Efficient in vivo manipulation of mouse genomic sequences at the zygote stage
(transgenic/Cre recombinase/loxP)

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ABSTRACT  We describe a transgenic mouse line carrying the cre transgene under the control of the adenovirus EIIa promoter that targets expression of the Cre recombinase to the early mouse embryo. To assess the ability of this recombinase to excise loxP-flanked DNA sequences at early stages of development, we bred EIIa-cre transgenic mice to two different mouse lines carrying loxP-flanked target sequences: (i) a strain with a single targeted neomycin resistance gene flanked by loxP sites and (ii) a transgenic line carrying multiple transgene copies with internal loxP sites. Mating either of these loxP-carrying mouse lines to EIIa-cre mice resulted in first generation progeny in which the loxP-flanked sequences had been efficiently deleted from all tissues tested, including the germ cells. Interbreeding of these first generation progeny resulted in efficient germ-line transmission of the deletion to subsequent generations. These results demonstrate a method by which loxP-flanked DNA sequences can be efficiently deleted in the early mouse embryo. Potential applications of this approach are discussed, including reduction of multicity transgene loci to produce single-copy transgenic lines and introduction of a variety of subtle mutations into the germ line.

The Cre/loxP system is rapidly becoming a major tool for modification of gene expression in mice (1–7). The approach uses the ability of the Cre recombinase of bacteriophage P1 to catalyze conservative reciprocal recombination events in mammalian cells (8). Genes whose function is to be altered carry inserts of 34 bp loxP sequences, the target sites of Cre-mediated recombination in bacteriophage P1. When Cre enzyme is provided, the sequences between directly repeated loxP sites are efficiently excised. In its convenient binary form, the experiment uses two strains of mice. One carries a transgene that is designed to express Cre enzyme in a tissue of interest. The other strain carries loxP sites at a transgene insertion or endogenous gene locus. The two strains are crossed to produce progeny that express Cre in the tissue of interest and that have excised chromosomal sequences between the loxP sites. Cre brought in by mating displays a remarkable efficiency of recombination in the developing animal, as tested in the context of Cre-mediated activation of a dormant transgene (1, 3) or Cre-mediated inactivation of a resident chromosomal gene (5, 7).

The present study addresses the efficiency of Cre-mediated DNA recombination in the specific context of the zygote stage of embryo development. We have generated transgenic mice in which the adenovirus EIIa promoter directs Cre expression selectively to preimplantation embryos (9). The ability of the EIIa-regulated recombinase activity to excise DNA sequences in vivo was assessed in two experiments. In the first experiment, we bred EIIa-cre mice to gene-targeted mice homozygous for the insertion of a loxP-neo-loxP cassette, a constitutively expressed marker commonly used in gene targeting (10, 11). The second experiment was aimed at varying transgene copy numbers at a given insertion site. EIIa-cre mice were mated with transgenic mice carrying multiple copies of maA-loxP-TAg, a lens-specific cassette in which the loxP site was placed between a maA-crystallin promoter sequence and simian virus 40 (SV40) sequences encoding the tumor antigens (TAg). Using the Cre/loxP system, we wished to determine whether changes in TAg transgene copy number at a fixed genomic integration site would affect the timing of TAg accumulation and the subsequent patterns of oncogenesis in the differentiating lens.

Our results show that sequences between loxP sites can be readily excised from developing mice exposed to Cre activity at an early stage of embryonic development. The resulting gene alterations are genetically fixed and therefore passed on to progeny. In the examples shown, this strategy allowed efficient excision of DNA sequences at a targeted chromosomal locus, and reduction of the transgene copy number at a preset transgene insertion site.

MATERIALS AND METHODS

Generation of EIIa-cre Transgenic Mice. The EIIa-cre plasmid was constructed by replacing the human cytomegalovirus promoter in pBS185 (12) with the adenovirus EIIa promoter from pEII-lacZ (9). The construct was microinjected into mouse zygotes for production of transgenic mice (13). Cre activity present in zygotes of the EIIa-cre genotype was measured as follows. EIIa-cre/EIIa-cre homozygous animals were mated with +/maA-loxP-STOP-loxP-TAg heterozygous animals (1, 3) and the presence of lens tumors in double transgenic progeny, indicative of Cre-mediated excision of STOP sequences and a concomitant activation of SV40 TAg, was scored.

