulted in only a very weak induction of recombination, whereas 4-OH-tamoxifen induced near complete recombination (Fig. 2B). Cre–EBD(G521R) was insensitive to the second major endogenous mammalian estrogen, estradiol, whilst responding efficiently to another synthetic antagonist, raloxifen (Fig. 2B). The synthetic antagonist nafoxidine (Fig. 2B) and the synthetic agonists diethylstilbestrol and hexesterol also did not induce recombination, whereas ICI 182,780 did (data not shown). Both raloxifen (Fig. 2C) and 4-OH-tamoxifen (data not shown) induced recombination with reasonable and equivalent kinetics. Titration experiments with 4-OH-tamoxifen (Fig. 2D), raloxifen or ICI 182,780 (data not shown) showed that Cre–EBD(G521R) displays ~100 times less apparent affinity for these ligands than expected from the affinities of the natural estrogen receptor for these ligands. Nevertheless, the Cre–EBD(G521R) protein used here shows the necessary properties of low background recombination, insensitivity to endogenous mammalian estrogens and efficient inducibility by several synthetic antagonists.

Previous work has indicated that ligand regulation of steroid receptor fusion proteins differs according to the length of linker peptide between the protein to be regulated and the ligand binding

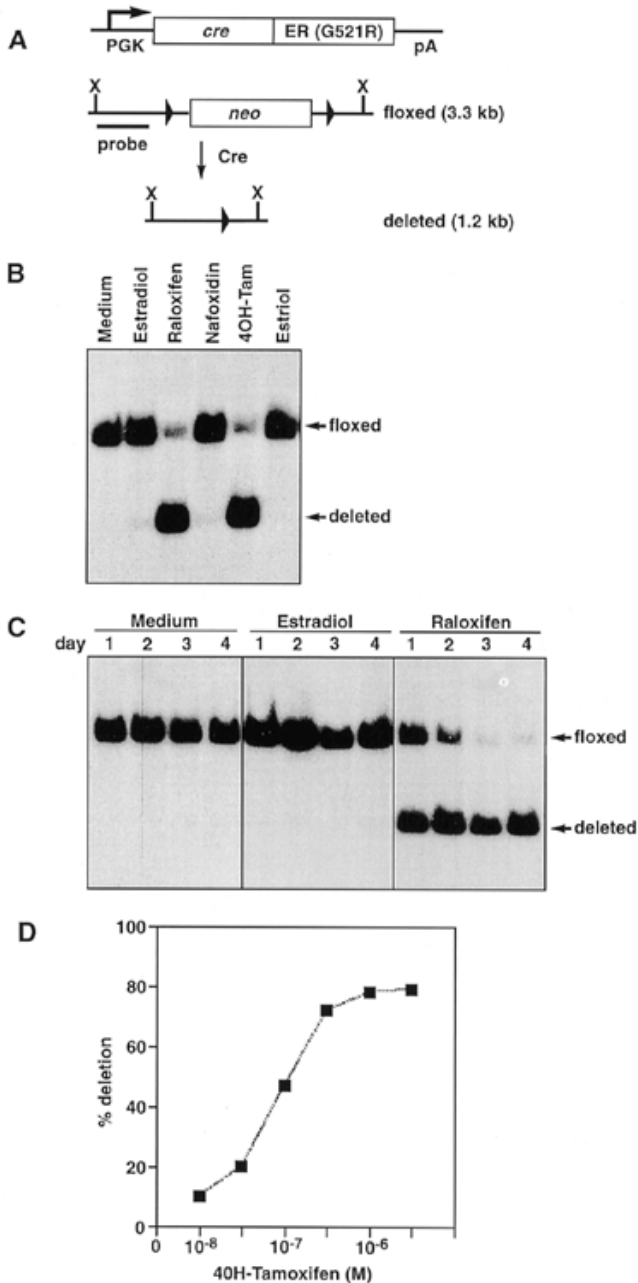


Figure 2. Characteristics of ligand-dependent recombination mediated by Cre-EBD(G521R) in a fibroblast cell line. (A) The transgene used to express Cre-EBD(G521R) constitutively and the floxed neomycin cassette (*neo*) stably integrated as a single copy in a fibroblast cell line are depicted. The Cre-EBD(G521R) fusion is composed of amino acids 303–595 of the human estrogen receptor with a G→R mutation at position 521. Cre-mediated deletion of the loxP-flanked *neo* gene was detected by Southern blot analysis of *Xba*I (X)-digested genomic DNA using the indicated probe. Upon Cre-mediated deletion the band detected by the probe is reduced by 1.2 kb. (B) Southern blot analysis of