

Null Mutant Mice for Thyroid Hormone Receptors

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1. Introduction

In mammals, thyroid hormones (TH) have been shown to control the post-natal development of many organs, such as brain, intestine and long bone, and to participate in the maintenance of homeostasis in adults by controlling basal metabolism, heart rate, and body temperature (*1,2*). To ensure this last role, circulating TH concentrations are maintained very stable by a tight control of TH production. Indeed, TH, which is primarily synthesized in the thyroid gland, represses the production of two peptidic hormones, thyrotropin-releasing hormone (TRH) in the hypothalamus and thyroid-stimulating hormone (TSH) in the pituitary. TRH normally stimulates the production of TSH, which, in turn, stimulates the thyroid gland and thus permits an efficient TH production (*3*).

TH are lipophilic molecules able to passively cross the membranes and bind to nuclear receptors, thyroid hormone receptors (TRs), which are transcription factors whose activity is modulated by ligand binding (*4*). Four TRs have been described to date, TR α 1, TR β 1, TR β 2 (*5*), and TR β 3 (*6*), encoded by two distinct loci *TR α* and *TR β* . In addition three other isoforms are generated from the *TR α* locus, TR $\Delta\alpha$ 2, TR $\Delta\alpha$ 1, and TR $\Delta\alpha$ 2, which do not bind TH and act in vitro as inhibitors of TR. Little is known on the mechanisms of action of TH in vivo, and even less is known about the specific roles played by each TR isotype or isoform in the transmission of TH signal. To address this question, different knock-out mice, in which the expression of one or more of the TRs is selectively abrogated, were generated by homologous recombination (*7–13*). A number of different alleles of the *TR α* locus have been generated in an attempt to better understand the role of the different isoforms. The comparative phenotypic analyses of these different mutant strains allowed to conclude that:

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- TR α 1 is the main receptor implicated in the transduction of TH signal during postnatal development, and particularly in the control of body growth, maturation of intestine and bone, and development of the immune system (9,10,14,15).
- TR β is the main receptor involved in the maturation of cochlea (16), and in the regulation of liver metabolism (17).
- TR α 1 cooperates with, respectively, TR β 2 to negatively control TSH production in the pituitary (10,11,18,19) and with TR β 1 to regulate body temperature and heart rate (13,20).

Knock-out is now a widely used technique based on homologous recombination (HR) performed in embryonic stem (ES) cells. These cells are pluripotent cells from the inner cell mass of E 3.5 blastocysts, able to grow in culture, and to participate to the development of the embryo when injected into a host blastocyst.

To specifically delete a gene (here one of the TRs), a recombination vector has to be introduced into ES cells in culture. This vector contains two arms of homology corresponding to the surrounding genomic regions of the region to be deleted and is separated by a positive selection cassette that encodes a protein conferring cell resistance to a toxic drug. This cassette allows one to identify cells in which the plasmid has been integrated. Since homologous recombination is a rare event, most of the clones, isolated after selection, are the result of a random integration in the genome. A specific screening of resistant cells is thus performed to identify the cells harboring the deletion of one allele of the targeted locus and its replacement by the selection cassette. These cells are then injected into host blastocysts, which are in turn, reimplanted in pseudopregnant females. These females give birth to some chimeric mice containing a mixture of grafted and host cells. These chimera are then crossed with wild-type mice. If a germinal transmission of the mutation occurs, some of the pups are heterozygous for the mutation in each cell of the whole body. Further crosses between heterozygous animals give rise to mice homozygous for the mutation. All these steps are summarized in **Fig. 1**.

In this chapter, we will describe how to perform a knock-out starting from the construction of the targeting vector to obtain ES cell clones harboring the mutation on one allele. Neither the production of mutant mice from these ES cells nor the different methods used to analyze these mutant strains will be discussed here.

The method developed here is the most classical one aimed at mutating a specific locus in all tissues of the mouse with the mutation occurring as early as the fertilized oocyte stage. New developments of this technique, using the Cre-*loxP* system, provide us with the possibility to perform the mutation in a time- and tissue-specific manner during mouse development. This system will only be described in the Notes section (*see* **Notes 1** and **5**).