Breeding of EIIa-cre Mice with loxP-neo-loxP Gene-Targeted Mice. The gene-targeted mice carry a specific gene-targeted mutation that replaces a region 8.5 Kb 3' of the immunoglobulin light chain kappa constant region (J.R.G., unpublished)

Abbreviations: SV40, simian virus 40; TAg, tumor antigen; E, embryonic day; ES cells, embryonic stem cells; maA, murine αA-crystallin gene promoter.
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5860
work) with a single neomycin phosphotransferase gene, flanked by loxP sites (loxP-neo-loxP). Female mice homozygous for the loxP-neo-loxP locus were bred with male mice homozygous for the EIIa-cre transgene. Deletion of the neo gene in F1 offspring was assayed by polymerase chain reaction (PCR) of tail DNA. The following primers were used in the assays of Fig. 1: a, 5'-gagcgtatattaactcattac-3'; b, 5'-ctcttcgagagcttggtcttctgag-3'; c, 5'-actaactattgcagggcaggtggttg-3'. PCR reactions were carried out as described (14) and included 200 ng of tail DNA and 2.5 units of AmpliTaq Polymerase (Perkin-Elmer). PCR conditions: 94°C, 2 min, followed by 45 cycles of 94°C, 30 sec; 50°C, 45 sec; and 65°C, 90 sec. Products were separated by electrophoresis on a 1.5% agarose gel, and were visualized by staining with ethidium bromide. F1 mice with complete deletion of the neo gene in the tail were interbred to obtain F2 mice that were subsequently assayed by PCR of tail DNA for deletion of the neo gene and for the presence or absence of the cre transgene.

Breeding of EIIa-cre Mice with Multicopy (maa-loxP-TAg)n Transgenic Mice. The maa-loxP-TAg cassette was derived by deleting STOP from the previously described construct maa-loxP-STOP-loxP-TAg (1). Multicopy (maa-loxP-TAg)n transgenic mice were generated by zygote injection of the maa-loxP-TAg cassette. Multicopy (maa-loxP-TAg)n heterozygous males were mated with homozygous EIIa-cre females to generate single-copy maa-loxP-TAg progeny. Pregnancies were timed by administration of gonadotrophins to females before copulation and embryonic eyes were collected at different developmental stages for morphological and immunohistochemical analyses. The embryonic torsos or extraembryonic membranes were used as a source of DNA for PCR and Southern blot analysis. PCR reactions were performed as described (1). For Southern blot analysis, genomic DNA (7 μg) was extracted from embryonic torsos or extraembryonic membranes of adult mice. Tail tips or digested embryos were transferred to nylon filters (DuPont), and hybridized as described (15). A 1169-bp HindIII fragment from SV40 TAg and a 400-bp fragment of the 5' portion of cre were used as hybridization probes for analysis of TAg and cre transgene sequences, respectively. Histological and immunohistochemical analyses of embryonic mouse eye sections was performed as described (3).

RESULTS

Cre Encoded by the EIIa-cre Transgene Efficiently Deletes a loxP-neo-loxP Cassette in Vivo. The EIIa-cre transgene was designed in an effort to direct Cre recombinase activity to the one-cell mouse embryo. The EIIa promoter confers the Cre action to a very early stage of preimplantation embryogenesis, most likely to one-cell embryos (9). Cre-mediated recombination of the genome, if indeed taking place in the zygote, should generate a gene alteration that would be present in all cells of the developing animal, including its germ cells, and that would be transmitted to progeny. To test whether the product of the EIIa-cre transgene would efficiently delete a single loxP-X-loxP cassette in vivo, we crossed homozygous EIIa-cre mice with homozygous mice carrying a targeted insertion of a single copy loxP-neo-loxP cassette in the immunoglobulin light chain kappa locus (J.R.G., unpublished work). Homozygous female loxP-neo-loxP mice were selected based on screening of tail DNA by Southern blot analysis (data not shown) and were bred with homozygous male Ia-cre transgenic mice.