Cre-EBD(G521R)-mediated gene deletion upon treatment with various ligands. Transfectant 4-4 was cultured for 4 days in medium alone or in medium containing estradiol (0.1 μM), raloxifen (0.5 μM), nafoxidin (0.1 μM), 4-OH-tamoxifen (0.1 μM) or estriol (100 μM). Cre-mediated deletion reached 90% upon induction with 4-OH-tamoxifen and raloxifen, as measured with a Fuji BAS 1000 Bio-Imaging Analyzer. (C) Time course of ligand-induced recombination in transfectant 4-4. Cells were cultured for the indicated number of days in medium alone or in medium containing 0.1 μM estradiol or raloxifen. (D) Titration of 4-OH-tamoxifen-induced recombination measured by quantification of Southern blots after 2 days induction.

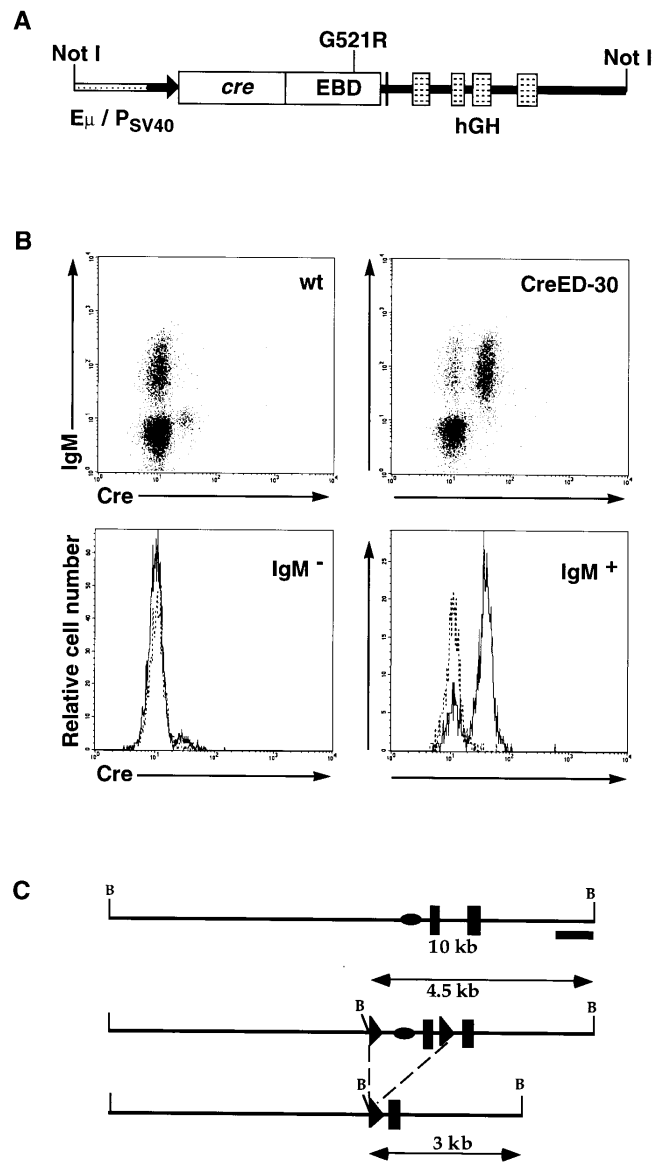


Figure 3. Characteristics of the Cre-EBD transgene and its expression. (A) Scheme of the transgene. The Cre-EBD(G521R) gene is expressed under control of the immunoglobulin heavy chain enhancer (E_μ) and the SV40 early minimal promoter (17). A 2.1 kb fragment of the human growth hormone gene (hGH) provides splicing and polyadenylation signals (18). (B) Flow cytometric analysis of Cre-EBD(G521R) expression in peripheral blood lymphocytes. Lymphocytes from wild-type (wt) and CreED-30 mice were isolated and stained intracellularly with the anti-Cre monoclonal antibody 7.23 (12) and for IgM on the cell surface. Quantitated Cre expression in IgM-positive B cells and IgM-negative non-B cells from wild-type (dashed lines) and Cre-ED-30 mice (solid lines) is shown below. (C) Strategy to distinguish the DNA polymerase β wild-type (upper line), loxP-flanked (middle line) and deleted (lower line) alleles. The promoter region (ellipses), the first two exons of the *polβ* gene (black rectangles) and an adjacent probe used for hybridization (bar) are shown. In the *polβ*^{lox} allele a 1.5 kb gene segment has been flanked by two loxP sites (triangles). The wild-type, loxP-flanked and deleted *polβ* alleles are represented by *Bam*HI (B) fragments of 10, 4.5 and 3 kb respectively.

