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Genetics

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 of development, we bred EIIa-*cre* transgenic mice to two different mouse lines carrying *loxP*-flanked target sequences: (*i*) a strain with a single gene-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 multicopy 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 strain 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 mαA-*loxP*-TAg, a lens-specific cassette in which the *loxP* site was placed between a mαA-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 +/mαA-*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

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Abbreviations: SV40, simian virus 40; TAg, tumor antigen; E, embryonic day; ES cells, embryonic stem cells; mαA, murine αA-crystallin gene promoter.

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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 F_1 offspring was assayed by polymerase chain reaction (PCR) of tail DNA. The following primers were used in the assays of Fig. 1: **a**, 5′-ggccgctaattccgatcatattc-3′; **b**, 5′-ccttcttgacgagttcttctgagg-3′; **c**, 5′-actaacatttgacaggtggatgc-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. F_1 mice with complete deletion of the *neo* gene in the tail were interbred to obtain F_2 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 (mαA-*loxP*-TAg)$_n$ Transgenic Mice. The mαA-*loxP*-TAg cassette was derived by deleting STOP from the previously described construct mαA-*loxP*-STOP-*loxP*-TAg (1). Multicopy (mαA-*loxP*-TAg)$_n$ transgenic mice were generated by zygote injection of the mαA-*loxP*-TAg cassette. Multicopy (mαA-*loxP*-TAg)$_n$ heterozygous males were mated with homozygous EIIa-*cre* females to generate single-copy mαA-*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 or adult mouse tails and digested with *Eco*RV, *Kpn*I, *Bgl*II (no restriction sites within the mαA-*loxP*-TAg sequence), and *Bam*HI (one single restriction site in mαA-*loxP*-TAg and EIIa-*cre*) (see Fig. 2). The digested DNA was separated in a 0.75% agarose gel, transferred to Gene Screen filters (DuPont), and hybridized as described (15). A 1169-bp *Hin*dIII 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 confines 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 IIa-*cre* transgenic mice.

The F_1 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 F_1 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 F_1 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 undeleted 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 F_1 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, F_1 mice that displayed complete *neo* excision in the tail were selected for breeding to obtain F_2 progeny.

The *neo* Excision Is Fixed in the Germ Line and Transmitted to F_2 Mice. Male and female F_1 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 F_2 mice as +/+, +/ΔNEO, 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 F_2 individuals derived from crosses of four F_1 males and nine F_1 females were analyzed. Mice of the +/+, +/ΔNEO, and 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 F_1 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 F_2 tail tissue reflected Cre action in the F_1 or in the F_2 zygote, F_2 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 F_2 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 F_1 parents. These in turn must have undergone Cre-mediated *neo* excision during or before germ cell formation. Our data indicate that F_1 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 mαA-*loxP*-TAg transgene uses the murine αA-crystallin gene promoter (mαA) to direct expression of TAg to the developing lens, resulting in lens oncogenesis (15). A 34-bp *loxP* site separates the mαA and TAg sequences in the transgene construct. We selected for our