The F1 offspring, all of which were heterozygous for the chromosome carrying the EIIa-cre transgene array and also for the loxP-neo-loxP targeted chromosome, were assayed by PCR and Southern blot analyses of tail DNA for the presence or absence of the neo gene on the targeted chromosome. The PCR assay was designed to detect both the loxP-neo-loxP targeted chromosome (called the NEO allele) and the loxP configuration that remains at the targeted locus after Cre enzyme has excised the neo gene (the ΔNEO allele). Representative results are shown in Fig. 1. In total, 56 F1 mice were screened. These mice contained a single target for Cre action in each cell. In each of these mice, the ΔNEO allele was detectable in tail DNA, indicating that all individuals had undergone Cre-mediated excision of the neo sequence in at least a portion of cells present in the tail tissue examined. Of the 56 F1 tail DNA samples, 28 (50%) showed both the NEO and ΔNEO PCR products. This finding indicates that a proportion of tail cells in this half of the cohort failed to undergo Cre-mediated neo excision. Persistence of the undetected NEO allele in a proportion of cells may reflect Cre action past the zygote stage, resulting in some mosaicism. The remaining 28 out of 56 F1 mice (50%) had undergone complete excision of the neo sequences as suggested by the fact that we detected only the ΔNEO PCR product. To test whether absence of a detectable loxP-neo-loxP cassette in tail DNA reflected complete excision in the germ cells, F1 mice that displayed complete neo excision in the tail were selected for breeding to obtain F2 progeny.

The neo Excision Is Fixed in the Germ Line and Transmitted to F2 Mice. Male and female F1 offspring judged by tail DNA PCR as ΔNEO, having lost the neo gene, were interbred to assay for germ-line transmission of the deletion. To unambiguously genotype F2 mice as +/+ or ΔNEO/ΔNEO, we assayed for the presence or absence of the untargeted wild-type (+) allele both by PCR (data not shown) and by Southern blot analyses. In all, 58 F2 individuals derived from crosses of four F1 males and nine F1 females were analyzed. Mice of the +/+, ΔNEO/ΔNEO genotypes were obtained in roughly Mendelian ratios (11:28:19, or 19%-48%-33%). None contained any detectable NEO PCR product nor were neo sequences detectable by Southern blot analysis. PCR was judged to be a more sensitive assay for excision or retention of the neo sequence because we had observed occasional F1 mice that appeared completely deleted for neo by Southern blot analysis but were still neo positive by PCR, indicating low levels of residual undeleted neo sequences. To assess whether neo excision observed in F2 tail tissue reflected Cre action in the F1 or in the F2 zygote, F2 mice were assayed by PCR for the presence or absence of the EIIa-cre transgene (Fig. 1). The EIIa-cre transgene was found to have segregated in roughly Mendelian fashion, with 42 of 58 individuals harboring and 16 of 58 lacking this transgene. The 16 F2 mice that lacked the EIIa-cre transgene encompassed both +/ΔNEO and ΔNEO/ΔNEO individuals. These 16 mice carried no detectable neo sequences and must therefore have inherited the ΔNEO allele from one or both F1 parents. These in turn must have undergone Cre-mediated neo excision during or before germ cell formation. Our data indicate that F1 mice that showed complete neo deletion in the tail had indeed undergone neo deletion in their germ cells as well.

