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B lymphocyte-specific, Cre-mediated mutagenesis in mice

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ABSTRACT

Adaptation of the P1 phage-derived Cre/*loxP* site-specific recombination system to the gene targeting technique allows for the conditional deletion of genes in mice. To selectively modify genes in B lymphocytes, we have generated mice (designated CD19-Cre) which express *cre* under the transcriptional control of the B lineage-restricted *CD19* gene. In a model system involving the cross of CD19-Cre mice with mice bearing a *loxP*-flanked substrate, we find a deletion efficiency of 75–80% in bone marrow-derived pre-B cells that increases to 90–95% in splenic B cells.

The gene targeting technique permits the introduction of defined mutations into the mouse germline and has greatly enhanced and expanded genetic approaches to examine gene function *in vivo* (1,2). Generally, this approach is used to inactivate an allele by homologous recombination in embryonic stem (ES) cells, followed by the derivation of mutant mouse lines through the generation of chimeric mice and subsequent breeding of mice bearing the transmitted mutant allele. Such gene ablation experiments are now near standard protocol as a means to understand the physiological role of a given gene product. Nonetheless, there are certain limitations in both the interpretation and application of congenital gene inactivation studies. This stems from the fact that the loss of a gene early in development often results in embryonic lethality or developmental compensation. Although informative, such results may preclude investigation of the intended biological problem.

Recently, the Cre/*loxP* recombination system of bacteriophage P1 (3,4) has been adapted to function in ES cells and shown to be a powerful system to mediate both subtle (5,6) and large scale mutations (7). This is made possible by the targeted insertion of recognition sites, termed *loxP*, at defined sites in the mouse genome. In the presence of Cre, the intervening DNA segment flanked by *loxP* sites ('floxed') is either deleted or inverted, depending upon the orientation of the *loxP* sites. This is the basis for the recent application of this system for the 'conditional' inactivation of genes (or activation of genes by removal of inhibitory sequences) in mice (8), which is achieved by the inducible (9) or cell type-specific (10–13) expression of *cre* and subsequent deletion of the floxed, but functional, gene segment. The option to generate germline mutations remains, particularly with the availability of transgenic mice that can introduce Cre-mediated modifications into the germline (14,15).

Here we report the creation of a system for genetic manipulation of cells of the B lineage in mice by expression of *cre* under the transcriptional control of the *CD19* promoter. CD19 was chosen since it is only transcribed in cells of the B lineage (16). Moreover, *CD19* is expressed at the earliest stages and throughout B cell development and differentiation (17). To obtain CD19-directed *cre* expression, a *cre* expression cassette was inserted into the second exon of *CD19* by homologous recombination in ES cells (18; Fig. 1). Thus, all the regulatory elements of the endogenous CD19 locus are present, whereas the addition of a heterologous β-globin intron and polyadenylation site was introduced to ensure appropriate splicing and polyadenylation of the *cre* RNA. Insertion of *cre* disrupts the *CD19* coding sequence, leading to a CD19 deficiency in the homozygous situation (18). Mice hemizygous for the *cre* insertion (designated CD19-Cre mice) which retain one functional CD19 allele are phenotypically normal and can be used for B lineage specific deletion of a floxed target gene.

To determine the efficiency and specificity of Cre-mediated deletion in B cells, CD19-Cre mice were crossed with mice bearing a DNA polymerase β gene (*polβ*) in a wild-type (*polβ*^{WT}), floxed (*polβ*^{flox}) or deleted (*polβ*$^{\Delta}$) germline configuration (12). Recombination of the introduced *loxP* sites excises the promoter and first exon of *polβ*, rendering the allele inactive (Fig. 2). Hence, somatic deletion of *polβ* can be monitored by Southern blot analysis of genomic DNA purified from the target cell population, and quantitation of the loss of the *polβ*^{flox} allele and acquisition of the *polβ*$^{\Delta}$ allele. In splenic B cells we consistently find a deletion efficiency of 90–95%, irrespective of whether the genotype of the mice is *polβ*^{flox}/*polβ*^{WT} or *polβ*^{flox}/*polβ*$^{\Delta}$ (Fig. 2). This result, and the presence of B cells in normal numbers in these mice (data not shown), suggest that pol β-deficient B cells are not significantly disadvantaged, despite the essential role of this repair enzyme during embryogenesis (11). Deletion was only evident in cells of the B lineage, since the *polβ* locus in T cells and cells from non-lymphoid tissues (liver, brain, intestine, kidney and lung) was maintained in germline configuration (Fig. 2 and data not shown). To determine the degree of deletion in developing B cells, DNA was prepared from bone marrow pre-B cells. Here the deletion efficiency of 75–80% is lower than in mature splenic B cells, indicating that Cre-mediated deletion is an ongoing process during B cell development. This is consistent with the known expression pattern of CD19, and correlates well with Cre expression in CD19-Cre mice as revealed through staining with Cre-specific antibodies (21).