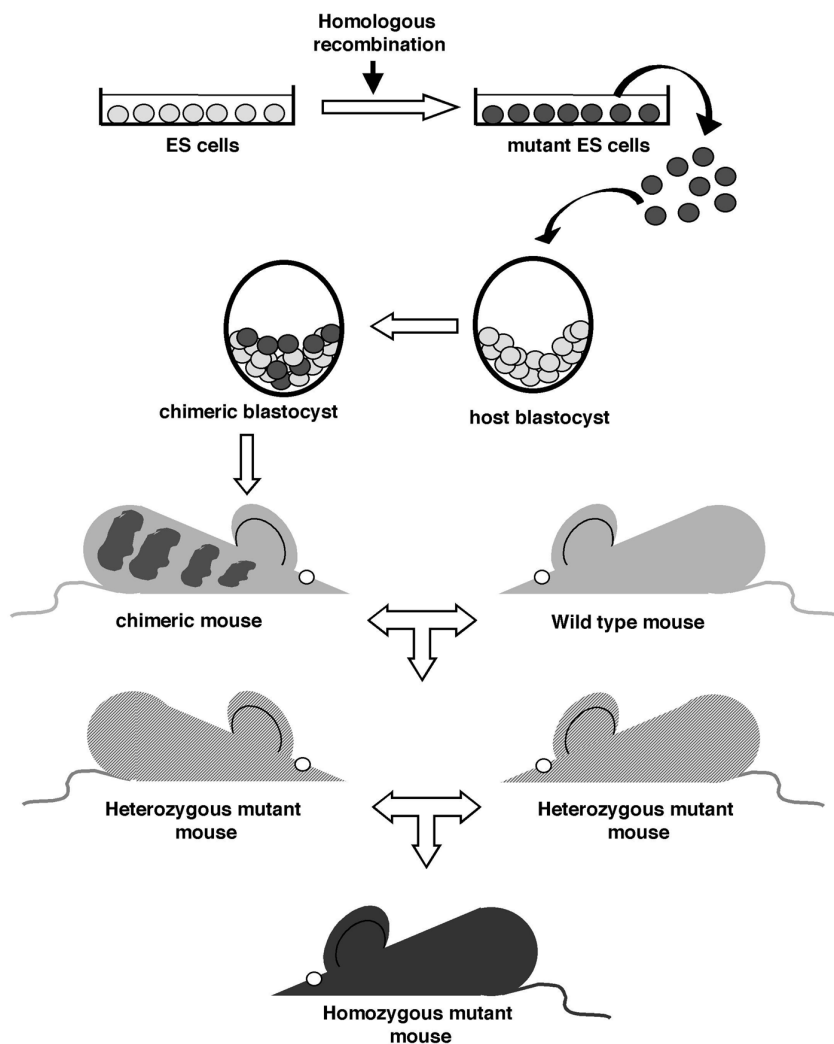


Fig. 1. Homologous recombination: from ES cells to mutant mice. Normal ES cells (figured light gray) are electroporated with the HR vector and selected for its integration at the right locus (cells in dark gray). Cells harboring the mutation on one of the two alleles of the targeted locus are injected into E 2.5 host blastocysts, which are then reimplanted in pseudopregnant females. The injected mutant ES cells participate to the development of the embryo, giving birth to chimeric mice composed of mutant and normal cells. Since ES cells and host blastocysts belong to two mouse strains recognizable by their hair color, the chimera can be easily identified. These chimera, usually males (because the ES cells used have a male genotype), are then crossed with wild-type females. Heterozygous animals are obtained and identified from their hair color. Heterozygous mice are intercrossed to generate homozygous animals in a mendelian ratio (1/4), provided that the mutation is not deleterious for embryonic development.

2. Materials

2.1. Construction of the Homologous Recombination Vector

1. Total genomic DNA from ES cells or a plasmid containing a fragment of genomic DNA covering the desired region.
2. Specific oligonucleotides designed to amplify the different arms of the homologous recombination vector.
3. A plasmid (pMC1Neo, Stratagene) containing the NeoR cDNA under the control of a promoter active in ES cells (e.g., phosphoglycerokinase [PGK]).
4. (Optional) A plasmid containing the herpes simplex thymidine kinase cDNA under the control of a promoter expressed in ES cells (e.g., PGK).
5. A PCR cloning kit (PGEMt, Promega; or Topo, Invitrogen; etc).
6. A *Taq* DNA polymerase able to amplify long DNA fragments with high fidelity (e.g., Expand Long Template, Roche).
7. 3 M Sodium acetate in ultrapure water. Store at room temperature (RT).
8. Ethanol 100%.
9. The restriction enzymes appropriate for the different cloning steps.

2.2. Homologous Recombination in ES Cells

Every material has to be sterile and tested for cell culture.

