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<p>13. ABSTRACT <i>(Maximum 200 words)</i></p> <p>Breast cancer is a genetic disease involving both gain and loss of function mutations in many different genes. It is important to define which genes are significant mutational targets in sporadic breast tumors so that treatments can be directed based on the knowledge of the genetic changes in the tumor. This proposal is focused on identifying tumor suppressor genes which are mutated in sporadic breast cancer. We proposed to identify these tumor suppressor genes by a novel genetic screen <i>in vivo</i>. This genetic screen involves the adaptation of our chromosome engineering technology to delete specific chromosomal regions in mouse mammary epithelial cells <i>in vivo</i>. We planned to use tissue specific expression of Cre in mammary epithelial cells to induce these deletion events <i>in vivo</i>. When combined with insertional mutagenesis this technology has the power to identify tumor suppressor genes mutated in breast cancer.</p> <p>In the first year of this proposal we have constructed several "pre-deletion" chromosomes, transmitted them into the germ line and tested recombination event <i>in vivo</i>. We have also evidence of tissue specific recombination in heart muscle. We have also generated a MMTV-Cre transgene in the <i>hprt</i> locus which is also in the mouse germ line.</p>			
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FOREWORD

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#### 5. Introduction

The purpose of this research is to develop new manipulative genetic tools which should enable recessive oncogenes to be functionally identified in breast cancer using mouse models. These new genetic technologies utilize the activity of *Cre-loxP* recombination to specifically delete regions of mouse chromosome 11 in breast epithelial cells. These regions are believed to include tumor suppressor genes based on mapping data of conserved linkage groups in human breast cancer. There are several steps in this process. First, pre-deletion chromosome tagged with *loxP* sites are generated by two rounds of gene targeting. Second, the chromosomes are crossed into a background of a mouse that expresses *Cre* in mammary epithelial cells. Third, these mice with large chromosomal deletions in a portion of their mammary epithelial cells will be infected with an insertional mutagen (such as MMTV) to generate recessive hits in tumor suppressor genes. These mice will be monitored for their tumor susceptibility and integration sites will be identified.

## 6. Body

### Statement of Work Completed May 1999

#### Year 1

- |       |   |   |                  |
|-------|---|---|------------------|
| (i)   | Construction of pre-deletion chromosomes                | - | <i>completed</i> |
| (ii)  | Construction of <i>Hprt-MMTV-Cre</i> targeted transgene | - | <i>completed</i> |
| (iii) | Germ line transmission of "pre-deletion" chromosomes    | - | <i>completed</i> |

#### Year 2

- |     |   |   |                  |
|-----|---|---|------------------|
| (i) | Germ line transmission of <i>Hprt-MMTV-Cre</i> allele | - | <i>completed</i> |
|-----|---|---|------------------|

The three goals of the first year of this proposal have been completed and also the first goal of Year 2. We have invested a substantial amount of effort in generating a variety of pre-deletion chromosomes. Most of these accomplishments are described in great detail in the attached manuscript (draft) which will be submitted to *Molecular and Cellular Biology*.

Because of the availability of these genetic reagents in mice, we have moved ahead with the *in vivo* testing of long range *Cre-loxP* recombination. We know from experiments in ES cells that these chromosomes can be recombined with *Cre* (see Zheng et al, Table 1). However, the *MMTV-Cre* allele only became available recently so an existing *Cre* transgene was used expressing *Cre* in the heart under a *Myosin Heavy Chain* promoter. This experiment revealed that we could obtain long range recombination at 10% frequencies in a specific cell type (see Zheng et al figure 6). Moreover, this deletion chromosome was tolerated somatically even though this was not tolerated in normal embryonic development. We do not yet know the relationship between recombination frequency and inter-*loxP* site separation *in vivo* nor how this might differ in various tissue types. In ES cells we have found that very long distance recombination will occur (see Zheng et al figure 5) but cells with substantial deletions (20 cM) are inviable and are lost in culture (see Zheng et al figure 4). We need to explore these questions *in vivo* both with the existing heart specific *Cre*, but also with the new *MMTV-Cre* transgenes.

The *MMTV-Cre* transgene targeted to the *Hprt* locus has been generated and is available in mice. Crosses are underway to evaluate whether this functions mammary epithelial cells. These experiments are facilitated by reporter mice which activate the expression of *lacZ* in cells where *Cre* is expressed. This provides a visual and easily quantifiable read out of the activity and specificity of our *MMTV-transgene*.

We need to generate a second *MMTV-Cre* allele because the original transgene was a multiple copy rather than the desired single copy in the *Hprt* locus. We also need to generate the alternative orientation in the *Hprt* locus. Each of these will be assessed for activity and specificity with the ROSA reporter mice. The strain with the highest and most specific expression will be selected for Year 2 goals (ii) and (iii).

**7. Key research accomplishments:**

- a. Construction of *MMTV-Cre* transgene at the *Hprt* locus in ES cells
- b. Germ line transmission of this transgene
- c. Construction of double *loxP* targeted chromosome
- d. Evaluation of *Cre-loxP* recombination efficiencies over large genetic distances in ES cells
- e. Demonstration of long-range tissue specific *Cre* recombination event *in vivo* for one of these chromosomes at 10% efficiency.

**8. Reportable outcomes:**

- a. Manuscript about to be submitted:

Zheng B, Sage M, Sheppard EA, Jurecic V, and Bradley A (1999). Engineering mouse chromosome with *Cre-loxP*: Range, efficiency and somatic applications. *Molecular and Cellular Biology*.

**9. Conclusions:**

The most important conclusion of this initial work is that long range *Cre* recombination is active *in vivo* in specific cell types determined by the promoter driving *Cre*. There are several immediate next steps that need to be accomplished.

- a. Testing of the *MMTV-Cre* transgenes, this will be accomplished by crossing with the ROSA26 inducible reporter mice. Cells which express *Cre* stain blue with X-gal staining. Breast epithelial cells will be examined.
- b. The *MMTV-Cre Hprt* targeting will be repeated because the first allele generated was a multiple copy transgene rather than the desired single copy.
- c. A cross between a pre-deletion chromosome and a p53 mutant allele that includes the *MMTV-Cre* transgene will be made with the goal of generating mammary cells with a p53 germ line mutation on one chromosome and a somatic induced deletion on the other. These will be monitored for tumor development.