Cre Activity from EIIa-cre Efficiently Reduces Transgene Copy Number at a Multicopy Integration Site. In the context of our ongoing study of oncogenesis in a differentiating epithelial tissue (1, 3, 15, 16), the second experiment was aimed at studying the effect of SV40 TAg dosage on oncogenesis in the developing lens of transgenic mouse embryos. The experiment, outlined in Fig. 2, calls for Cre-mediated reduction of transgene copy number at a multicopy TAG integration site. Excision of TAG transgene copies is designed to occur between the loxP sites present in each TAg copy. The maa-loxP-TAg transgene uses the multiple cloning site and loxP sequences to direct expression of TAg to the developing lens, resulting in lens oncogenesis (15). A 34-bp loxP site separates the maa and TAg sequences in the transgene construct. We selected for our
Fig. 1. (A) Restriction map of the targeted region in the immunoglobulin light chain kappa locus. Restriction enzyme sites are represented by letters: B, BamHI; Xh, XhoI; R, EcoRI. The XhoI site is located 8.5 kb 3' of the exon encoding the kappa constant region. (B) Scheme of the targeted locus with the neo cassette replacing the region between the XhoI site and the EcoRI site of the endogenous locus. Solid triangles flanking the neo gene indicate loxP sites in direct repeat orientation. Arrows and lowercase letters represent described PCR primers; forward primer a is located adjacent to the 5' loxP site and forward primer b corresponds to a sequence within neo. Reverse primer c is located 3' of the neo cassette. The ac sequence, amplified between the a and the c primer, is too large to amplify in the assay, whereas bc represents a 700-bp neo sequence between primers b and c on the loxP-neo-loxP targeted product. (C) Targeted locus after in vitro Cre-mediated excision of the neo gene. Only the ac sequence is amplified in this Δneo constellation. (D) PCR assay of tail DNA. (Left) The products of a PCR mix containing primers a, b, and c. The undeleted neo cassette and/or the deleted product will be amplified if present. Lanes 1–4, tail DNA from four F1 offspring carrying a + allele and variable amounts of NEO and/or ΔNEO alleles, depending on the efficiency of neo excision; lanes 5–8, DNA from four F2 offspring; lane 9, DNA from +/ΔNEO ES cells in which the neo gene had been deleted in vitro by transient transfection with a Cre expression vector; lane 10, +/NEO ES cells from which the homozygous loxP-neo-loxP mice used in this study were generated (note that the genotype of the mouse represented in lane 6 is +/+ based on data not shown, so no ΔNEO product is amplified; also note that lanes 5, 7, and 8 represent ΔNEO/ΔNEO mice, i.e., only the ΔNEO product is amplified). (Right) The 269-bp PCR amplification products of the cre transgene. Lane 12 represents an F2 mouse not shown at Left, whereas lanes 5–8 show PCR data for the mice analyzed in the corresponding lanes at Left. Location and size of molecular weight markers are shown to the left of each panel. In this panel, as in E, the alleles scored, + (the wild-type allele), N (NEO), or Δ (ΔNEO), are indicated below the lanes numbers. (E) Southern blot analysis of ES cell and tail DNA. BamHI-digested DNA was blotted and probed with a 500-bp XhoI/NcoI fragment located just 5' of the XhoI site in the genomic locus. Lanes 1–4, ES cell DNA; lanes 5–7, offspring of a +/neo × +/neo cross; lanes 8–11, F1 offspring of a neo/neo × El/lacZ/El/lacZ cross; lanes 12–14, F2 offspring of interbred F1 mice (only homozygotes are shown); lanes 15–18, thymus DNA from F2 mice. Expected allele sizes are shown to the right.
FIG. 2. DNA constructs used to generate Elia-cre and (maA-loxP-TAg)n, transgenic mice and schematic representation of matings that result in maA-loxP-TAg progeny. Elia-cre mice carry a transgene encoding the bacteriophage P1 recombinase under the control of an adenovirus Elia promoter that directs Cre action to the earliest stages of embryonic development (9). The maA-loxP-TAg transgene contains the 34-bp loxP site between the maA-crystallin promoter and the SV40 large TAg gene derived from pA366a-T (16). The (maA-loxP-TAg)n strain carries multiple copies of the transgene in head-to-tail arrangement. The +/(maA-loxP-TAg), animals have lens tumors and die young. Crosses of Elia-cre/Elia-cre females and +/(maA-loxP-TAg)n males resulted in a Mendelian distribution of transgenes among offspring. Those mice that harbored both the Elia-cre and the maA-loxP-TAg transgene displayed eye cataracts and had a life span that was close to normal. DNA fragments used as probes in Southern blot analyses are shown. B, BamHI restriction site.

study a strain of mice that carries multiple copies of the maA-loxP-TAg transgene at a single integration site. Lens tumor progression in these (maA-loxP-TAg)n mice is fulminating, and their life span is less than 3 months. In fact, to be able to propagate the strain, we resorted to ovariectomy from young adult transgenic to nontransgenic females. We crossed male +/(maA-loxP-TAg)n and female Elia-cre/Elia-cre mice to obtain zygotes in which the multicopy maA-loxP-TAg site would be exposed to Cre activity. If recombination were to occur at all loxP sites, the number of transgenes present at the multicopy transgene integration site should be reduced to a single copy. Observing lens development in embryos derived from these zygotes would allow us to describe the phenotypic consequences of a reduction in oncogene copy number without alteration of the chromosomal position of the transgene.

