Increasingly, the Cre-loxP system is being used to generate both null and conditional mutations in mice by homologous recombination in embryonic stem (ES) cells (1,3–5). The advantage of the Cre-loxP approach in generating mutations in mice, is that the gene of interest can be inactivated in any organ at a given time during the mouse’s life, thus bypassing developmental and/or pleiotropic effects inherent in the classical knock-out approach. Furthermore, this strategy allows for the generation of mutant alleles, as well as “floxed” alleles (genomic DNA flanked by loxP sites) by using just one construct for gene targeting, since one parental homologous recombinant ES clone can yield subclones with both null and floxed alleles, following transient transfection with a Cre-recombinase expressing plasmid. Genotype analysis of mutant mice, generated by homologous recombination is done using genomic DNA for either Southern analysis or by use of the polymerase chain reaction (PCR). PCR is preferred since it rapidly yields the required results and allows for a larger throughput of samples. For classical knock-out techniques, where a large marker gene such as a neomycin-resistance gene or the lacZ gene (or both) is either inserted into or replaces an exon(s) (2), PCR analysis requires the use of three primers (see below). In our experience, amplifying mouse genomic DNA is not straightforward, because determining the correct conditions with respect to annealing temperature, magnesium concentration, quality and amount of template, are all critical factors in allowing for the development of a robust assay. The more primers used for this type of assay, the more difficult the task of determining the optimal PCR conditions. We have been using the Cre-loxP system to generate both null and conditional mutations in a number of genes, including the following transcription factors: cAMP responsive element binding protein (CREB), glucocorticoid receptor (GR) and hepatocyte nuclear factor 4γ (HNF4γ). Here, we describe the PCR strategy we use in analyzing the genotypes of our floxed mice, using only two primers, making the development of assays simpler, quicker and more robust. During our analysis of CREB and HNF4γ ES cell clones, we realized that this approach also allowed rapid screening for multiplicity insertions of the targeting construct in putative homologous recombined ES clones.

For the classical knock-out approach, analysis of mouse genotypes by PCR requires use of three primers, one common to both mutant and wild-type (WT) alleles and one specific to each (Figure 1A; primers 1, 2wt and 3mut) allowing for the amplification of relatively short products in each case. The amplification yields two different sized products, one mutant specific (null) and one WT specific. Use of only two primers on either side of the selection cassette in this case is problematic, because the size of the reporter gene(s) is relatively large (>1 kb), making routine amplification of large genomic fragments in multiple heterogeneous samples difficult. Secondly, the large size difference, hence amplification rate difference, between the mutant and WT products in this case would further complicate the determination of suitable PCR conditions.

For floxed mice, genotyping by PCR is possible using only two primers flanking one loxP site (Figure 1B; primers 1 and 2). This allows for the amplification of two different sized products depending on the presence or absence of the loxP site. The size difference between the floxed allele and WT allele is due to the extra nucleotides that comprise the loxP sequence (34 bp) and the surrounding restriction sites used for constructing the targeting construct. Because the size difference is relatively small, differential amplification of one product over the other is avoided. Moreover, by designing primers outside of both loxP

### Figure 1. Strategies for genotype analysis by PCR. (A) Both WT and null alleles are shown, as generated by the classical knock-out strategy; in this case, the gene is silenced by the in-frame insertion-replacement into the indicated exon by a neomycin-resistance and lacZ reporter gene. Positions of primers used for the amplification and detection of differences between the two alleles are shown. Primer 1 is common to both loci, the 2wt primer is WT-specific, and the 3mut primer is mutant-specific. (B) Both WT and corresponding floxed alleles are shown. The loxP sites are shown as gray rectangles, whereas primers are shown as arrows and exons as black rectangles. See text for further explanation.