**Engineering Mouse Chromosomes with Cre-*loxP*: Range, Efficiency and Somatic Applications**

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## ABSTRACT

Chromosomal rearrangements are important resources for genetic studies. Recently, a *Cre-loxP* based method to introduce defined chromosomal rearrangements (deletions, duplications and inversions) into the mouse genome (chromosome engineering) has been established. To exploit the full potential of this technology, we have applied this strategy to various parts of mouse Chromosome 11. Although the efficiency of *Cre-loxP* mediated recombination decreases with increasing genetic distance when the two endpoints are on the same chromosome, virtually any rearrangement can be made. Rearrangements encompassing up to three quarters of Chromosome 11 have been constructed in mouse embryonic stem (ES) cells. While larger deletions may lead to ES cell lethality, smaller deletions can be produced very efficiently both in ES cells and *in vivo* in a tissue/cell type specific manner. We conclude that any chromosomal rearrangement can be made in ES cells with the *Cre-loxP* strategy provided that it does not affect cell viability. *In vivo* chromosome engineering can be potentially used to achieve somatic losses of heterozygosity in creating mouse models of human cancers.

## INTRODUCTION

Specific chromosomal rearrangements can be engineered in mice to model human chromosomal disorders, such as those associated with deletions or duplications of chromosomal segments (for example, Smith-Magenis syndrome, Downs syndrome, Charcot-Marie-Tooth type 1A) (5, 7, 10). Chromosomal rearrangements also facilitate genetic studies (2, 14). Inversion chromosomes can be used to establish balanced lethal systems to facilitate stock maintenance. Deletions can be used for mapping and for genetic screens of recessive mutations.

In *Drosophila* there is a wealth of chromosomal rearrangements that are widely used as genetic tools. In particular, chromosomal deletions (deficiencies) which collectively cover approximately 60-70% of the genome have been indispensable in mapping recessive mutations and in region specific mutagenesis screens. The use of deletions in mice, however, has been much more limited because of the paucity of chromosomal deletions which until recently were restricted to a few regions of the mouse genome flanking visible genetic markers (14). The application of the Cre-*loxP* recombination system over large distances in mouse embryonic stem (ES) cells has made it possible to engineer specific chromosomal rearrangements in the mouse (13, 17). This chromosome engineering strategy involves three steps of manipulations in ES cells: 1) one *loxP* site is targeted to one endpoint along with the 5' half of an *Hprt* gene (5' *hprt*); 2) another *loxP* site is targeted to a second endpoint with the 3' half of the *Hprt* gene (3' *hprt*); 3) transient expression of Cre recombinase results in *loxP* site-specific recombination, leading to the desired rearrangement. Reconstitution of a full-length *Hprt* gene provides a selection for the recombination products in cell culture in HAT medium. Using this technology, deletions, duplications, inversions

or translocations can be generated depending upon the relative position and orientation of the two *loxP* sites (13, 17).

The Cre-*loxP* chromosome engineering strategy provides a unique and unprecedented opportunity to manipulate the mouse genome. However, several critical questions remain to be answered in order to exploit fully the potential of this technology. First, is there any limit as to the kind and size of any rearrangements that can be made with this technology? While there are likely to be biological limits in mice, ES cells harboring large chromosomal deletions offer an opportunity to perform haploid genetic screens *in vitro*. For such applications, the larger the deletion, the more powerful the screen. Second, what is the efficiency of Cre-mediated recombination for substrates of different genetic distances? This will be pertinent to the scope and applicability of this technology. Third, can this strategy be used to engineer chromosomes somatically, that is, in a tissue/cell type specific manner without the strong positive selection schemes that are used in cell culture? Tissue specific deletions also enable recessive genetics to be employed somatically, for instance, to induce loss of heterozygosity (LOH) to model genetic changes in human cancers, or to conduct screens for novel tumor suppressor genes in combination with point mutagenesis. Somatic deletions may avoid heterozygous lethality associated with larger germline deletions and consequently a larger chromosomal region can be studied in a single animal.

To address these questions, we applied the Cre-*loxP* chromosome engineering strategy to various parts of mouse Chromosome (Chr) 11 in ES cells and *in vivo*. With an improved selection cassette, we obtained an 11% deletion efficiency for a two centiMorgan (2 cM, equivalent to 4 Mb, or megabases) deletion substrate in murine ES cells. Rearrangements of up to three quarters of Chr 11 have been made, demonstrating that there appears to be no recombination-based restriction as to

what type of rearrangements can be made provided that ES cells tolerate the genetic change. We found that the efficiency of Cre-mediated recombination between two *loxP* sites on the same chromosome (*cis*) decreases with increasing genetic distance. We found that large chromosomal deletions may be deleterious to ES cells and that deletions which were lethal to developing embryos could be engineered somatically at high efficiencies, breaking ground for somatic chromosome engineering.

## MATERIALS AND METHODS

**Construction of targeting vectors.** The *Hsd17b1* targeted cell line has been described (13). The targeting vectors for *Wnt3* (modified from a previous version, (8)) and *p53* will be described elsewhere (B. Z. and A. B., manuscript in preparation). All microsatellite markers were targeted with insertion vectors. The targeting vectors for *D11Mit199* and *D11Mit69* were modified from previous versions (8), replacing the mutant 3' *hprt* cassette with the wild type sequence.

The *D11Mit142* and *D11Mit71* loci were targeted with insertion vectors generated from a targeting-ready genomic library that contains the puromycin resistance gene, a *loxP* site, 3' *hprt* cassette, and an agouti coat color transgene in the vector backbone (22). Clones isolated from this library were mapped, and a gap was created in the region of homology which was used as the probe to detect targeting by Southern analysis. The targeting vectors for *D11Mit142* have been described (22). A clone with a 10.9 kb genomic insert at *D11Mit71* was isolated from the 3' *hprt* library and mapped with several restriction enzymes. The insert consists of two flanking (2.3 kb and 3.9 kb) and three internal (0.8 kb, 3.3 kb and 0.6 kb) *NcoI* fragments. The internal fragments were deleted from the clone to create a gap in the region of homology, resulting in targeting vector pTVD11Mit71F. The insert was then flipped using rare cutter *AscI* sites that flank the insert, resulting in targeting vector

pTVD11Mit71R which was used to deliver the *loxP* site to the *D11Mit71* locus with the reverse orientation. The 3.3 kb internal *NcoI* fragment was used as the probe in mini-Southern analysis to detect gap repair dependent targeting events (20, 22). This probe hybridizes to a 6 kb and a (weak) 2.8 kb wild type *EcoRI* fragment and in targeted clones, an additional 18.6 kb targeted fragment resulting from the insertion of the vector sequence into the targeted locus.