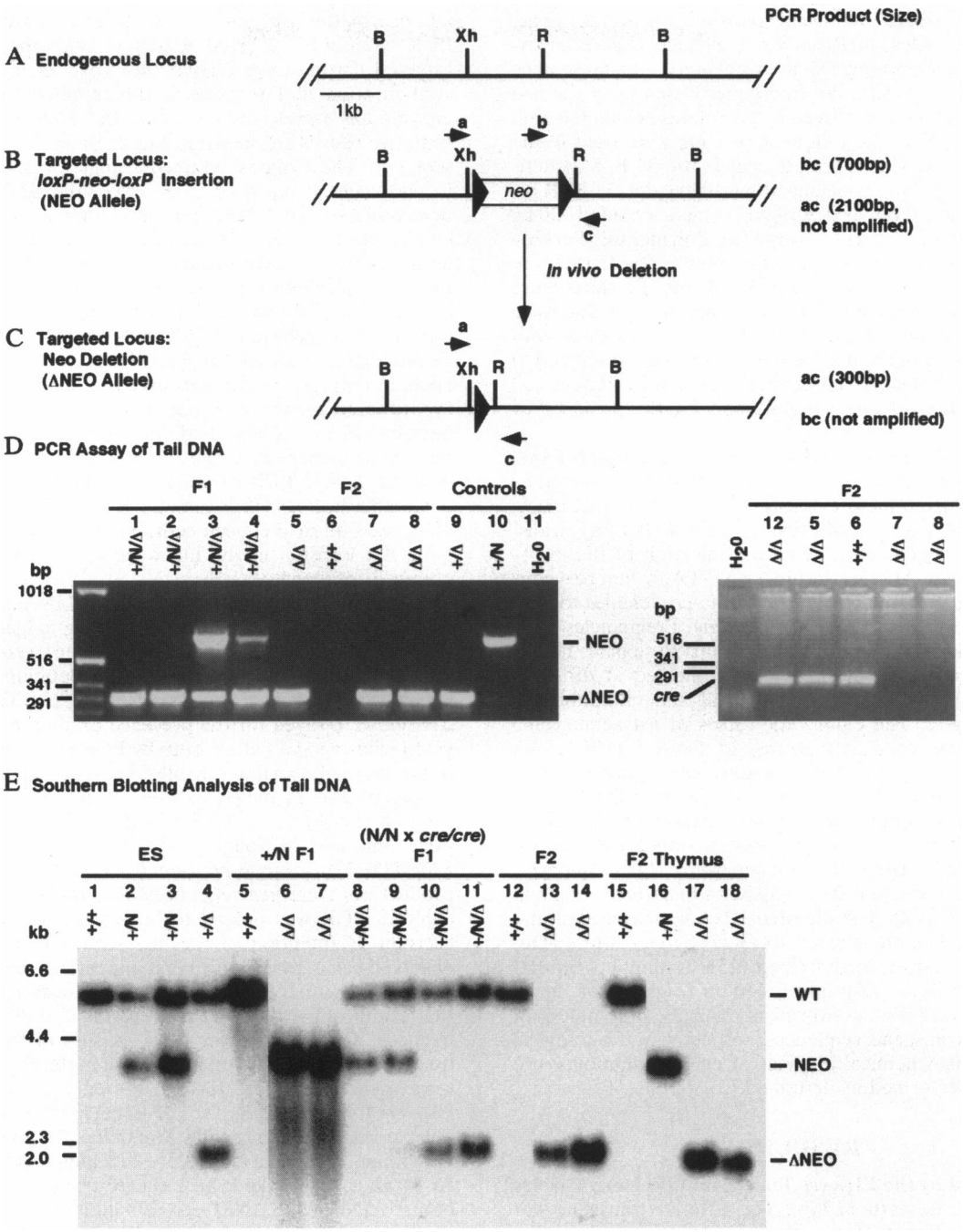

FIG. 1. (*A*) Restriction map of the targeted region in the immunoglobulin light chain kappa locus. Restriction enzyme sites are represented by letters: B, *Bam*HI; Xh, *Xho*I; R, *Eco*RI. The *Xho*I site is located 8.5 kb 3′ of the exon encoding the kappa constant region. (*B*) Scheme of the targeted locus with the *neo* cassette replacing the region between the *Xho*I site and the *Eco*RI 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 chromosome. (*C*) Targeted locus after *in vivo* 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 F_1 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 F_2 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 F_2 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 lane numbers. (*E*) Southern blot analysis of ES cell and tail DNA. *Bam*HI-digested DNA was blotted and probed with a 500-bp *Xho*I/*Nco*I fragment located just 5′ of the *Xho*I site in the genomic locus. Lanes 1–4, ES cell DNA; lanes 5–7, offspring of a +/NEO × +/NEO cross; lanes 8–11, F_1 offspring of a NEO/NEO × EIIa*cre*/EIIa-*cre* cross; lanes 12–14, F_2 offspring of interbred F_1 mice (only homozygotes are shown); lanes 15–18, thymus DNA from F_2 mice. Expected allele sizes are shown to the right.