1. Fetal calf serum (FCS) tested for toxicity and cloning efficiency on ES cells.
2. Mouse embryonic fibroblasts (MEF) resistant to the antibiotic used for the positive selection (Gibco).
3. An ES cell line. We use ENS ES cells (**10**).
4. Gelatin solution: 0.1% (w/v) tissue-culture grade gelatin mixed in ultrapure water and sterilized by autoclave. Store at RT.
5. Standard culture medium: Glasgow-modified essential medium (GMEM). Store at 4°C.
6. Penicillin–Streptomycin (PS): stock solution 100X, 10 g/L. Store at –20°C.
7. Glutamine (G): stock solution 100X, 200 mM. Store at –20°C.
8. Sodium pyruvate (NaP): stock solution 100X, 7.5% NaP. Store at 4°C.
9. Nonessential Amino Acids (NEAA). Stock solution 100X. Store at 4°C.
10. β -Mercaptoethanol : Stock solution 1000X, 10^{-1} M β -Mercaptoethanol in phosphate-buffered saline (PBS). Store at –20°C.
11. PBS without Ca^{2+} and Mg^{2+} . Store at room RT.
12. Mouse ESGRO™ LIF 10^6 μmL (Gibco-BRL): stock solution 1000X. Alternatively, supernatant from transfected COS7 cells expressing the human recombinant leukemia-inhibitory factor (LIF), sterilized by filtration (0.22 μm). The amount of supernatant required has to be evaluated. Store at –20°C.
13. ES medium: GMEM, FCS 10%, PS 1X, G 1X, NaP 1X, NEAA 1X, β -Mercaptoethanol 1X, LIF 1X. Store at 4°C for a maximum of 15 d.
14. Freezing medium (2X): 80% (v/v) FCS, 20% (v/v) dimethyl sulfoxide (DMSO). Extemporaneously prepared.

15. Trypsin solution for ES cells (TES): 70% (w/v) NaCl, 10% (w/v) D-glucose, 3% (w/v) Na₂HPO₄, 3.7% (w/v) KCl, 2.4% (w/v) KH₂PO₄, 4% (w/v) EDTA, 30% (w/v) Trizma base in ultrapure water. pH has to be adjusted to 7.6 with HCl. Add 25% (w/v) trypsin (Gibco) in this solution preheated at 37°C, under stirring. Filter-sterilize on a 0.22-μm membrane. Store at -20°C.
16. G418: stock solution 1000X, 200 mg/mL in ES medium (Roche). Filter-sterilize on a 0.22-μm membrane. Store at -20°C. The dose used for selection has to be determined for each cell line and G418 batch: the minimal dose necessary to kill 100% of nonresistant cells (200 μg/mL for our ES cell line).
17. Gancyclovir: stock solution 20 mM. Used at 0.2 μM. Store at -20°C.
18. Culture plates (Corning): diameter 100 mm (B100), 60 mm (B60), 96- and 24-well plates.
18. Electroporation apparatus: Bio-Rad Gene Pulser™ with a capacitance extender.
19. Gamma ray irradiation apparatus.

2.3. Screening of the Resistant Clones

1. PCR lysis buffer (Tween buffer): 50 mM KCl, 10 mM Tris-HCl, pH 8.3, 1.5 mM MgCl₂, 0.5% (v/v) Tween-20 in ultrapure water. Sterilize by autoclave. Store at 4°C. Add 0.05% (w/v) proteinase K (PK) extemporaneously.
2. *Taq* DNA polymerase.
3. Polymerase chain reaction (PCR) machine.

2.4. Amplification and Further Characterization of the Positive Clones

2.4.1. Southern Blot

1. Southern blot lysis buffer: 100 mM NaCl, 10 mM Tris, pH 8.0, 10 mM EDTA in ultrapure water. Sterilize by autoclave. Extemporaneously add 0.5% (w/v) sodium dodecyl sulphate (SDS), 0.05% (w/v) PK.
2. Prehybridization (and hybridization) solution: 0.25% (w/v) fat-free milk powder, 4X sodium chloride sodium phosphate EDTA (SSPE [4X SSPE: 600 mM NaCl, 40 mM NaH₂PO₄, 5 mM EDTA]), 1% (w/v) SDS, 0.01% (w/v) denatured salmon sperm in ultrapure water. To be prepared extemporaneously.
3. For hybridization, just add the denatured radioactive probe in the prehybridization solution.

2.4.2. Karyotype

1. Colcemid stock solution 25X (2 μg/mL): demecolcin tested for cell culture (Sigma) diluted in PBS. Store at 4°C.
2. Hypotonic solution: KCl 0.56%. Store at RT.
3. Fixation solution: methanol (3 vol)/ acetic acid (1 vol). To be prepared extemporaneously and kept at RT.
4. Giemsa staining solution: add in this order, 9 mL of water, 1 mL of Giemsa R colorant, and 0.1 mL of Wright. Prepare extemporaneously.
5. Eukitt (O. Kindler GmbH & Co) for slide mounting.

2.4.3. *Mycoplasma* Detection

1. Standard Mycotect assay from Gibco-BRL.

3. Methods

3.1. *Construction of the Homologous Recombination Vector*

3.1.1. *Structure of the Construction*

The vector contains different components (**Fig. 2**):

- A backbone plasmid containing a resistance gene to ampicillin or kanamycin and a replication origin for amplification in bacteria.
- The two arms of homology, which are the DNA genomic sequences surrounding the chromosomal region to be destroyed. The size of the two fragments have to be different: 1–2 kb for the shorter one, 3–6 kb for the longer one. It is easier for the following if one knows the partial or entire sequence of these regions, but this is not absolutely necessary.
- A selection cassette that enables the autonomous expression of a positive selection marker, most of the time a cDNA, providing the resistance to Neomycin (NeoR) under the control of a PGK promoter.