**Generation and analysis of chromosomal rearrangements.** ES cell cultures, gene targeting and germline transmission were performed as described (11, 12). AB2.2 ES cells were used in most experiments except in a few cases where a hybrid ES cell line (between 129S7 and C57BL/6-*Tyr<sup>c-Brd</sup>*), ER3.4, was used (E. Regel and A. Bradley, unpublished). Electroporation of the Cre expression plasmid (POG231), selection of Cre recombination products with HAT medium and drug (neomycin and puromycin) resistance tests were performed as described (8, 13) with some modifications. In a typical transient Cre expression experiment, 25  $\mu$ g of pOG231 was electroporated into  $1 \times 10^7$  ES cells. HAT selection was initiated about 48 hours after electroporation and maintained for 10 days before counting and picking the colonies. For the 2 cM substrates, electroporated cells were subject to serial dilution before plating to enable counting of the HAT resistant colonies. In all experiments a  $10^4$  dilution was also plated under no selection to count and calculate the number of colonies that survived electroporation.

**Fluorescence *in situ* hybridization.** Metaphase chromosome spreads from ES cells were prepared as described (15). FISH was performed with phage or BAC probes (isolated from a mouse genomic BAC library, Research Genetics) following a standard protocol (3). The *mPerl* phage clone, BAC 330H2 were labeled with digoxigenin and detected by anti-digoxigenin-rhodamine. BAC 293C22 and BAC 330P14 were labeled with biotin and detected with FITC-

avidin. BAC 232M23 was labeled with a mixture of digoxigenin and biotin. The chromosomes were stained with DAPI. The images were taken as monotonic pictures and the composites were made with artificial coloration for clarity.

**PCR and sequence analysis.** Primer BZL40, 5'-AGG ATG TGA TAC GTG GAA GA (*Hprt* intron, forward) and primer BZL37, 5'-GCC GTT ATT AGT GGA GAG GC (PolIII promoter in the neomycin resistance gene, reverse) were used to specifically PCR amplify a fragment containing exon 3 sequence in the 5' *hpert* cassette. Primer BZL40 and primer BZL41, 5'-CCA GTT TCA CTA ATG ACA CA (*Hprt* exon 9, reverse) were used to specifically amplify exon 3 sequence in the 3' *hpert* cassette. Primer BZL24, 5'-GCA TTG TTT TGC CAG TGT C (*Hprt* exon 6, reverse) was used to sequence exon 3 in the PCR products.

The primers used to detect the cardiac specific 2 cM deletion are: P1, 5'-CCT CAT GGA CTA ATT ATG GAC (*Hprt* exon 2, forward) and P2, the same as BZL41 (*Hprt* exon 9, reverse). The primer pair used to detect the  $\alpha$ MyHC-Cre transgene has been described (1).

## RESULTS

### **High efficiency Cre-*loxP* based chromosomal engineering with an improved vector in mouse embryonic stem cells**

Sequence analysis identified a frame shift mutation in the coding portion of the 3' *hpert* selection cassette (Fig. 2) previously successfully used for chromosome engineering (13), leading to a translation stop codon nine codons downstream of the mutation (Fig 2A). This mutation should render a reconstituted *Hprt* minigene non-functional, yet HAT resistant colonies were obtained with this cassette. These may have resulted from a repair event during or following Cre

recombination (see below). Since the events we have scored to date required selection, the efficiency of Cre mediated *loxP* site-specific recombination on multi-megabase substrates may be greater than that scored by the number of selected HAT resistant clones. Because the recombination efficiency is pertinent in applications of the Cre-*loxP* based chromosome engineering strategy, we reassessed this efficiency for a 2 cM interval between *Hsd17b1* (*E<sub>2</sub>DH*) and *D11Mit199* on Chr 11 (8) using cassettes without the frameshift mutation. The *D11Mit199* locus was re-targeted with the corrected 3' *hpvt* cassette in an ES cell line that had been targeted at the *Hsd17b1* locus with the 5' *hpvt* cassette (13) so that the *loxP* sites were in the same orientation (8). The double targeted cell lines were electroporated with a Cre expression plasmid and the recombination efficiency assessed (defined here as the number of HAT resistant colonies per cell surviving electroporation). Approximately half of the double targeted clones yielded recombination efficiencies of approximately 11% while the rest had efficiencies of approximately 0.047%, Table 1. Cre recombination products from the former group are puromycin sensitive (indicating that these cell lines have a deletion) while those from the latter group are puromycin resistant (indicating that these cell lines have a deletion and a duplication). Therefore, the two different efficiencies reflect the configuration of the targeted *loxP* sites on the Chr 11 homologues in the parental cell lines, in *cis* which is resolved into a deletion and in *trans* which is resolved into a deletion and a duplication (8, 13). The *cis* events occurred several hundred times more efficiently than the *trans* events. As a control, the 2 cM substrate with the mutant 3' *hpvt* cassette gave a Cre recombination efficiency of 0.007% for *cis* and 0.0001% for *trans* (8). Thus, the Cre recombination efficiency is improved by approximately three orders of magnitude after correction of the 3' *hpvt* selection cassette.

### **Coupled Cre-*loxP* recombination and gene conversion**

The *Hprt* cassette reconstructed by Cre-*loxP* recombination from the mutant 3' *hprt* selection cassette should be non-functional. However, HAT resistant colonies were readily obtained (8, 13). This raised the question as to what event leads to the HAT resistant colonies in these experiments. The frequency of spontaneous reversion is too low to explain the observed frequency of HAT-resistant clones from the mutant cassette. The frame shift mutation is located in a 2 kb region that overlaps between the 5' and the 3' *hprt* cassettes, and therefore the mutation in the 3' cassette may be corrected by homologous recombination using sequences in the 5' cassette. We hypothesized that Cre brings the two *loxP* sites together to promote site-specific recombination and during or immediately after this process the endogenous homologous recombination machinery repairs the mutation (Fig. 2B). This would predict that all recombination products would have the wild type exon 3 sequence rather than a correcting single nucleotide insertion resulting from a spontaneous reversion.