domain of the steroid receptor (5,15,16). Therefore, we tested two Cre-EBD(G521R) fusion proteins. In addition to the one employed here (composed of amino acids 303–595 of the human estrogen receptor), a fusion protein that additionally included estrogen receptor amino acids 252–303 between Cre and the EBD was tested. This longer fusion protein showed significant recombination in the absence of added ligand in both the stable fibroblast cell system and transgenic mice (data not shown).

Generation of Cre-EBD(G521R) transgenic mice

Mice transgenic for the E_{μ}/P_{SV40} Cre-EBD(G521R) construct illustrated in Figure 3A were established. Expression of the Cre transgene was limited to B lymphocytes by placing it under control of the immunoglobulin heavy chain enhancer (E_{μ}) linked to a minimal SV40 promoter (17). Of 14 lines two expressed Cre-EBD(G521R) only in B lymphocytes (strains CreED-24 and CreED-30) and showed similar properties of expression and ligand regulation. FACS analysis of Cre-EBD(G521R) expression in CreED-24 and CreED-30 peripheral blood lymphocytes showed that $75 \pm 5\%$ of IgM^{+} cells expressed Cre (Fig. 3B and data not shown). Thus the E_{μ}/P_{SV40} Cre-EBD(G521R) transgene delivered mosaic expression to the B lymphocytes of the CreED-24 and CreED-30 strains.

Ligand-inducible, B cell-specific gene deletion in mice

The CreED-30 mouse line was crossed with a mouse line carrying a floxed allele of the DNA polymerase β gene ($pol\beta^{lox}$; 9). This allele was chosen because its heterozygotic deletion in B cells shows no deleterious effects on cell survival, thereby permitting analyses of recombination uncomplicated by cellular selection. This was important, since we wanted to detect background recombination that might occur in the absence of ligand induction. No recombination was detected in B lymphocytes before ligand induction by Southern analysis (Fig. 4A–C). However, a low amount of background recombination (less than $\sim 1\%$ total theoretical deletion of the floxed allele) in B lymphocytes before ligand induction could be detected by PCR (data not shown).

Ligand-inducible activation of Cre was tested in $pol\beta^{lox}$ heterozygotes (Fig. 4A–D) and homozygotes (Fig. 4D). Ligand was administered by daily i.p. injections of 0–8 mg ligand solubilized in oil. A variety of experiments showed that maximal recombination plateaued at $\sim 65\%$ and that dosages of >4 mg/injection were not saturating. Five consecutive daily injections of 1, 2, 4 or 8 mg produced 17, 44, 62 or 64% recombination (Fig. 4A). Analysis of different mice showed that percent induced recombination with different administration protocols was accurately reproducible (Fig. 4B). A single injection of apparently saturating concentrations of 4-OH-tamoxifen (5 mg) induced 16% recombination of the floxed allele (Fig. 4C). Further injections induced more recombination up to a maximum of $\sim 65\%$ within 5 days (Fig. 4C). Induction kinetics elicited by 4-OH-tamoxifen or raloxifen were very similar (data not shown). No recombination was detected in any tissue other than B lymphocytes in mice treated with 4-OH-tamoxifen (Fig. 4E and data not shown). As Cre was not expressed in all IgM^{+} cells (Fig. 3B), the 65% maximum of observed recombination reflects $>80\%$ recombination in Cre-expressing cells. This was confirmed by experiments on mice carrying two floxed alleles. Both alleles in $pol\beta^{lox}$