3.1.2. *Strategy for the Construction*

The different components have to be inserted into the backbone plasmid: the short homology region should be inserted first, in order to avoid the accumulation of restriction sites and to work as long as possible with small plasmids. The different elements in the vector should be ordered as follows, from 5' to 3' (**Fig. 2**): the 5' arm of homology, the positive selection cassette (preferably in the opposite orientation), the 3' arm of homology. A unique restriction site is absolutely required, positioned at either end of the block containing the above elements, for linearization of the construct before electroporation (*see Note 2*).

3.1.3. *Obtaining the Two Arms of Homology*

The simplest way (and the only one developed here) to obtain the fragments is to use PCR amplification on a genomic DNA preparation or on a plasmid containing a fragment of genomic DNA covering the desired region. Cohesive ligations have to be used for all the cloning steps, either taking benefit of some naturally occurring sites or introducing them in the primers used for PCR. When designing the short arm, keep in mind that you have to know a sequence upstream of it, if it is located 5' relative to the region to be deleted (downstream of it, if it is in the 3' position), in order to design a primer for PCR screening of the transfected ES clones.

1. Amplify the two arms by PCR using a reagent able to amplify long DNA fragments with high fidelity (for example Template Long expand, Roche). The

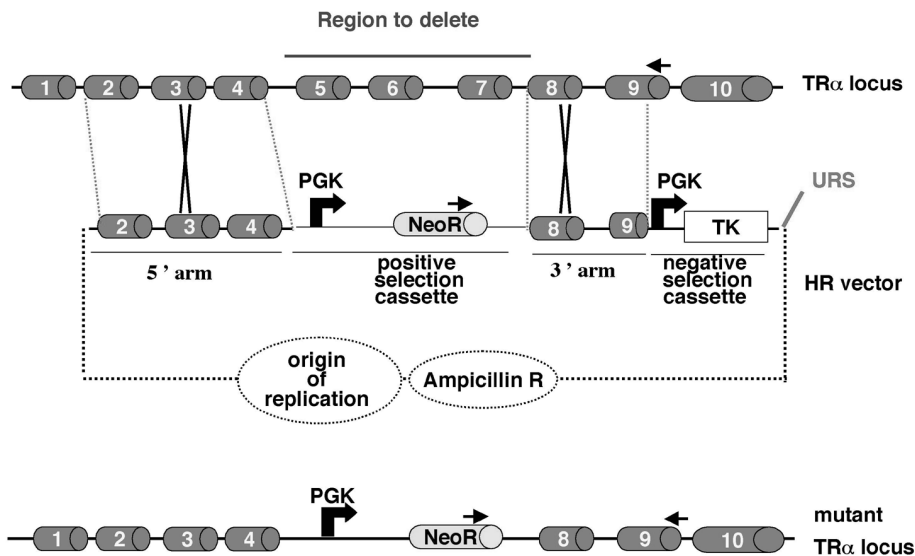


Fig. 2. The homologous recombination vector. The backbone plasmid is figured as a dotted line (.....). For genomic regions, numbered gray cylinders represent the exons, and the thick black line represents the intronic regions. The positive selection cassette contains a cDNA encoding the resistance to G418 (NeoR) (green cylinder) under the control of the PGK promoter (thick arrow). The upper arrows represent the primers used for screening the resistant clones: one in the HR vector, one outside of the short arm. A negative selection cassette can optionally be placed outside, figured as the TK box. A unique restriction site (URS) is placed outside of the arms to linearize the vector before electroporation.

template can either be 10 ng of plasmid or 100 ng of genomic DNA. Elongation time has to be long, approx 1–2 min/kb to be amplified. 25 Cycles are sufficient for plasmid as starting material, 35 cycles are required using genomic DNA preparation.

2. Clone each arm using a TA cloning system and sequence the exonic portions.
3. Insert, one-by-one, the different components by cohesive ligations. The long arm and selection cassette can be cloned into the vector containing the short arm. However, it may be more convenient to sequentially transfer each of the arms into a vector containing the selection cassette. The strategy must be chosen according to the availability of restriction sites. Remember, you have to introduce a unique restriction site at the 3' or 5' position of the recombination block.
4. Digest 40 μ g of the final plasmid (the vector for homologous recombination) with the restriction enzyme chosen for linearization for 3 h at 37°C. After checking for a complete cutting, precipitate DNA adding 0.1 vol of sodium acetate 3 M and 2.5 vol of ethanol 100%. Wash with ethanol 70% and let the pellet dry in a sterile environment. Resuspend in 40 μ L of ultrapure sterile water.

