Microinjection of Cre recombinase RNA induces site-specific recombination of a transgene in mouse oocytes

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ABSTRACT

We have developed a strategy for producing single copy transgenic mouse lines using *Cre-loxP* site specific recombination. The method is based on transient expression of the recombinase after injection of *in vitro* transcribed mRNA into the cytoplasm of fertilised eggs containing multiple copies of the transgene. The success rate of the recombination event is 100% (15 out of 15).

The Cre-*loxP* recombination system of Bacteriophage P1 is a powerful tool for targeting and excising DNA sequences from the genome of higher eukaryotic organisms both *in vitro* (1–3) and *in vivo* (4–6). Cre recombinase is a 38 kDa protein that mediates site specific intramolecular and intermolecular recombination between 34 bp repeats termed *loxP* sites, in the absence of additional cofactors. The process is reversible, but the integration is less efficient due to ongoing excision. In the case of intramolecular recombination, DNA flanked by two directly orientated *loxP* sites is efficiently excised leaving a single *loxP* site in the genome. If the *loxP* sites are in opposite orientation the segment is inverted.

In transgenic mice, the expression level of the transgene depends on the regulatory elements in the construct, it's integration site in the genome and the number of integrated transgenes. However, in many cases it is necessary to compare the expression level between single copy transgenic mice (such as the testing of gene therapy constructs which would be present in the host genome as single copies) (7). Thus, it has become common practice to introduce a *loxP* site into the DNA construct that is microinjected to obtain transgenic mice. Multiple copy transgenic lines are usually reduced in copy number by injecting a plasmid expressing Cre (8) into their fertilised eggs. The disadvantage of this method is the possibility that the plasmid sequences may be present for many generations and integrate into the mouse genome. In addition, and in contrast to published results, in our hands it does not have a 100% efficiency. Finally, the recombination event has to take place as soon as possible to avoid mosaicism. We therefore tested the injection of Cre RNA as an alternative.
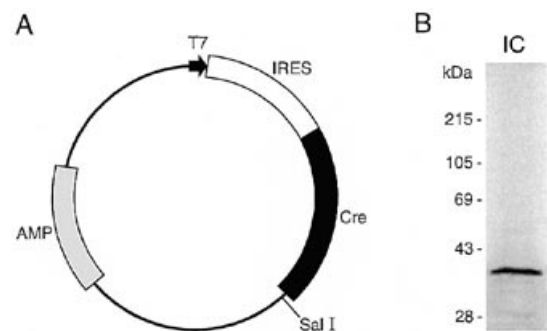

Figure 1. (**A**) Map of T7-IC used for the *in vitro* transcription of IRES-Cre RNA. The Cre cDNA was amplified via PCR and cloned in the vector pGEM-T (Promega). The IRES was derived from IRES-β-geo (17) and introduced 5′ of the Cre gene. (**B**) Analysis of translation product of IRES containing mRNA. RNA template was translated in a rabbit reticulocyte lysate and the translation product was analysed on a 12.5% SDS-polyacrylamide gel. The migration of proteins of known molecular weight is indicated in kDa.

It has been shown previously that picorna virus mRNAs have a specialised 5′ non-translated region called the internal ribosomal entry site (IRES) that allows cap-independent translation (9–12). *In vitro* studies have been done using the picorna virus 5′ NTR and heterologous protein-coding sequences under the control of a bacteriophage T7 or T3 promoter (13,14). The EMCV 5′ NTR IRES was shown to function in mouse embryos as well as in embryonic stem cells (15). Here, we present a method for efficient removal of transgenic sequences from fertilised mouse oocytes by site specific recombination occurring after cytoplasmatic microinjection of an *in vitro* transcribed IRES-Cre mRNA.

Cre cDNA was PCR amplified using pMC-Cre as a template (16; gift from K.Rajewsky) and subcloned into pGEM-T vector (Promega, Madison, WI) resulting in the plasmid pGEM-T-Cre. From the plasmid IRES-βgeo (17; gift from P.Mountford) the IRES was cut out as a 600 bp *Not*I-blunted *Nco*I fragment and subcloned into *Sph*I-blunted *Nco*I site of pGEM-T-Cre, resulting in the plasmid T7-IRES-Cre (T7IC) (Fig. 1A).