Genomic analysis of progeny derived from such crosses verified that the multicopy maA-loxP-TAg transgene integration site can indeed be reduced to a single-copy site. An immediate indication of success was the birth of F1 progeny that showed lens cataracts, indicative of TAg oncosgenesis, but that appeared healthy, unlike their +/(maA-loxP-TAg)n parents. They stayed healthy for at least 8 months, after which time some of them developed lens tumors. Southern blot analysis of tail DNA gave evidence of efficient, and possibly complete, excision of sequences flanked by loxP sites. In Fig. 3A, the transgene locus of (maA-loxP-TAg)n parents is compared with that of F2 progeny of an Elia-cre/(maA-loxP-TAg)n transgenic mouse. Genomic tail DNA was restricted with EcoRV that cuts outside the transgene sequences. The size (>30 kb) and intensity (determined by densitometric analysis) of the bands in lanes 1 and 3 suggest that the (maA-loxP-TAg)n parent locus carries more than 10 copies of the transgene, probably in a tandem head-to-tail arrangement (13). By contrast, the size and intensity of the TAg-specific bands in F2 progeny DNA (lanes 5, 6, and 8) corresponds to a single transgene arrangement. A more extensive analysis, using additional restriction enzymes, confirmed this observation (data not shown). A comparable reduction of transgene copies was observed in progeny of at least three additional Elia-cre × (maA-loxP-TAg)n crosses (data not shown). We conclude that the germ cells that gave rise to the F2 progeny analyzed here carried a single copy of the maA-loxP-TAg transgene. The distribution of cre-specific bands of BamHI restricted tail DNA is consistent with the segregation pattern of a nonlinked Elia-cre allele (Fig. 3B). These results indicate efficient recombination by the Cre enzyme in Elia-cre/(maA-loxP-TAg)n early embryos and show that single-copy maA-loxP-TAg progeny lacking the Elia-cre allele can readily be obtained.

Cre-Mediated Reduction of maA-loxP-TAg Transgene Copy Number Leads to a Delay in Lens Tumorigenicity. The efficiency of Elia-cre-mediated transgene excision allowed us to examine whether a reduction of maA-loxP-TAg transgene copy number affects the rate of oncosgenesis in the developing embryonic lens. In Fig. 4, we compared multicyclic and single copy maA-loxP-TAg lenses by studying cell morphology in H&E and in TAg immunostained sections. Our previous studies have shown that the onset of TAg accumulation in the maA-TAg transgenic lens coincides with embryonic lens cell differentiation at midgestation (15, 16). In the multicyclic (maA-loxP-TAg)n lens observed at day 11.5 of embryonic development (E11.5) nuclei of posterior epithelial cells that are about to differentiate into primary fibers readily stain for TAg (Fig. 4A). This nuclear TAg immunostaining is absent from the single copy maA-loxP-TAg lens at E11.5 (Fig. 4B), but readily visible at embryonic day E13.5 when lens epithelial cells have already undergone fiber elongation (Fig. 4D). At this stage of lens development, it appears that the multicyclic (maA-loxP-TAg)n lens is filled with largely undifferentiated, TAg transformed cells likely to be tumorigenic (Fig. 4C). By contrast, cells in the single copy maA-loxP-TAg lens, especially in the posterior hemisphere (Fig. 4D) appear to have undergone fiber elongation before TAg accumulation and may thus not be able to participate in the process of lens tumor development.
formation (see also ref. 3). These conclusions are supported by a study of the morphology of hematoxylin/eosin stained E13.5 (Fig. 4E–G) and E17.5 (Fig. 4H–J) lens sections. Normal lens development is characterized by orderly lens fiber elongation throughout these stages of development (Fig. 4F and I). By comparison, cell arrangement is highly disorganized both in the multicopy (maA-loxP-TAg)ₙ lens (Fig. 4E and H) and in the single copy maA-loxP-TAg lens (Fig. 4G and J). In addition, the process of TAg-mediated lens transformation is clearly accelerated in the (maA-loxP-TAg)ₙ lens as evidenced by a nearly uniform population of small, round, rapidly dividing cells, whereas the single-copy maA-loxP-TAg lens shows more protracted proliferation of cells that retain some morphologic features of elongated fiber cells (compare Fig. 4E with G). By E17.5, the (maA-loxP-TAg)ₙ lens showed small rapidly dividing cells with compacted nuclei that surround a necrotic lens center (Fig. 4H), whereas the maA-loxP-TAg lens still retained morphological features of fiber elongation (Fig. 4J). The difference between the rate of TAg accumulation and cell proliferation in the multicopy (maA-loxP-TAg)ₙ and the single copy maA-loxP-TAg lens strongly suggests a quantitative connection between transgene dosage and TAg output. The results presented here extend our earlier investigations and confirm our conclusions that rapid transformation is the outcome of an early and rapid accumulation of TAg, whereas late and slow accumulation leads to a less malignant lens phenotype (3, 16).