3.2. Homologous Recombination in ES Cells

3.2.1. General Recommendations for ES Cell Culture

ES cells are cultured in complete ES medium on a feeder layer. These feeders are MEFs, which have been irradiated at 45 Gy and seeded in 100 mm culture dishes coated with 0.1% gelatin. Irradiated MEFs cannot be stored more than 1 wk. ES cells have to be trypsinized every 2 d and seeded at a density of 5×10^6 cells per 10 mL. The day after, the medium has to be changed.

3.2.2. Electroporation of ES Cells and Selection of the Resistant Colonies

1. Trypsinize ES cells.
2. Inactivate the trypsin with normal ES medium and spin for 5 min at 800g.
3. Wash the pellet twice with GMEM or OptiMEM.
4. Count the washed cells. Mix 5×10^6 cells with 40 μ g of the linearized recombination vector in a total volume of 800 μ L GMEM or OptiMEM.
5. Transfer the mixture into a 4-mm electroporation cuvette and perform the electroporation at 260 V and 500 μ F.
6. Wait for 20 min before seeding these cells on 5 B100 plates on a MEF layer resistant to the antibiotic used for the positive selection and add 8×10^5 nonelectroporated ES cells per plate.
7. Seed 8×10^5 nonelectroporated ES cells on a MEF layer in a B100 plate as a control.
8. Replace the ES medium 24 h after seeding.
9. Replace the ES medium 14 h later and add the antibiotic used for the selection (200 μ g/mL for G418).
10. During the first 3 d of selection, wash the cells with PBS before replacing the medium, in order to discard the maximum of dead cells. During the rest of the selection period, only aspirate the medium and replace it with some fresh medium supplemented with antibiotic everyday.
11. After 4 or 5 d of selection, there should not be any cells left in the control plate, and colonies should appear in the plates seeded with electroporated ES cells.
12. Let the colonies grow up until they occupy the entire field observed using the 100X objective of the microscope, a size usually obtained after 7 to 9 d of selection (see **Note 3**).

3.2.3. Isolation and Amplification of the Resistant Colonies

Each colony of resistant ES cells has then to be cloned and amplified (see **Note 4**).

1. To pick up the clones, settle the microscope under the laminar flow hood.
2. Aspirate the medium of the B100 plate, wash with PBS, and again add 10 mL of PBS.
3. Each colony has then to be mechanically detached from the plate by scraping around with a tip of a P20 Gilson pipetman. When it is partially detached, just aspirate it in a maximum volume of 15 μ L.

4. Mix with 40 μL of TES in an eppendorf tube, dissociate actively by gently pipeting up and down, and wait for 20 min at RT.
5. Each dissociated colony is then individually seeded over into a well of a 96-well plate on a MEF layer full of ES medium.
6. The clones have to be amplified. Just change the ES medium everyday until you estimate that cells are at a normal density in the well (usually 2 to 4 d depending on the initial size of the colony).
7. Trypsinize the cells in the well with 50 μL of TES, inactivate with 100 μL of ES medium, transfer into a well of a 24-well plate on a MEF layer, and fill up the well with ES medium.
8. Change the ES medium every day until you estimate that cells are at a normal density (usually 2 to 3 d).
9. For amplification, trypsinize the cells in the well with 100 μL of TES and add 1 mL of ES medium. 200 μL of this suspension are transferred into a well of a 24-well plate full of ES medium and containing a MEF layer for maintenance. For the screening procedure, the remaining cells are transferred into a well of the same size precoated with 0.1% gelatin, without MEF layer. At this stage, each clone has to be individually identified.
10. One day later, lyse the cells in the screening well (see the protocol below) and replace the medium in the amplification well.
11. The day after, freeze the cells in the amplification well. Trypsinize cells with 100 μL of TES, resuspend them in 400 μL of cold ES medium (4°C), put the plate on ice for 15 min, then slowly add 500 μL of freezing medium, and gently mix. Tightly seal the plate with parafilm and store it at -80°C in polystyrene box for up to 15 d.

3.2.4. Screening of the Resistant ES Cell Clones

3.2.4.1. LYSIS OF THE CELLS

1. Aspirate the medium and wash with PBS.
2. Replace PBS with 200 μL of PCR lysis buffer.
3. Transfer immediately into an Eppendorf and incubate overnight at 56°C under agitation.

3.2.5. PCR Screening (see **Note 5**)

1. The lysate (1 μL) is then used to perform the PCR in a total volume of 50 μL .
2. The kind of polymerase used for the reaction and the specific amplification program depend on the size of the fragment to be amplified and should have been set up previously.
3. PCR mixture (15 μL) is then loaded onto an agarose gel.

3.2.6. Amplification and Further Characterization of the Positive Clones

3.2.6.1. AMPLIFICATION

1. Thaw the positives clones as soon as possible after identification.
2. Take the 24-well plate out of the freezer and add 500 μL of prewarmed ES medium (37°C) in the wells containing the positives clones.