Sequence analysis demonstrated that all HAT resistant colonies had acquired a wild type sequence in the reconstituted full length *Hprt* minigene (n = 10). *Trans* events generate an *Hprt*<sup>+</sup> deletion chromosome and the reciprocal product, a duplication chromosome, which retains the recombined overlapping region between the 5' and 3' cassettes. Sequence analysis of PCR products from exon 3 in the reciprocal product revealed that this exon 3 remained wild type in all cases analyzed (n = 17), indicating that the repair results from a gene conversion event.

### **Long range chromosomal rearrangements can be made in embryonic stem cells**

Our chromosome engineering strategy has primarily focused on deletions, duplications and inversions of a few centiMorgans (8, 13). The ability to manipulate a larger region of the chromosome is desirable in many instances. For example, large inversions, when marked with a

recessive lethal mutation, can be used as balancer chromosomes (2). ES cells with a large deletion may be useful in screens for recessive mutations *in vitro*. Since the apparent Cre recombination efficiency was dramatically increased with the corrected 3' *hpvt* cassette, we tested whether long range (defined here as tens of megabases) deletions can be made in ES cells.

A deletion of 22 cM between *Hsd17b1* and *D11Mit69* on Chr 11 was used for this test. Previous attempts to generate this deletion in ES cells with the mutant 3' *hpvt* cassette had failed (8). The *D11Mit69* locus was targeted with the 3' *hpvt* cassette oriented for a deletion in an ES cell line that had been targeted at *Hsd17b1* (8). Fifteen double *loxP* targeted cell lines were transiently transfected with a Cre expression plasmid and HAT resistant colonies were counted after 12 days. Drug resistance tests indicated that four parental cell lines were double targeted in *cis* and eleven were in *trans*. Unlike previous *cis-trans* tests, however, Cre recombination for both configurations occurred at a similar efficiency of approximately  $3 \times 10^{-5}$  (Table 1 and see below). We further successfully generated a number of long range rearrangements on Chr 11 (Fig. 3, Table 1). The most dramatic example is illustrated in Fig. 4D, where Cre recombination between two *loxP* sites targeted in *trans* to *Hsd17b1* and *D11Mit71* that are 60 cM away from each other on Chr 11 leads to a mini deletion chromosome and a large duplication chromosome. Therefore, long range chromosomal rearrangements, including deletions and deletion/duplications, can be generated with the improved selection cassette.

### **Large chromosomal deletions may cause ES cell lethality**

The Cre mediated deletion efficiency for the *cis* configuration differs by more than three orders of magnitude between a 2 cM (*Hsd17b1* - *D11Mit199*) and a 22 cM (*Hsd17b1* - *D11Mit69*) substrate (Table 1). The reduced Cre recombination efficiency for a larger substrate may simply reflect a

lower efficiency of Cre-*loxP* juxtaposition with greater physical separation. However, it is also possible that ES cells with larger deletions may be selected against if the deletion has deleterious effects on cell viability or growth. In this scenario, only cells that have undergone a compensatory genetic change would survive. To test this, the deletion cell lines were analyzed by fluorescence *in situ* hybridization (FISH) with probes both internal and external to the deletion interval.

Intriguingly, of five recombination products derived from three independent *cis* double targeted parental cell lines, all were trisomy 11 with two wild type and one deletion chromosome. The two wild type chromosomes were found to exist as two separate chromosomes (as in Fig. 4B, three out of five analyzed), or as a Robertsonian fusion in other cases (as in Fig. 4C, two out of five analyzed, both of which derived from an independent double targeted parental cell line). In contrast, the majority (three out of four) of the *trans* recombination products analyzed contain the expected single deletion and duplication chromosomes. The remaining *trans* product contained a duplication chromosome and two deletion chromosomes in the Robertsonian configuration. All double targeted parental cell lines analyzed, irrespective of *cis* or *trans*, contain two wild type chromosomes (data not shown). These results indicate that the deletion in *cis*, which leads to a single copy of the 22 cM region of Chr 11, is haploinsufficient in ES cells. Consequently rare variants are selected in which the remaining wild type chromosome is duplicated. Thus, the hemizygous 22 cM deletion causes ES cell lethality or a severe growth disadvantage.

### **Cre-*loxP* recombination efficiency decreases over increasing genetic distances**

The Cre recombination efficiency is an important consideration in designing Cre-*loxP* based chromosome engineering experiments. To provide a framework for future experiments, we determined this efficiency for *cis* events at different genetic distances. Since a 22 cM deletion had been observed to cause cell death or a growth disadvantage, we assessed the efficiency of

inversions as the indicator of Cre recombination efficiency for the larger intervals. Four rearrangements were included in this analysis: 1) a 2 cM deletion between *Hsd17b1* and *D11Mit199*, Del(11)4Brd ; 2) a 24 cM inversion between *p53* and *Wnt3*, In(11)8Brd; 3) a 30 cM inversion between *Hsd17b1* and *D11Mit142*, In(11)6Brd; 4) a 60 cM inversion between *Hsd17b1* and *D11Mit71*, In(11)7Brd (Fig. 3). When the two *loxP* sites are in opposite orientations, approximately half of the independent double targeted cell lines give HAT resistant colonies (interpreted as *loxP* sites in *cis*) and the other half do not give any colonies (interpreted *loxP* sites in *trans*) presumably due to the formation of dicentric and acentric chromosomes. FISH analysis confirmed that the relevant inversions had occurred in representative clones from all three large genetic intervals (data not shown). As shown in Fig. 5, between 2 cM and 60 cM, the logarithm of the Cre recombination efficiency is inversely proportional to the genetic distance between the *loxP* sites.