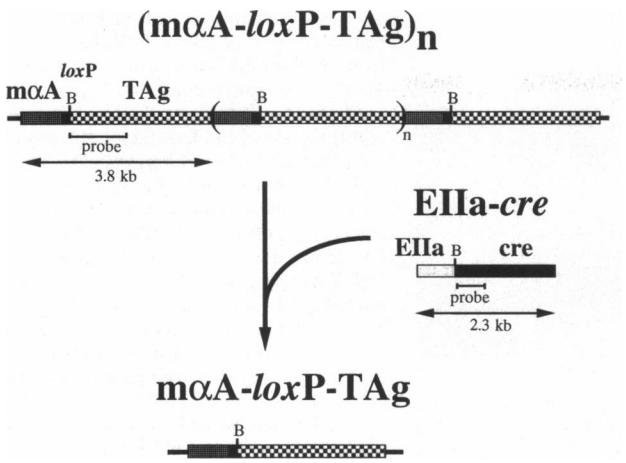

FIG. 2. DNA constructs used to generate EIIa-*cre* and (mαA-*loxP*-TAg)$_n$ transgenic mice and schematic representation of matings that result in mαA-*loxP*-TAg progeny. EIIa-*cre* mice carry a transgene encoding the bacteriophage P1 recombinase under the control of an adenovirus EIIa promoter that directs Cre action to the earliest stages of embryonic development (9). The mαA-*loxP*-TAg transgene contains the 34-bp *lox*P site between the mαA-crystallin promoter and the SV40 large TAg gene derived from pαA366a-T (16). The (mαA-*loxP*-TAg)$_n$ strain carries multiple copies of the transgene in head-to-tail arrangement. The +/(mαA-*loxP*-TAg)$_n$ animals have lens tumors and die young. Crosses of EIIa-*cre*/EIIa-*cre* females and +/(mαA-*loxP*-TAg)$_n$ males resulted in a Mendelian distribution of transgenes among offspring. Those mice that harbored both the EIIa-*cre* and the mαA-*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, *Bam*HI restriction site.

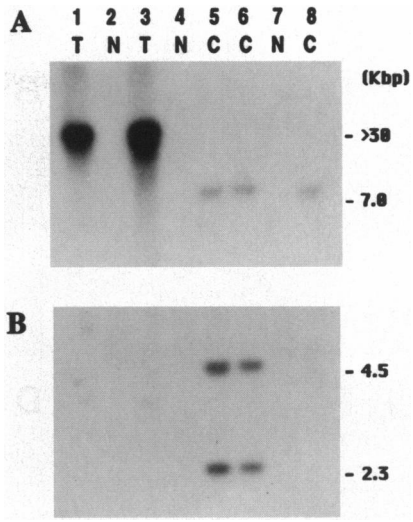

FIG. 3. Southern blot analysis of tail DNA and eye phenotype of (mαA-*loxP*-TAg)$_n$ and mαA-*loxP*-TAg mice. (A) *Eco*RV digests probed with the 1169-bp *Hin*dIII fragment of the TAg plasmid (see Fig. 2). Note that there are no *Eco*RV sites inside the mαA-*loxP*-TAg transgene. (B) *Bam*HI digested DNA hybridized with a 400-bp fragment of *cre* sequence (see Fig. 2). Because *Bam*HI cuts once in the EIIa-*cre* sequence covered by the probe, the blot shows two bands of hybridizing fragments. Lanes 1 and 3, (mαA-*loxP*-TAg)$_n$ DNA; lanes 2 and 4, nontransgenic control DNA; lanes 5–8, DNA from F_2 offspring derived from the progeny of a cross between EIIa-*cre*/EIIa-*cre* females and +/(mαA-*loxP*-TAg)$_n$ males. Letters below the lane numbers refer to the eye phenotype observed in young adults. T, bilateral lens tumors; N, normal clear eyes; C, bilateral lens cataracts.

study a strain of mice that carries multiple copies of the mαA-*loxP*-TAg transgene at a single integration site. Lens tumor progression in these (mαA-*loxP*-TAg)$_n$ mice is fulminant, and their life span is less than 3 months. In fact, to be able to propagate the strain, we resorted to ovary transfer from young adult transgenic to nontransgenic females. We crossed male +/(mαA-*loxP*-TAg)$_n$ and female EIIa-*cre*/EIIa-*cre* mice to obtain zygotes in which the multicopy mαA-*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 mαA-*loxP*-TAg transgene integration site can indeed be reduced to a single-copy site. An immediate indication of success was the birth of F_1 progeny that showed lens cataracts, indicative of TAg oncogenesis, but that appeared healthy, unlike their +/(mαA-*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. 3*A*, the transgene locus of (mαA-*loxP*-TAg)$_n$ parents is compared with that of F_2 progeny of an EIIa-*cre*/(mαA-*loxP*-TAg)$_n$ double transgenic mouse. Genomic tail DNA was restricted with *Eco*RV 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 (mαA-*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 F_2 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 EIIa-*cre* × (mαA-*loxP*-TAg)$_n$ crosses (data not shown). We conclude that the germ cells that gave rise to the F_2 progeny analyzed here carried a single copy of the mαA-*loxP*-TAg transgene. The distribution of *cre*-specific bands of *Bam*H1 restricted tail DNA is consistent with the segregation pattern of a nonlinked EIIa-*cre* allele (Fig. 3*B*). These results indicate efficient recombination by the Cre enzyme in EIIa-*cre*/(mαA-*loxP*-TAg)$_n$ early embryos and show that single-copy mαA-*loxP*-TAg progeny lacking the EIIa-*cre* allele can readily be obtained.