3. Move the cell suspension up and down with the pipetman until complete thawing.
4. Place the whole content of the well into a new well on a MEF layer and fill up with ES medium.
5. Replace the medium the next day in order to eliminate the DMSO.
6. Amplify in standard ES cell culture conditions and freeze a few samples in freezing tubes for storage in liquid nitrogen.

3.2.7. Characterization of the Positive Clones by Southern Blot

1. A large amount of ES cell DNA has to be prepared, therefore 2×10^6 – 10^7 cells should be used as starting material.
2. Seed ES cells from a positive clone on a B100 plate precoated with 0.1% gelatin.
3. When cultures reach high density, wash the plate with PBS, and lyse with 1 mL of Southern blot lysis buffer, into which PK has just been added, transfer into an Eppendorf tube, and incubate overnight at 56°C under stirring.
4. Add 1 mL of phenol-chloroform (v/v) and 100 μ L of sodium acetate 3 M, shake, spin for 10 min at 1200g, and transfer the supernatant into a new Eppendorf tube.
5. Add 1 mL of isopropanol, shake and transfer the DNA precipitate into an Eppendorf tube full of ethanol 70%.
6. Transfer the DNA pellet in an empty Eppendorf tube, let it dry, and resuspend it in Tris 5 mM EDTA, 0.1 mM Rnase, 10 μ g/mL, pH 7.5, for 1 h at 37°C.
7. Digest 10–15 μ g of this genomic DNA with 40 U of the appropriate enzyme in a total volume of 70 μ L. Dithiothreitol (DTT) (1 mM) and 1 mM spermidine are added to stabilize some restriction enzymes and to avoid star activity. Incubate at least for 3 h (or overnight) at 37°C.
8. Run the samples (after loading buffer addition) on an agarose gel. Incubate the gel for 15 min in a 0.25 M HCl solution, and then transfer it on a Hybond N⁺ membrane (Amersham) by capillary transfer under alkaline conditions (0.4 N NaOH) overnight.
9. Wash the membrane twice with a 0.2X SSPE solution and prehybridize it for at least 1 h.
10. Hybridize overnight, with a radiolabeled probe denatured for 5 min at 100°C, wash, and expose. The probe is usually one of the vector arms labeled by random priming–extension (Pharmacia Ready-to-go).

3.2.8. Checking for the Karyotypes

In cell culture, aberrant Karyotypes can arise. Since such abnormality will prevent recombinant ES cells to generate gametes in chimeric animals, it is better to check for the Karyotype of the selected ES clones before they are injected into host blastocysts.

1. Karyotype analysis has to be performed on a subconfluent B60 plate of ES cells cultured on a feeder MEF layer.
2. Replace the medium at least 1 h before beginning the experiment.
3. Add colcemide (0.08 μ g/mL) into the medium and incubate from 30 min to 1 h at 37°C.

4. Trypsinize the cells, inhibit the trypsin with ES culture medium, and spin for 5 min at 800g. Resuspend the cell pellet in PBS and spin again. This step has to be repeated twice. The pellet is finally resuspended in 2 mL of hypotonic solution.
5. Let the cells blow up for 10 min at RT and add 2.5 mL of fixative solution.
6. Spin for 5 min at 800g, resuspend the cells in 6 mL of fixative solution at RT, and wash the pellet twice in fixative solution at RT. The pellet is finally resuspended in 0.5 mL of fixative solution.
7. Place the cells for at least 2 h at -20°C .
8. Burst the cells as soon as they are out of the freezer, by letting a few droplets of cell suspension fall onto an tilted slide, pretreated with 70% ethanol.
9. Let the slide dry and stain it with Giemsa for 15 min. Wash and let dry again.
10. Mount the preparation for the observation with Eukitt or aquavitrex and cover with a coverslip.

3.2.9. Checking for the Presence of Mycoplasmas

Mycoplasma infection in ES cells may prevent them, after injection into blastocysts, to efficiently colonize the germline. Mycoplasma infection in positive clones can be checked using the standard MycoTect assay from Gibco-BRL. One confluent well from a 24-well plate of ES cells is sufficient to perform the test.

3.3. Conclusion

The efficiency of homologous recombination varies a lot depending on the locus to be targeted and the specific region to be destroyed within this locus. One should also keep in mind that the density of ES cells, when trypsinized for electroporation, and the time when selecting drugs are added, highly influence the ratio of homologous recombinations nonspecific integration, using the same RH vector. For example, the rate of positive clones ranged from 1/300 to 1/5 when we performed the generated the TR α allele.

Thus, so far there is no way to predict the efficiency of homologous recombination. Nevertheless, taking care of some details will help to increase your chances.