### **Tissue specific chromosome engineering**

Several deletions of a few centiMorgans around the *Hsd17b1* locus on Chr 11 are heterozygous lethal (8). Although this underscores the developmental importance of this chromosomal region, lethal deletions cannot be used for genetic screens. However, if the deletion can be made somatically, for instance, in a tissue or cell type specific manner, the problem of heterozygous lethality can be partially circumvented. To test this possibility, we generated a 2 cM *Hsd17b1* – *D11Mit199* double targeted mouse line (deletion substrate) and crossed it to a cardiac specific Cre (Cre coding sequence under the control of the  $\alpha$ -Myosin Heavy Chain promoter, abbreviated as  $\alpha$ MyHC-Cre) (1). The  $\alpha$ MyHC-Cre line had previously been used to make cardiac specific deletion of several kilobases with an up to 90% efficiency (1). Tissue DNA was isolated from two progeny that inherited both the  $\alpha$ MyHC-Cre transgene and the 2 cM substrate. PCR analysis with

primers specific to the reconstituted *Hprt* minigene was performed to determine whether the Cre-mediated recombination had occurred (Fig. 6A). This analysis demonstrated that the Cre recombination occurred in heart, but not in muscle, liver, lung or spleen (Fig. 6A). To provide a more quantitative measure of Cre recombination, Southern analysis was performed on two animals using restriction digestions and a probe at *Hsd17b1* that would distinguish the wild type allele, the (double) targeted allele and the rearranged allele (Fig. 6B). Deletion occurred exclusively in heart but not in other organs tested (Fig. 6B). Based on the ratio of intensity of recombined fragment vs. pre-recombined allele for both animals tested, the deletion efficiency in heart is about 10%.

## DISCUSSION

The organism that the Cre-*loxP* system is derived from, bacteriophage P1, uses the system to resolve its ~100 kb genome into monomeric circular form (18). The Cre-*loxP* site-specific recombination system has been extensively used for conditional genetic technology, namely, the temporal and spatial control of gene expression in mice (16). In these applications, the genetic material involved (as determined by the distance between the two *loxP* sites) is usually a few kilobases. We have previously shown that this system can be adapted for substrates of several megabases by incorporating a positive selection scheme (8, 13). In the present study, we redefined the Cre recombination efficiency for a 4 Mb substrate, after correcting a mutation in the selection cassette. Surprisingly, the efficiency for this substrate is approximately 11% by transient Cre expression. This efficiency approaches that obtained with substrates of several kilobases and indicates that between several kilobases and several megabases the Cre-*loxP* recombination occurs at comparable efficiencies. This might reflect aspects of chromatin domain organization such that sequences that are 1 kb to 1 Mb apart may have similar separations in 3 dimensional space.

In addition, since our *in vivo* efficiency using a cardiac specific Cre is similar to that observed in the cell culture assay, the Cre recombination efficiency obtained in cell culture appears to be a reasonable estimate for the efficiency *in vivo*. In this aspect, the fact that tissue/cell type chromosomal deletions can be achieved at a 10% efficiency is of particular significance. In many cancers, interstitial deletions are the dominant mode for loss of the remaining allele of a tumor suppressor gene (6). Therefore, *in vivo* chromosomal deletions can be used to mimic somatic LOH in human cancers and in searches for novel tumor suppressor genes in combination with point mutagenesis.

The 22 cM deletion between *Hsd17b1* and *D11Mit69* on Chr 11 appear to cause ES cell lethality or a severe growth disadvantage because deletion products for this interval exclusively carry an additional wild type Chr 11. This may be due to a dosage effect of one or multiple genes in this interval such that a single copy of these genes cannot support the normal growth of ES cells (haploinsufficiency). The Cre-*loxP* mediated deletion of this 22 cM region therefore selects for cells that have duplicated the wild type Chr 11. This result underscores the tight control of the euploid ES cell genome. A region of haploinsufficiency has also been proposed to reside on Chr 9 in studies on a radiation induced deletion complex (19). This is in direct contrast with many cancer cells that are associated with large chromosomal deletions and chromosomal losses. Such a unique feature of ES cells may be further studied by isolating suppressors of this lethality caused by the deletions. On the other hand, these data indicate that duplications are tolerated better than deletions in ES cells. This is consistent with the notion that monosomies rarely, if ever, exist while trisomy 8, 11, 15 and several other chromosomes have been observed in ES cells (9). The relatively frequent occurrence of trisomy 11 is further suggested by our observation that one out of four 22

cM deletion/duplication products analyzed by FISH contain one duplication and two deletion chromosomes where the deletion chromosome is presumably not required to be duplicated for cell survival or growth. The lethality caused by large deletions in ES cells precludes a straightforward approach to use the deletion as a partial haploid reagent in mutagenesis screens. However, this may be circumvented by schemes that introduce point mutations followed by inducing deletions in the ES cells.

It is possible that the partial trisomy ES cells selected by the 22 cM *cis* deletion are derived from an underlying trisomy 11 population in the ES cells transfected with Cre. Although these cells are not detected by analysis of double targeted clones, extrapolation of the inversion recombination efficiencies suggest that either these cells are present at  $10^{-2}$  frequencies in the transfected clones, or that this non-disjunction event is induced by the Cre-*loxP* recombination event itself.

Large deletion associated ES cell lethality can obscure the Cre recombination efficiency. We therefore determined the Cre efficiency using large inversion substrates. This analysis indicates that Cre recombination efficiency decreases over increasing genetic distances. However, in all cases, the recombination products (HAT resistant colonies) are readily obtained in a single experiment except when inviable products are generated (dicentric and acentric chromosomes). For multi-megabase substrates, the logarithm of the Cre recombination efficiency is approximately inversely proportional to the genetic distance (Fig. 5). This can be used as a guide for future experiments using Cre-*loxP* based chromosome engineering. However, other factors such as chromosomal locations and differences in experimental manipulations may affect the Cre recombination efficiency. For deletions, the Cre recombination drops more precipitously as the genetic distance increases for two reasons. First, the physical barrier Cre has to overcome to bring the two *loxP*

sites together is greater as the distance between the two *loxP* sites increases, as in inversions. Second, larger deletions may cause ES cell lethality or a growth disadvantage and are consequently selected against after Cre recombination. In the *trans* configuration where a deletion and a duplication are the product, Cre recombination efficiency is moderately reduced with an increasing genetic distance (Table 1). If *trans* recombination occurs mainly in G2 and recombined sister chromatids tend to segregate away from each other, as reported in *Drosophila* (4), the HAT resistant deletion products will frequently contain a wild type chromosome instead of the duplication chromosome. In this scenario, *trans* deletion/duplication events involving a larger distance will occur at a lower frequency due to the production of haploinsufficient deletions.