### Figure 2. Amplification of DNA. Amplified products from PCR assays containing genomic DNA from CREB-loxP mice (lanes 1–3), ES cells (lanes 4–6) and HNF4γ-loxP ES cells (lanes 7–9) as template. A 10-µl sample of each PCR was electrophoresed on a 1.2% agarose gel containing 0.1 µg/mL EtdBr. Lane 1, homozygous (floxed); lanes 2, 5 and 8, heterozygous; lanes 3, 4 and 7, WT; lanes 6 and 9, heterozygous with multiple copies of loxP targeting vector. PCR primers used: CREB-loxP; sense, 5'-TAGACATACTTGACCATT3'; antisense, 5'-TTGAGTGTCTTCCATTTAC3'; HNF4γ-loxP; sense, 5'-TGCATATTCCATCAAC3'; antisense, 5'-CTGGATCTCTACTAC3'.
sites, one can use the assay for determining recombination mediated by Cre recombinase (not shown). Results of PCR assays are shown for genomic DNA from mouse tails and ES cells for the CREB-loxP allele, and from ES cells for the HNF4γ-loxP allele. The DNAs from both ES cells (approximately 10⁶ cells) and mouse tails (5-mm biopsy) were prepared using the same method. We chose to use a method that allows for the production of relatively large amounts of DNA, since we analyze the samples for other transgenes using other techniques. Alternative methods, requiring much less starting material and less handling could be used. The DNA preparations were made and analyzed as follows: (i) Cells or tail material were suspended in 0.8 mL lysis buffer (1% sodium dodecyl sulfate [SDS], 50 mM Tris- HCl, pH 8.0, 100 mM EDTA, 200 µg/mL proteinase K) and incubated at 56°C for 16 h. (ii) A 0.25-mL vol of saturated NaCl was added, and the samples were mixed on a Thermomixer (Eppendorf, Hamburg, Germany) for 5 min. (iii) After centrifugation for 10 min (15 000×g), 0.8 mL of the supernatant was precipitated with 0.6 mL isopropanol. (iv) The DNA was resuspended in 0.1 mL TE (50 mM Tris- HCl, pH 8.0, 10 mM EDTA). (v) For amplification, 1 µL DNA, (1:100 dilution) was added to a mixture containing PCR buffer (Boehringer Mannheim GmbH, Mannheim, Germany), 2.5 mM magnesium chloride, 0.2 mM of each dNTP (Boehringer Mannheim); 25 pmol of each primer and 0.5 U Taq DNA Polymerase (Boehringer Mannheim). Amplification was performed in a Gradient 96 Robo-Cycler® (Stratagene, La Jolla, CA, USA) using the following conditions: 95°C for 5 min, followed by 35 cycles of 95°C for 40 s, 60°C for 1 min and 72°C for 40 s. (vi) Ten microliters of the sample were then electrophoresed on a 1.2% agarose gel (Life Technologies, Paisley, Scotland, UK) containing 0.1 mg/mL ethidium bromide (EtBr). For CREB-loxP, the amplified product of the floxed allele was 568-bp long; while for the WT allele, the band seen migrated at 500 bp. For HNF4γ-loxP, the corresponding bands were 512 and 400 bp, respectively (Figure 2). The larger size of the loxP products compared with WT was due to the loxP sites (34 bp) and sequences surrounding the loxP sites, which include restriction sites coming from the parental plasmids. The assay has proven to be robust and consistent, given that we can routinely screen one hundred or more samples in one assay.

A further advantage of this PCR approach is the rapid identification of undesirable multicopy insertions of the loxP targeting vector when incorporated into putative homologously recombined
ES cells. This phenomenon has been observed in our laboratory in a number of cases. Clones putatively identified as homologous recombinants by Southern analysis can be quickly checked for random insertion of extra copies of the targeting vector by PCR. Given the small difference in size between the two alleles and use of only two primers common to both WT and mutant DNA, the amplification rate of both products is similar. Therefore, any significant difference in signal intensity between the WT and floxed products indicates multiple integration of the targeting vector, which can be later confirmed by Southern analysis. The presence of multicopy insertions in CREB-loxP ES cells and HNF4-γ-loxP ES clones resulted in over-amplification of the mutant band (Figure 2, lanes 6 and 9). Presence of multiple copies of the mutant constructs was confirmed by Southern blot analysis (data not shown).

To summarize, with the ever increasing use of the Cre-loxP system studying genetic mutations in the mouse, we present a generally applicable PCR-based approach for genotyping genomic DNA harboring loxP sites. Unlike previously reported knock-out detection methods, where PCR analysis for genotyping requires use of three primers, only two primers are required here, allowing for robust PCR assays to be set up rapidly. Due to the use of identical primers in the detection of both WT and loxP alleles, the method also allows for accurate detection of copy number differences when screening ES cells.

**REFERENCES**