4. Notes

1. The Cre-*loxP* system is derived from the P2 bacteriophage. Cre is a recombinase that recognizes some specific sequences, the *loxP* sites, and is able to catalyze the excision of a DNA fragment present between two of these sites arranged as direct repeats (**Fig. 3**). This system is now frequently used to perform tissue- and/or time-specific knock-out.

Two *loxP* sites are introduced in tandem by homologous recombination in such a way they will flank the region to be deleted, without interfering with gene expression (placed in introns for example). Mice harboring this mutation are then crossed with transgenic mice expressing Cre in a tissue-specific manner. The

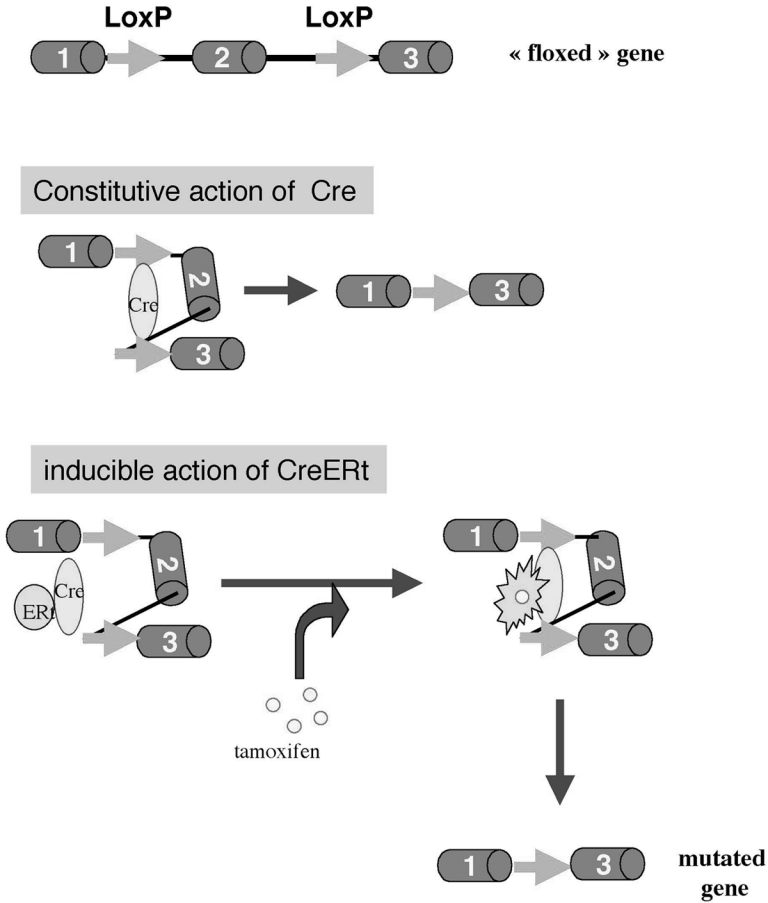


Fig. 3. A technological improvement : the CreLoxP system. LoxP sites are figured as green arrows. The Cre recombinase recognizes two of them arranged as direct repeats and excises the fragment in between. The activity of this enzyme becomes inducible when fused to the ligand-binding domain of the estrogen receptor (ERt) modified to bind only tamoxifen. In the absence of tamoxifen, the enzyme is inactive.

time specificity is obtained using a Cre fused to the estrogen receptor (ER) ligand-binding domain modified to respond only to tamoxifen (CreER^t). In mice expressing this chimeric protein, recombinase activity can be induced by tamoxifen administration (21).

This system can also be very useful to perform some more precise mutations without modifying the structure of the entire locus. It has been particularly helpful in the case of *TRα*, to study the in vivo functions of TRΔα1 and TRΔα2, by preventing their production without altering the expression of neither TRα1 nor TRα2. To do so, a specific deletion of one part of intron 7 containing the pro-

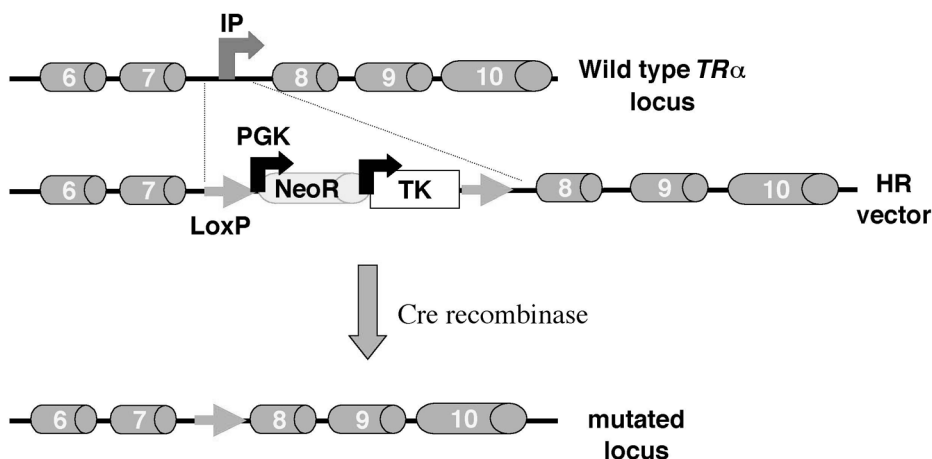


Fig. 4. Homologous recombination for the deletion of the internal promoter (IP) of the *TRα* locus. Signs and symbols are the same as in **Fig. 2**.