**DISCUSSION**

Our results show that targeted endogenous genes or transgenes bearing loxP sites can be efficiently modified in vivo. Mating of Elia-cre mice with gene-altered mice that carry DNA sequences flanked by loxP sites readily generates progeny in which excision of sequences between the loxP sites has taken place and is genetically fixed. We can infer from what is known about the adenovirus Elia promoter activity in the mouse (9) that much of this efficient Cre action is confined to the one-cell zygote stage of embryonic development. The efficient excision that we observed in cell populations as divergent as germ-line
cells, tail tissue, and lens tissue certainly supports this view and can be fully explained by Cre activity targeted to the first cell of the developing organism. The frequency and extent of excision events may depend on a variety of factors, among them the genomic site of Cre action (17) and the length of the sequences to be excised. Use of these Ela-cre mice to bring about gene modifications at loxp-targeted loci in a variety of experimental settings will allow the general applicability of the procedure to be further assessed in the near future.

The two experiments reported here point to a number of potential applications. In the first experiment, excision of the neo expression cassette from a targeted gene removes a functional promoter and a selectable gene product after they have served their purpose. This is done to prevent these elements from exerting potentially unwanted effects on the targeted tissue. A similar need was realized in earlier experiments where direct Cre action on targeted embryonic stem (ES) cells led to the in vitro removal of neo cassettes (4). Our approach to accomplish this goal in vivo obviates the need for extended culture of ES cells in order to expose them to Cre activity before germ-line transmission. It also affords direct comparisons between animals that underwent Cre-mediated gene modification and those that did not.

By a slight modification of the conventional breeding strategy for germ-line transmission, in vivo Cre-loxP-mediated deletion of DNA sequences could be accomplished in a manner that would reduce in vitro ES cell manipulation without requiring any extra breeding steps. Conventional gene targeting technology involves injection of gene-targeted ES cells into blastocysts to generate chimeras carrying the targeted locus. Male chimeras are subsequently crossed with normal females to test for germ-line transmission of the targeted allele, and offspring that have transmitted the gene-targeted allele are interbred to produce second generation offspring homozygous for the targeted locus. If Ela-cre mice are substituted for the normal females in this strategy, loxp-flanked sequences present at the targeted locus could be deleted as the male chimeras are tested for germ-line transmission. Progeny of crosses between males chimeric for the gene-targeted allele and females carrying the Ela-cre transgene should include offspring that have deleted loxp-flanked sequences at an early embryonic stage, and can transmit the Cre-modified targeted allele. These progeny would be interbred to obtain a fraction of offspring that lack the Ela-cre transgene and are homozygous for the Cre-modified allele. Obtaining these mice after two successive crosses would provide an attractive alternative to schemes of Cre-mediated in vitro excision of loxp-flanked sequences from ES cells. Examples of applications include introduction of a variety of subtle mutations into the germ line, replacement of mutated alleles with corrected sequences, replacement of genes of one species with those from another, and gene targeting of cis-acting elements.

In our second experiment, a multicopy gene locus in which the TAg transgene was flanked on one side by a loxp site was reproducibly reduced to one transgene copy by breeding with Ela-cre transgenic mice. This reduction in TAg copy number demonstrated a correlation between transgene copy number and lens oncogenicity. Production of transgenic mice by current approaches results in multicopy tandem arrays of transgenes whose number can only be varied by generating a set of founders that differ by the number of copies they carry. However, they also differ by integration site and this introduces an unwanted variable. The crosses between Ela-cre mice and strains that contain loxp sites next to a transgene of choice allow reproducible and efficient generation of mouse strains that differ by the number of transgene copies which they carry at a given integration site. This approach has obvious and far-reaching applications. The system of Cre-mediated gene modification described here leaves room for technical improvements. For example, the first of the two experiments that we describe would benefit from a further increase in Cre efficiency, whereas the second experiment would benefit from a graded administration of Cre activity to produce more variation in transgene copy number. Such titration of Cre activity and a resulting spread of transgene copy number among progeny has been achieved (unpublished work) using direct zygote microinjection with circular plasmid DNA encoding Cre; this goal has been independently pursued elsewhere (18). The direct microinjection approach, while labor intensive, demonstrates the ability to titrate the degree of Cre-mediated DNA excision by varying the amount of Cre enzyme delivered. A similar result could conceivably be achieved with the help of a set of Ela-cre strains that vary in their ability to deliver Cre activity to the early embryo.

**Note Added in Proof:** A relevant study entitled a cre-transgenic mouse strain for the ubiquitous deletion of loxp-flanked gene segments including deletion in germ cells has recently been published by F. Schwenk, U. Baron, and K. Rajewsky.

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