Cre-Mediated Reduction of mαA-*loxP*-TAg Transgene Copy Number Leads to a Delay in Lens Tumorigenicity. The efficiency of EIIa-*cre*-mediated transgene excision allowed us to examine whether a reduction of mαA-*loxP*-TAg transgene copy number affects the rate of oncogenesis in the developing embryonic lens. In Fig. 4, we compared multicopy and single copy mαA-*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 mαA-TAg transgenic lens coincides with embryonic lens cell differentiation at midgestation (15, 16). In the multicopy (mαA-*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. 4*A*). This nuclear TAg immunostaining is absent from the single copy mαA-*loxP*-TAg lens at E11.5 (Fig. 4*B*), but readily visible at embryonic day E13.5 when lens epithelial cells have already undergone fiber elongation (Fig. 4*D*). At this stage of lens development, it appears that the multicopy (mαA-*loxP*-TAg)$_n$ lens is filled with largely undifferentiated, TAg transformed cells likely to be tumorigenic (Fig. 4*C*). By contrast, cells in the single copy mαA-*loxP*-TAg lens, especially in the posterior hemisphere (Fig. 4*D*) appear to have undergone fiber elongation before TAg accumulation and may thus not be able to participate in the process of lens tumor

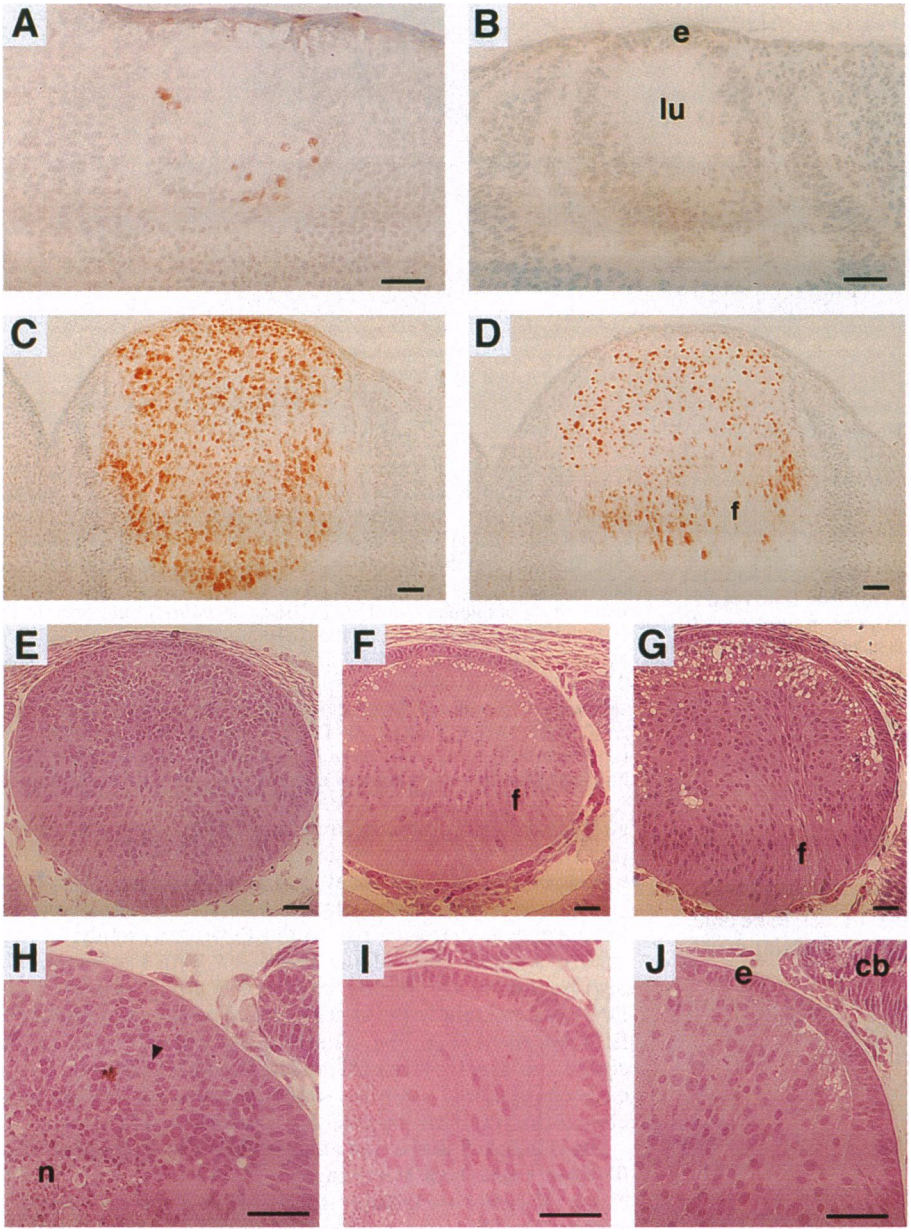

FIG. 4. Histology and TAg immunohistochemistry of tissue sections derived from embryonic multicopy (mαA-*loxP*-TAg)$_n$ and single-copy mαA-*loxP*-TAg eyes. All sections are oriented with the anterior pole of the lens facing the top of the page. lu, Lumen of lens vesicle; cb, iris and ciliary body primordia; f, fiber; e, anterior epithelial layer; n, necrosis. (Bar = 50 μm.) (*A–D*) Frozen sections of embryonic lenses derived from multicopy (mαA-*loxP*-TAg)$_n$ (*A* and *C*) and single-copy mαA-*loxP*-TAg individuals (*B* and *D*), respectively. The tissue sections were immunostained with an antibody specific for TAg using an indirect peroxidase staining procedure. (*A* and *B*) E11.5; (*C* and *D*) E13.5. The lens vesicle in *A* shows strong nuclear staining in differentiating cells located in the posterior lens hemisphere. There is a barely visible and diffuse TAg signal in the corresponding area of *B*. At E13.5, all lens cells of both the multicopy (*C*) and the single-copy genotype (*D*), show strong nuclear TAg staining, with the exception of the anterior epithelial layer. Note that the nuclei of cells differentiating into primary fibers (f) in the posterior half of the lens displayed in *D* show a weaker and more diffused TAg signal. (*E–J*) Methacrylate-embedded, hematoxylin/eosin stained sections of transgenic and normal eyes, derived from E13.5 (*E–G*) or E17.5 embryos (*H–J*). At E13.5, the