The Cre recombination efficiency for large deletion/duplication is probably comparable to that for translocations between non-homologues. In some of our experiments, we analyzed some random integration clones when targeting the second *loxP* site. Upon Cre expression, approximately half of these clones give HAT resistant colonies and the other half do not give viable HAT resistant colonies. The former group presumably yields translocations while the second group yields dicentric and acentric products. The efficiency of generating these translocations is about  $10^{-5}$ . It has been reported that Cre recombination efficiency for a translocation between chromosomes 12 and 15 using a similar strategy occurs at about  $10^{-7}$  (17). The higher efficiency in our experiments may be due to the Cre plasmid, the tissue culture conditions and/or electroporation procedures used. It remains possible that the two kilobase homology between our 5' *hpvt* and 3' *hpvt* cassette assists the Cre-*loxP* recombination by recruiting the homologous recombination machinery to help secure the *loxP* site recombination synapse.

The mutant 3' *hpvt* cassette used in previous chromosome engineering experiments provides a unique opportunity to study a potential interaction between homologous and site-specific recombination. Sequence analysis of Cre recombination products indicates that the mutation is repaired by homologous recombination with the wild type template in the 5' *hpvt* cassette. This homologous recombination can not occur in absence of site-specific recombination since the homology is only 2 kb and the distance between the substrates is large (several megabases along the chromosome). Therefore, it must have occurred during or immediately after the *Cre-loxP* recombination. It is possible that the Holliday junction structure created by Cre (21) can be resolved by homologous recombination machinery. This scenario would suggest that the two recombination events are not mutually exclusive and can be coupled under specific circumstances. The other possibility is that *Cre-loxP* recombination facilitates gene conversion merely by bringing the two substrates together. Immediately after site-specific recombination, homologous recombination occurs. As HAT resistant colonies for a 2 cM substrate are obtained with an efficiency of approximately three orders of magnitude higher with the wild type 3' selection cassette than with the mutant version, homologous recombination responsible for repairing the mutation occurs at about 0.1% of the time after Cre recombination.

Taken together, the *Cre-loxP* chromosome engineering strategy provides a powerful tool for genetic studies and for genome manipulation. We explored the possibility and determined the efficiency of generating various chromosomal rearrangements on mouse Chr 11. We conclude that any desired rearrangement can be made with the *Cre-loxP* system provided that the rearrangement does not have any deleterious effect on the ES cells. *Cre-loxP* recombination is very efficient for substrates of a few centiMorgans both in tissue culture and *in vivo*. This efficiency decreases over

increasing genetic distances between the two *loxP* sites. The work presented here provides a framework for future applications of chromosome engineering.

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## FIGURE LEGENDS

FIG. 1. The Cre-*loxP* based chromosome engineering strategy

5' *hprt* is previously named *hprt* $\Delta$ 3'; 3' *hprt* is previously name *hprt* $\Delta$ 5'. A neomycin (*Neo*) or a puromycin (*Puro*) resistance gene is linked to the first or the second *loxP* site respectively for positive selection during gene targeting. In this case, Cre recombination between two *loxP* sites targeted in the same orientation in *cis* (on the same chromosome) leads to a deletion that is neomycin and puromycin sensitive due to the loss of the *Neo* and *Puro* carrying reciprocal product, a ring chromosome. If the two *loxP* sites are on the two different chromosome homologues (or in *trans*), a deletion and a duplication will be produced. The rearrangements can then be transmitted through the mouse germline if viable.

FIG. 2. A frame shift mutation in the original 3' *hprt* cassette used in chromosome engineering

(A) Partial exon 3 sequence of the wild type (WT) and mutant 3' *hprt* cassette with conceptual translation. The thymidine residue in the wild type sequence that is deleted in the mutant is in bold and underlined. The altered amino acid residues affected by the mutation is in bold italicized. "\*" = stop codon. (B) A proposed mechanism by which HAT resistant colonies were obtained by a combination of Cre-*loxP* site-specific recombination and homologous recombination. P = promoter. 1 – 2, 3 – 6 and 3 – 9 refer to exons. The portion of the coding sequence affected by the mutation in exons 3 – 9 is shaded. The polyadenylation signal, neomycin and puromycin resistance genes are not shown for simplicity.

FIG. 3. Genetic intervals of rearrangements made on mouse Chr 11 in this study

2 cM, *Hsd17b1* – *D11Mit199*, deletion, deletion/duplication; 22 cM, *Hsd17b1* – *D11Mit69*, deletion, deletion/duplication; 24 cM, *Wnt3* – *p53*, inversion, deletion/duplication; 30 cM, *Hsd17b1* – *D11Mit142*, inversion, deletion/duplication; 60 cM, *Hsd17b1* – *D11Mit71*, inversion, deletion/duplication. The total genetic distance from centromere (Cen) to telomere (Tel) is about 80 cM.

FIG. 4. FISH analysis of long range Cre recombination products on Chr 11

(A) Del(11)5Brd/Dp(11)5Brd, a 22 cM deletion chromosome and a 22 cM duplication chromosome produced by a *trans* event between *Hsd17b1* and *D11Mit69*. Red = an *mPer1* phage clone (external probe); Yellow = BAC 292C22 (*D11Mit199*, internal probe). (B) Del(11)5Brd/+/+, a 22 cM deletion chromosome produced by a *cis* event between *Hsd17b1* and *D11Mit69* while the remaining wild type chromosome is duplicated to survive. Same probes as in (A). (C) The same as in (B) except that the duplicated wild type chromosomes are in a Robertsonian configuration. (D) Del(11)8Brd/Dp(11)7Brd, a 60 cM deletion chromosome and a 60 cM duplication chromosome produced by a *trans* event between *Hsd17b1* and *D11Mit71*. Yellow = BAC 232M23 (*D11Mit320*, internal probe 1); Red = BAC 330H2 (*D11Mit263*, internal probe 2); Green = BAC 330P14 (*D11Mit11*, external probe). Vertical lines within the chromosomes indicate the location of *loxP* sites.

FIG. 5. The efficiency of Cre recombination over genetic distance

Percentage of Cre recombination efficiency (Y-axis, in log scale) is plotted against the genetic distance (X-axis, in linear scale). The first data point represents a deletion. The other three with larger genetic distances represent inversions.

FIG. 6. Cardiac specific 2 cM deletion between *Hsd17b1* and *D11Mit199*

(A) PCR analysis of the deletion products. P1 and P2, primers used to specifically amplify the full-length *Hprt*. PCR reactions on  $\alpha$ MyHC-Cre serve as a control. N – negative control; P – positive control; H – heart; M – muscle; Li – liver; Lu – lung; S – spleen. (B) Southern analysis of the deletion products. C – control cell line that contains a deletion and a wild type chromosome. The cardiac specific deletion band is indicated by an asterisk (\*). *N* = *NheI*; *S* = *SfiI*. Solid triangle = *loxP* site.