T7IC DNA was *Sal*I linearized and transcribed *in vitro* with T7 RNA polymerase as described (18). The DNA template was

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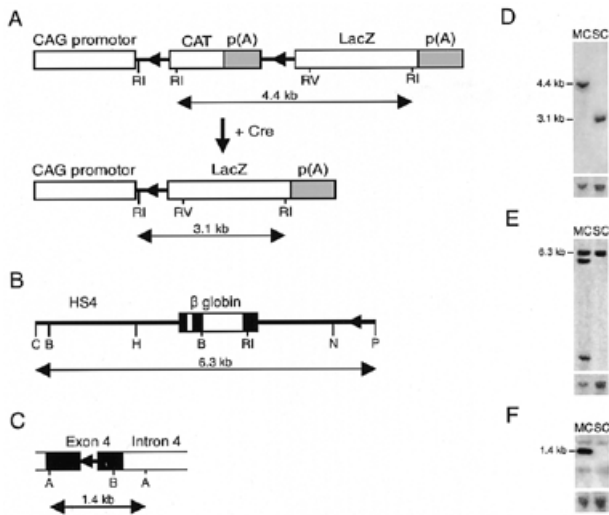
Figure 2. (**A**) CAG-CAT-Z construct (8) containing the chicken β-actin (CAG) promoter separated from the lacZ gene by the CAT gene, before and after recombination. *Eco*RI (RI) and *Eco*RV (RV) sites are indicated. Arrow heads represent the *loxP* sites. (**B**) HS4-β-*loxP* construct shown as a 6.3 kb *Cla*I–*Pvu*II fragment, containing hypersensitive site four of the human β-globin LCR and the β-globin gene with –800 promoter. *Cla*I (C), *Bam*HI (B), *Hind*III (H), *Eco*RI (RI), *Not*I (N) and *Pvu*II (P) sites are indicated. (**C**) Thy-*loxP* construct shown as a 1.4 kb *Apa*I fragment, containing parts of exon 4 and intron 4 of the mouse Thy 1.2 gene. *Apa*I (A) and *Bam*HI (B) sites are indicated. (**D**) Southern blot analysis of *Eco*RI digested tail DNA from multi copy (mc) and single copy (sc) animals of line CAG-CAT-Z16. Before recombination the 4.4 kb band corresponds to an *Eco*RI fragment of the unrecombined transgene and after recombination the 3.1 kb *Eco*RI band indicates that the CAT gene was recombined out. A 2.0 kb *Eco*RV–*Eco*RI LacZ fragment was used as a probe. The Southern blot was rehybridized with a Thy 1.2 probe as an internal control for loading (lower panel). Please note that lane sc contains twice the amount of DNA as lane mc. (**E**) Southern blot analysis of *Eco*RI digested tail DNA from mc and sc animals of line HS4-β-*loxP*. After hybridisation with a 900 bp *Bam*HI–*Eco*RI fragment of the human β-globin gene, a 6.3 kb band that represents the head-to-tail repeats of the transgene (approximately two copies) and two smaller fragments are seen. After recombination only the 6.3 kb band is seen, with an intensity compatible with a single copy. Lower panel shows loading control as in (D). (**F**) Southern blot analysis of *Bam*HI digested tail DNA from mc and sc animals of lineThy-*loxP*. Before recombination, a 1.4 kb band is seen representing the head-to-tail arranged repeats of the transgene (~10 copies) after hybridisation with the complete transgene. After recombination only the two end-fragments are left. Lower panel shows loading control as in D.

removed by treatment with 10 U RNase-free DNase for 15 min at 37°C and the reaction was terminated by adding stop mix (10 mM EDTA and 0.4% SDS final concentration). The transcript was purified by sequential phenol/chloroform extraction and ethanol precipitated twice with NH₄Ac at a final concentration of 0.4 M. To confirm that the transcription product encoded a protein of the expected molecular mass, the RNA was translated in a reticulocyte lysate system (Promega). A band of ~38 kDa was detected correlating with the correct size of the Cre protein (Fig. 1B).