multicopy (mαA-*loxP*-TAg)$_n$ lens (*E*) is completely filled with rounded, dividing cells that are poorly differentiated, compared with the elongated, postmitotic fiber cells (f) in the lens of an age-matched normal littermate (*F*). In the single-copy mαA-*loxP*-TAg lens (*G*), there is an intermediate phenotype of rounded and dividing cells alternating with partially elongated fiber-like (f) cells. At E17.5, necrosis (n) becomes visible in the center of the multicopy (mαA-*loxP*-TAg)$_n$ lens (*H*). The cells surrounding this necrotic center are rapidly dividing (the arrowhead points to a mitotic figure). (*J*) The single-copy mαA-*loxP*-TAg lens at E17.5. Disorganization of lens tissue is less severe than in *H*, but nonetheless quite noticeable when compared with the normal situation (*I*).

formation (see also ref. 3). These conclusions are supported by a study of the morphology of hematoxylin/eosin stained E13.5 (Fig. 4 *E–G*) and E17.5 (Fig. 4 *H–J*) lens sections. Normal lens development is characterized by orderly lens fiber elongation throughout these stages of development (Fig. 4 *F* and *I*). By comparison, cell arrangement is highly disorganized both in the multicopy (mαA-*loxP*-TAg)$_n$ lens (Fig. 4 *E* and *H*) and in the single copy mαA-*loxP*-TAg lens (Fig. 4 *G* and *J*). In addition, the process of TAg-mediated lens transformation is clearly accelerated in the (mαA-*loxP*-TAg)$_n$ lens as evidenced by a nearly uniform population of small, round, rapidly dividing cells, whereas the single-copy mαA-*loxP*-TAg lens shows more protracted proliferation of cells that retain some morphologic features of elongated fiber cells (compare Fig. 4 *E* with *G*). By E17.5, the (mαA-*loxP*-TAg)$_n$ lens showed small rapidly dividing cells with compacted nuclei that surround a necrotic lens center (Fig. 4*H*), whereas the mαA-*loxP*-TAg lens still retained morphological features of fiber elongation (Fig. 4*J*). The difference between the rate of TAg accumulation and cell proliferation in the multicopy (mαA-*loxP*-TAg)$_n$ and the single copy mαA-*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 EIIa-*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 EIIa 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 EIIa-*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 EIIa-*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 EIIa-*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 EIIa-*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 EIIa-*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 EIIa-*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 EIIa-*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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