## TABLE

Table 1. Efficiency of Cre mediated *loxP* site-specific recombination over different genetic distances

Interval (5' <i>hprt</i> - 3' <i>hprt</i> )	Genetic distance	Cre recombination efficiency <sup>a</sup> (Number of colonies in HAT/No Drug)		
		<i>loxP</i> sites in opposite orientation	<i>loxP</i> sites in the same orientation	
		<i>cis</i> (inversion) <sup>b</sup>	<i>cis</i> (deletion)	<i>trans</i> (deletion/duplication)
<i>Hsd17b1</i> - <i>D11Mit199</i>	2 cM	ND <sup>c</sup>	$1.1 \pm 0.5 \times 10^{-1}$ (n = 8)	$4.7 \pm 0.7 \times 10^{-4}$ (n = 5)
<i>Hsd17b1</i> - <i>D11Mit69</i>	22 cM	ND <sup>c</sup>	$3.7 \pm 2.4 \times 10^{-5}$ (n = 4)	$3.1 \pm 1.1 \times 10^{-5}$ (n = 11)
<i>Wnt3</i> - <i>p53</i>	24 cM	$2.2 \pm 0.6 \times 10^{-3}$ (n = 5)	$2.9 \times 10^{-5}$ (n = 1) <sup>d</sup>	$8.2 \pm 0.9 \times 10^{-5}$ (n = 5)
<i>Hsd17b1</i> - <i>D11Mit142</i>	30 cM	$3.2 \pm 0.7 \times 10^{-4}$ (n = 3)	$9.8 \pm 1.7 \times 10^{-6}$ (n = 2) <sup>d</sup>	$5.9 \pm 4.7 \times 10^{-5}$ (n = 5)
<i>Hsd17b1</i> - <i>D11Mit71</i>	60 cM	$8.3 \pm 0.8 \times 10^{-5}$ (n = 2)	$9.5 \pm 2.1 \times 10^{-7}$ (n = 2) <sup>d</sup>	$1.4 \pm 0.2 \times 10^{-5}$ (n = 4)

<sup>a</sup> Numbers are in mean  $\pm$  standard deviation

<sup>b</sup> No colonies were obtained with *trans* configuration because dicentric and acentric chromosomes are produced

<sup>c</sup> ND - not determined

<sup>d</sup> not confirmed by FISH analysis

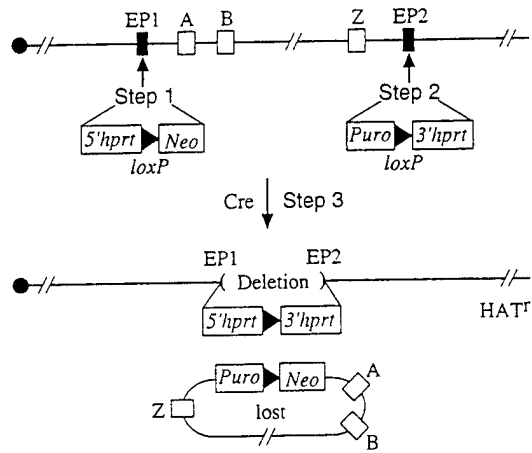


Fig. 1, Zheng et al.

**A** exon 3 →

WT GACTGAACGTCTTGCTCGAGATGTGATGAAGGAGATGGGA  
 T E R L A R D V M K E M G  
 GGCCATCACATTGTA..  
 G H H I V ..

Mutant GACTGAACGTCTTGCTCGAGATGTGAGAGAAGGAGATGGGAG  
 T E R L A R D V R R R W E  
 GCCATCACATTGTAG..  
 A I T L \*

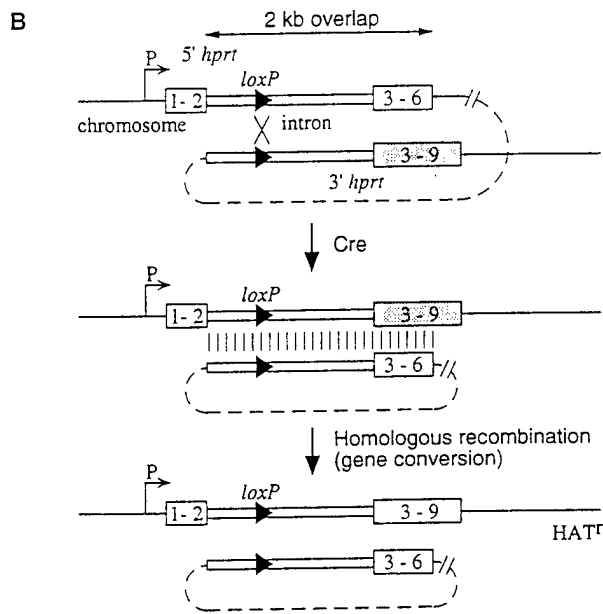


Fig. 2, Zheng et al.

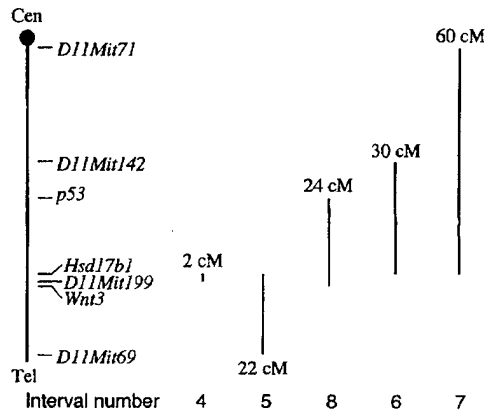


FIG. 3. Genetic intervals of rearrangements made on mouse Chr 11 in this study. 2 cM, *Hsd17b1-D11Mit199*, deletion, deletion-duplication; 22 cM, *Hsd17b1-D11Mit69*, deletion, deletion-duplication; 24 cM, *Wnt3-p53*, inversion, deletion-duplication; 30 cM, *Hsd17b1-D11Mit142*, inversion, deletion-duplication; 60 cM, *Hsd17b1-D11Mit71*, inversion, deletion-duplication. The total genetic distance from centromere (Cen) to telomere (Tel) is about 80 cM.