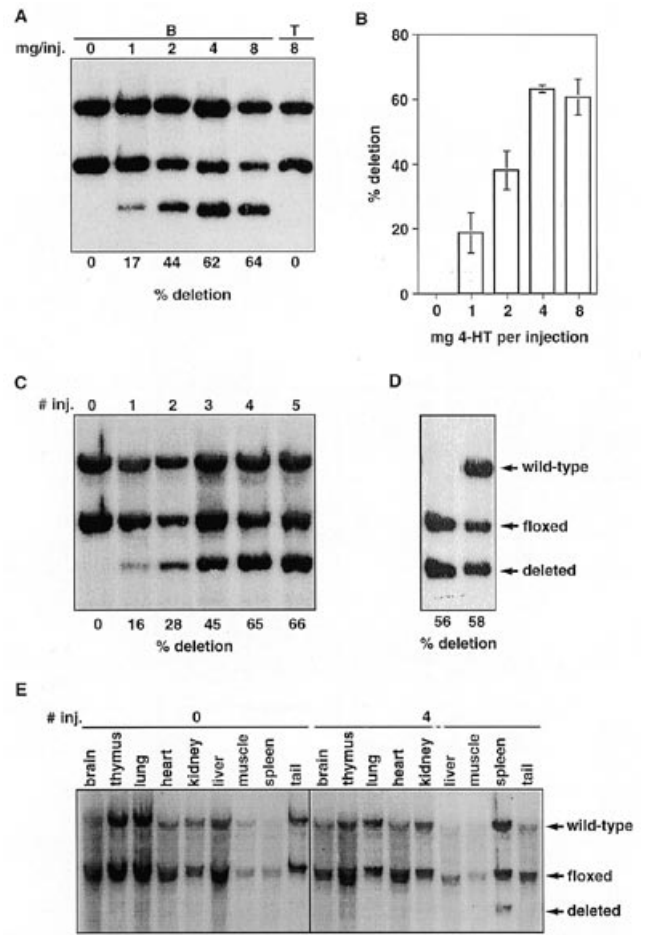


Figure 4. Southern analyses of ligand-inducible, B cell-specific gene deletion in mice. (A) Southern blot analysis of genomic DNA from B and T cells of mice given daily i.p. injections of various doses of 4-OH-tamoxifen as indicated for 5 days. Genomic DNA was prepared from splenic B and T cells 1 day after the last injection. Deletion of the $pol\beta^{lox}$ allele was detected by Southern blot analysis using a $pol\beta$ -specific probe. The positions of the fragments derived from the wild-type, loxP-flanked (floxed) and deleted $pol\beta$ alleles and the percentages of deletion of the floxed allele, as quantified with a Bio-Imaging Analyzer, are indicated. Since $\sim 25\%$ of B cells do not express Cre-EBD(G521R) (Fig. 3B), 64% recombination represents 85% recombination in Cre-EBD(G521R)-expressing cells. (B) Reproducibility of Cre-mediated deletion of the loxP-flanked DNA polymerase β ($pol\beta^{lox}$) allele in CreED-30 mice. Groups of two or three mice were each treated with 0, 1, 2 or 4 mg as indicated. Injections and quantifications were as in (C). Percent deletion (mean \pm SD) of the floxed $pol\beta$ allele is presented in the histogram. (C) Kinetics of 4-OH-tamoxifen-inducible gene deletion. CreED-30 mice were injected with 5 mg 4-OH-tamoxifen daily for 1–5 days and analyzed 1 day after the last injection. (D) Analysis of Cre-mediated deletion in homo- and heterozygous $pol\beta^{lox}$ mice. Littermates were injected with 4 mg 4-OH-tamoxifen daily for 4 days and were analyzed 1 day later. (E) Southern blot analysis of genomic DNA from various organs of an untreated (left) or 4-OH-tamoxifen-treated mouse (daily i.p. injections of 5 mg 4-OH-tamoxifen for 4 days; right).

homozygotes showed percent induced recombination to the same extent as the single allele in heterozygotes (Fig. 4D). A similar degree of 4-OH-tamoxifen-inducible recombination was obtained in B cells from mice harboring either a floxed allele of the C-terminal src kinase gene (*csk*) or two heterozygotic floxed alleles (*pol\beta* and *csk*; F.Schwenk, C.Schmedt and A.Tarakovsky, unpublished results). We observe therefore that recombination

can be induced in ~80% of Cre-expressing B lymphocytes to the same extent at one or two floxed loci. This suggests that no recombination is inducible in the remaining 20% of Cre-positive B lymphocytes as determined by FACS analysis (Fig. 3B), possibly due to insufficient levels of Cre expression.