The blastocyst complementation system using severe combined immunodeficiency (SCID, 19) mice or recombination-activating

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Figure 1. *CD19-Cre* allele. Targeting of the CD19 locus has been described in detail elsewhere (18). In brief, the *cre* gene was modified using a PCR based method as described (5), whereby a eukaryotic translation initiation consensus sequence and the nuclear localization signal of the SV40 large T antigen were introduced into the *cre* gene at the 5′-end using the following 5′ and 3′ primers, respectively: TTAGTCGACCATGCCCAAGAAGAAGAGGAAGGTGTC-CAATTTACTGAC and CTGAAGATATAGAAGAT. An 1180 bp fragment containing the rabbit β-globin intron (Stratagene, La Jolla, CA, USA) was inserted at the 3′-end of the *cre* gene followed by the neomycin resistance gene flanked by FRT sites (22). Although deletion of the FRT-flanked *neo*^{r} gene (18) by the Flp recombinase may be desirable, it appears that its presence does not have an appreciable effect on *cre* expression.

gene (RAG)-deficient mice (20) provides a means to study the effect of targeted mutations specifically in the lymphoid compartment. SCID or RAG blastocyst-derived B and T lineage cells are blocked at an early stage of development due to the inability to rearrange their immunoglobulin and T cell receptor gene segments. Thus, in chimeric mice generated by injection of manipulated ES cells the only mature B or T cells present should be ES cell derived. Although this system provides for rapid phenotypic analyses by avoiding time consuming breeding of homozygous mutant mice, it is laborious in that homozygous mutant ES cells need to be generated *in vitro*, and blastocyst injection is an ongoing necessity to produce chimeric animals for analysis. Moreover, the variable degree of chimerism is a parameter difficult to control, and blastocyst-derived normal lymphoid progenitors may out compete ES-derived mutant cells.

In the context of the Cre/*loxP* system, the CD19-Cre mice offer an alternative to the RAG/SCID complementation systems, allowing for conditional deletion in B cells but leaving T cells unaffected. The fact that the efficiency of Cre-mediated deletion in CD19-Cre mice is not complete may be a factor of concern because 'wild-type' cells which escape Cre activity may selectively expand. Functionally 'neutral' floxed substrates or the deletion of heterozygous floxed alleles allow to evaluate the principal efficiency of cre-mediated deletion under non-selecting conditions. The comparison of the latter with deletion resulting in the complete absence of a gene would be informative as to the role of the gene in a given biological context, as we have shown here for *pol*β. As it is likely that the efficiency of Cre-mediated recombination *in vivo* will vary with respect to the floxed substrate and the source of Cre expression, a challenge for the future is to devise a means to introduce an indicator gene into the target gene in such a way that cells which have undergone a deletion event could be identified directly.

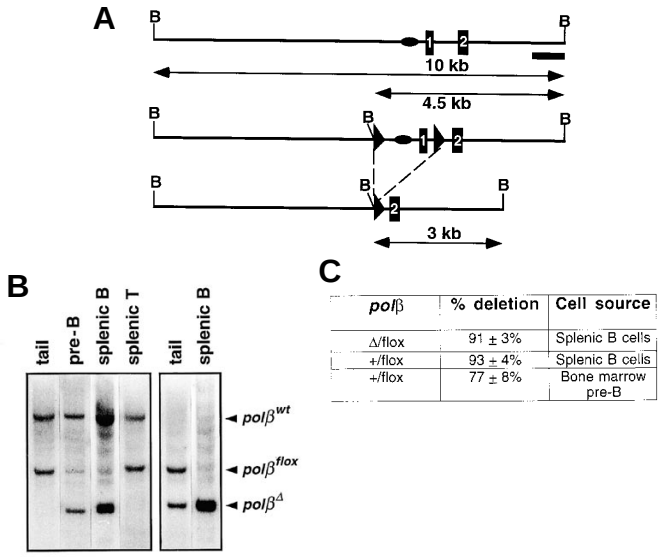

Figure 2. Cre-mediated deletion. (**A**) *pol*β locus and Southern blot strategy to distinguish *pol*β^{WT} (upper), *pol*β^{flox} (middle) and *pol*β^{Δ} (lower) alleles after *Bam*HI digestion and transfer of genomic DNA. The genomic probe is depicted as a black bar. This scheme is reproduced from ref. 14. (**B**) Hybridization with the *pol*β probe. Splenic B cells (CD45R/B220$^+$) were isolated by positive enrichment using magnetic bead separation (MACS, Miltenyi Biotec, Cologne); T cells by MACS enrichment for Thy1.2$^+$ cells; and pre-B cells by direct flow cytometric sorting of CD45R/B220$^+$, CD43/S7$^-$ and IgM$^-$ cells. Mature B and T populations were from single mice, whereas pre-B cells were from a group of four mice. (**C**) Quantitation of deletion with Bio-Imaging analyzer (Fuji Bas 1000). The percent deletion (mean ± SD) was calculated as described (9) and normalized for the purity of the sorted population, which was always >95%.

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