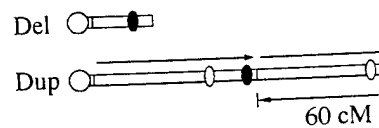
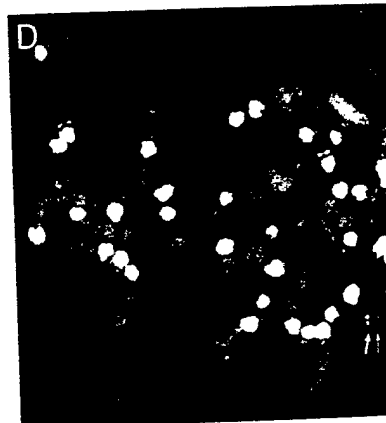
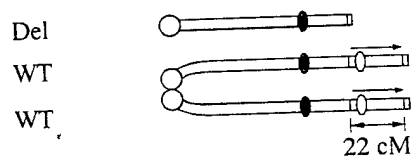
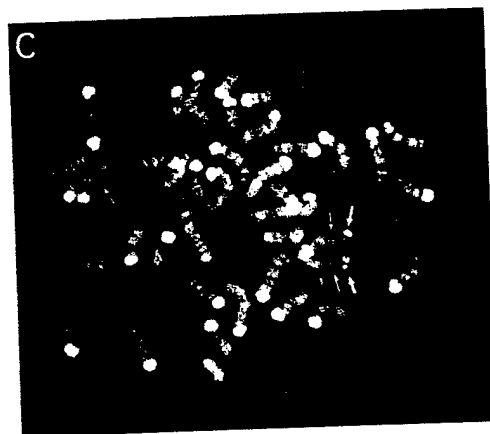
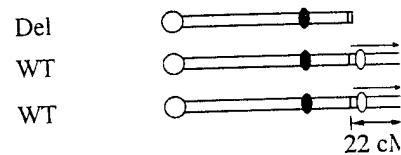
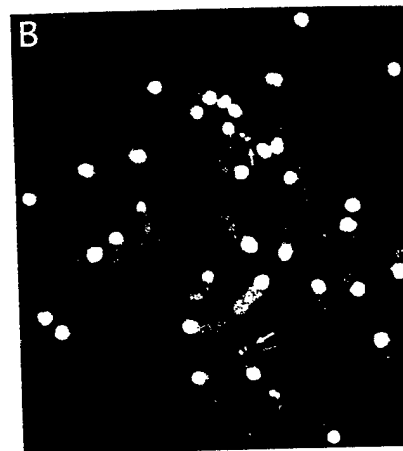
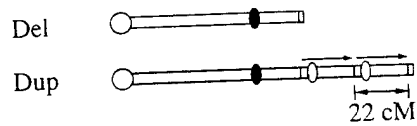
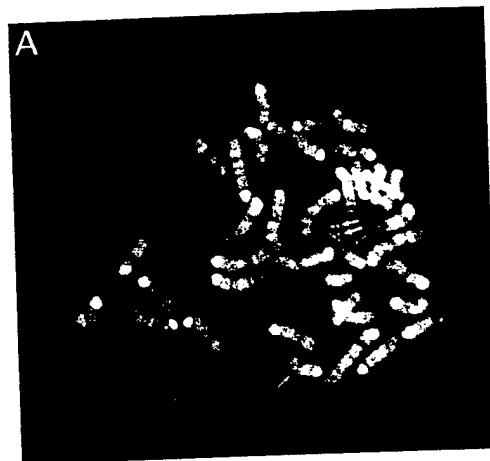


Figure 4

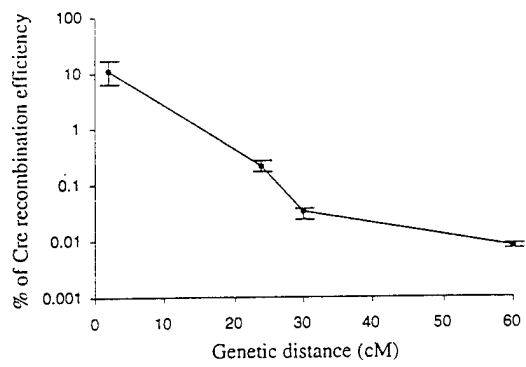


Fig. 5, Zheng et al.

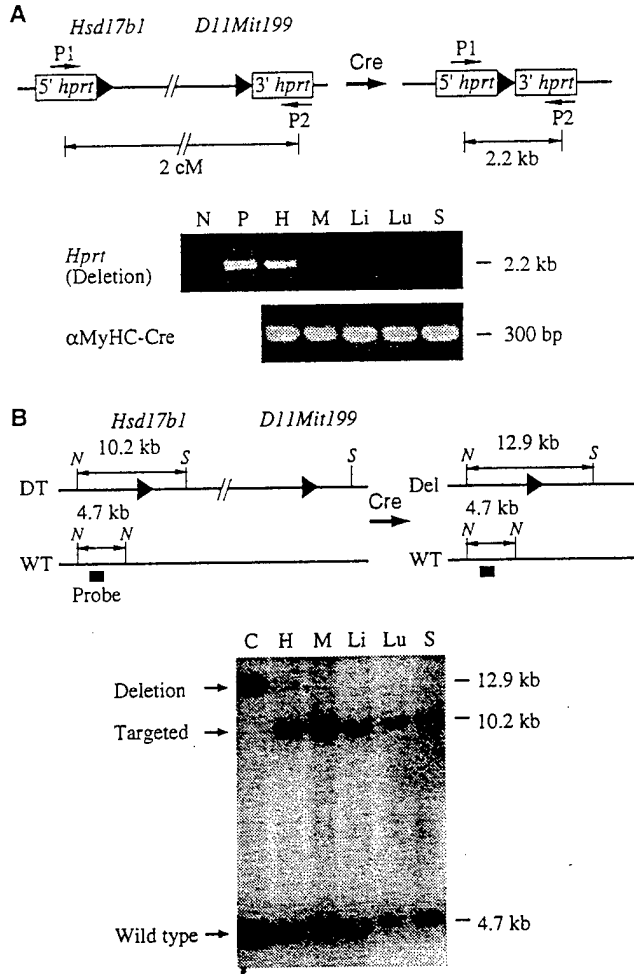


Fig. 6, Zheng et al.