DISCUSSION

Contemporary analysis of gene function in mammals rests predominantly on the technology of genomic manipulations of mice. DNA delivery to mouse oocytes or embryonal stem cells permits stable alteration of the mouse genome, however, strategies of somatic mutagenesis are required so that gene function can be studied in a chosen cell type at a chosen time. Previous work to develop somatic mutagenesis in mice employed site-specific recombination, either by restricting expression of Cre activity to a chosen cell type (8–11) or in a variety of tissues upon induction (7,18,19). Ideally, somatic mutagenesis should be exclusively confined to a chosen place and time. We show here a strategy that delivers this precision in B lymphocytes by combining tissue-specific and ligand-dependent regulation. These two regulatory modes were readily combined in a single transgene. Consequently, precise somatic mutagenesis in B lymphocytes was accomplished by use of a single effector mouse line. The simplicity of the strategy recommends its use in other applications. Other applications will require, as was the case here for B lymphocytes, enhancer/promoter combinations that deliver the desired tissue-restricted expression pattern at reasonable expression levels. Currently there is an ongoing, collective effort to establish a range of transgenic mouse lines that express Cre recombinase in different tissues (<http://www.mshri.on.ca/develop/nagy/Cre.htm>). One outcome of this effort will be identification of useful enhancer/promoter combinations for Cre expression in restricted patterns in mice. We suggest that these Cre transgenes are good candidates for the simple addition of the ~1 kb DNA fragment that encodes a steroid receptor LBD so that temporal regulation can be added. It is also worth considering that addition of a second level of regulation may overcome the occasional problem of Cre recombinase transgene expression very early in development. In such cases very early expression of Cre in development can result in widespread, undesired deletion of the floxed allele before the intended time and place. The additional level of regulation conferred by inclusion of an LBD should help to overcome this problem and thereby expand the repertoire of useful enhancer/promoter combinations for Cre recombinase expression.

There have been three previous publications describing systems of temporally regulated somatic mutagenesis in mice (7,18,19). Either because of implicit strategy limitations or by choice, none of these studies employed combinatorial use of temporal and spatial regulation to enhance mutagenic precision. Consequently, these approaches have been limited by unpredictable or unwanted consequences of SSR expression in a range of different tissues. In contrast, our combinatorial use of a tissue-specific transgene with a Cre-EBD fusion protein permitted greater mutagenic precision, thereby avoiding the complications inherent in widespread recombination of differing efficiencies in many tissues.

Future developments with this strategy of somatic mutagenesis should address two issues. First, we did not observe complete recombination after induction in the chosen cell type. The inability to induce complete recombination in a chosen cell population represents a limitation to certain experiments, such as

those involving gene knock-outs of non-cell autonomous factors. However, gain-of-function experiments or knock-outs with cell autonomous factors do not require 100% recombination in the target tissue. In the latter case the presence of a mixed population of wild-type and recombined cells may offer the advantage of allowing wild-type/mutant comparisons in the same animal. An internal comparison can be varied by use of different ligand administration regimes to generate populations displaying different ratios of wild-type and recombined cells. Nevertheless, it would clearly be desirable to express the SSR-LBD in 100% of the cell type being investigated. In common with some other cases of transgenesis generated by oocyte injection (20), the transgene employed here appears to express in a mosaic fashion (Fig. 3B). This problem may be avoidable by 'knock-in' strategies, where the SSR-LBD is directed to an endogenous locus known to be expressed in the desired cell-restricted manner (21).

Second, high doses of 4-OH-tamoxifen, or other estrogen antagonists, were required for induction. This is because the G521R mutation reduces β -estradiol binding by >10 000-fold, but also reduces binding by the synthetic antagonists by ~100-fold (M.Nichols, J.M.J.Rientjes, and A.F.Stewart, unpublished results). Furthermore, these estrogen antagonists will affect the endogenous estrogen receptor. Although we did not observe any obvious effect on adults or any change in lymphocyte populations at even the highest doses administered here, antagonist action on the endogenous estrogen receptor will significantly limit certain applications, such as induction of recombination *in utero* (22). Better ligand/steroid receptor pairs, ideally ones that are completely independent of activities in mice, are needed to extend the usefulness of LBD regulation.

The effective combination of spatial and temporal regulatory modes presented here has implications for regulatory design in other contexts. Since LBD regulation appears to function in all eukaryotes (23–25), the combinatorial principle of this work is broadly applicable, particularly to plants. Similarly, a combinatorial approach in medicinal gene therapy could also be used to enhance control of expression of the exogenous gene(s).

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