Multiple copy transgenic lines were established by injecting three different DNA constructs. A 7 kb *Sal*I–*Pst*I fragment of pCAG-CAT-Z (Fig. 2A) (8; gift from P.Vassalli) resulted in three lines (#12, 16 and 18) having three, two and three copies respectively in a head to tail arrangement. One HS4-β-*loxP* transgenic mouse line (having two copies of the transgene in a head to tail arrangement), was generated using a 6.3 kb
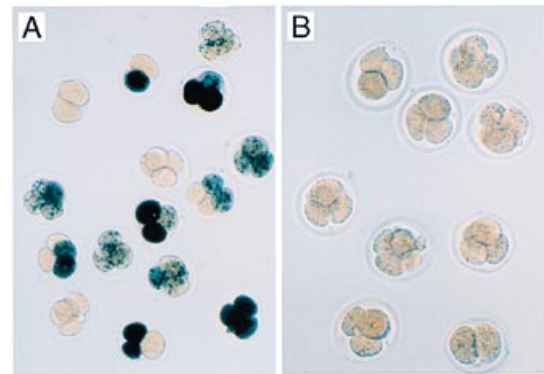

Figure 3. X-Gal staining of two- and four-cell stage embryos of CAG-CAT-Z16, recombined or not recombined. Fertilised eggs were collected from FVB females mated with heterozygote CAG-CAT-Z16 males and IC mRNA was injected into the cytoplasm at a concentration of 5 ng/µl. After incubation (5% CO₂, 37°C) for 24 or 48 h, two- and four-cell stage embryos were stained. (**A**) Injected transgenic eggs (12) were positive and injected non-transgenic eggs (3) as well as (**B**) uninjected transgenic eggs (9) were negative for X-gal staining.

*Cla*I–*Pvu*II fragment containing hypersensitive site 4 of the human β-globin LCR, the β-globin gene with –800 promoter followed by a *loxP* site downstream of the gene (Fig. 2B). One Thy-*loxP* transgenic mouse line (having ~10 copies of the transgene in a head to tail arrangement), was generated using a 1.4 kb *Apa*I fragment containing parts of exon 4 and intron 4 of the mouse Thy 1.2 gene with a *loxP* site cloned in the *Stu*I site (Fig. 2C). All fragments were injected as described (7).

Non-transgenic FVB females, 3 weeks old, were superovulated and mated with heterozygous multi copy transgenic (CAG-CAT-Z, HS4-β-*loxP* or Thy-*loxP*) males. Fertilised oocytes were collected and IRES-Cre (IC) mRNA was microinjected into the cytoplasm at a concentration of 5 ng/µl. During microinjection the RNA was kept on ice and the needle was changed every 25–30 eggs. Injected and non-injected eggs from CAG-CAT-Z lines, were incubated in M16 medium for 24 h (two-cell stage) or 48 h (four-cell stage) at 37°C in 5% CO₂ (50% survival rate). Recombination results in removal of the CAT gene between the *loxP* sites and β-galactosidase expression (8) (Fig. 2A). From seven independent injection experiments in the three different CAG-CAT-Z transgenic lines, 45% of the eggs injected with IC mRNA developed to two- or four-cell stage and stained for β-galactosidase as expected from Mendelian distribution (Fig. 3A). Non-transgenic eggs (Fig. 3A) or non-injected transgenic eggs (Fig. 3B) were negative for β-galactosidase. These results show that by injecting IC mRNA into murine oocytes, Cre recombinase is expressed at a sufficient level to perform site-specific recombination. In experiments with all three lines, we have seen that not all the cells of the dividing embryo were β-galactosidase positive, as previously reported (8). This was seen in both two- and four-cell stage embryos. When transferred into pseudopregnant BCBA foster mothers and stained for β-galactosidase at day 16 p.c. embryos showed a ubiquitous staining pattern (data not shown). This suggests that the recombination event is an early process although not instantaneous.

IC mRNA injected eggs were also transferred into the oviducts of pseudopregnant female BCBA mice. Tail DNA was examined

by Southern blotting. Complete recombination occurred in all the lines (15/15) and resulted in single copy transgenic mice. The IC sequences were not integrated into the genomes of any of these transgenic mouse lines.

We have found that injecting IC mRNA is more efficient than injecting the plasmid pCAGGS-Cre as previously described (8). Using the 'plasmid method' and injecting DNA at a concentration of 5 ng/µl we have seen complete recombination but also a significant number of partial recombination events (3 out of 11) using the same multicopy transgenic lines.

Using RNA (by injection or otherwise) to deliver genetic material to cells, rather then DNA, would also be of great interest for gene therapy protocols. In this way, the necessity of delivering DNA to the nucleus, currently one of the main obstacles in non-viral therapy protocols to achieve successful gene expression, could be avoided.

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