motor activity was performed (22). The structure of the RH vector was classical apart from the *loxP* sites flanking the selection cassette (**Fig. 4**). Once positive recombinant ES cell clones were identified (after G418 selection), they were electroporated with 5 μ g of a Cre-expressing vector (in the conditions described) to remove the *loxP*=NeoR(TK)=*loxP* cassette, which contained some autonomous promoters and stop signals likely to interfere with the normal transcription and/or translation of the *TRα1* and *TRα2* products. Gancyclovir was used here as a positive selection, in order to select clones in which TK had been excised. In our hands, excision occurred in 100% of the resistant clones. We have verified that mice homozygous for this mutation, present normal level of *TRα1* and *TRα2*, and strongly reduced level of *TRΔα1* and *TRΔα2*.

This strategy has also been used by Kaneshige et al. (23) to introduce a point mutation in the coding region of *TRβ*, to transform this receptor into a transdominant negative form responsible for the generalized resistance to thyroid hormone syndrome (24). In this case the strategy was quite simple. The easiest way was to clone a very large genomic fragment of the locus (10–15 kb) in a backbone plasmid, to introduce the point mutation (changing 2 or 3 bp) with a recombinant PCR strategy in this genomic sequence, and then to introduce a *lox*=Neo(TK)=*lox* cassette in an intron for recombinant ES cell selection. It is important to place the resistance cassette as close as possible to the point mutation in order to minimize the risk of a recombination event taking place in between. To verify within recombinant ES cells that the point mutation has been retained, it is recommended to introduce a restriction site, either in the mutation or just beside it. Just amplify a DNA fragment surrounding the mutation and cut the PCR product with the appropriate restriction enzyme.

The excision of the selection cassette can either be performed in the ES cells (as

described for the deletion of the internal promoter of *TRα*) or in vivo, by crossing mice homozygous for the mutation with transgenic mice expressing the Cre during the very early embryonic stages (25) as performed by Kaneshige et al. (23).

For all these uses, it is absolutely necessary to sequence all the coding regions present in the RH vector to be sure that the phenotype observed is due to the introduced mutation and not to *Taq* DNA polymerase mistakes.

2. The construction of the homologous recombination vector is a crucial step. The arms of homology in the HR vector have to be as homologous as possible to their cellular genomic counterparts. Use genomic DNA prepared from the same mouse strain as that of the ES cells in which the recombination will be performed. Use a reagent with high fidelity to amplify fragments of genomic DNA. A negative selection cassette can be placed either upstream of the 5' arm or downstream of the 3' arm. This cassette must enable the autonomous expression of a negative selection marker. The most frequently used is the cDNA encoding the herpes simplex thymidine kinase under the control of a PGK promoter. TK will transform the innocuous gancyclovir into a toxic derivative. Gancyclovir is added during the course of the positive selection, but for 5 d only. If integration takes place at a nonspecific site by nonhomologous integration, TK will usually be integrated, resulting in death of the cells upon gancyclovir treatment. In contrast, if homologous recombination occurs, the TK-containing cassette is discarded with the rest of the vector, and the cells are thus insensitive to the addition of gancyclovir in the selection medium. A LacZneo fusion (β geo) can be used instead of Neo as positive selection gene. The expression of β -galactosidase (β -gal), revealed by 5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside (X-gal) staining can be used to screen the mutant mice. Alternatively, this β geo cassette can be used without promoter, fused to the ATG start site of the targeted cellular gene, present in the 5' arm. With such a construct, the homologously recombined β geo transcript will be expressed with the same pattern as that of the targeted locus. X-gal staining allows one to follow its expression in ES cells and in the mouse derived from them. However, this strategy is inefficient if the transcription of the targeted gene is too low in ES cells, since it will result in weak resistance of the recombined ES cells to G418 and obviate efficient selection.
3. It is very important to wait until the colony is large enough before cloning them. Look carefully at your plates during the selection. It sometimes happens that colonies stop growing in diameter and grow in thickness. The cells at the periphery then start to differentiate, and the clone will never cover the expected surface. This type of clone should be picked up early. As a general rule, clone only compact colonies, composed of small cells with as few differentiated cells as possible.
4. The number of clones to pick up depends on how many resistant colonies arise and on how many colonies you are able to handle for amplification. Considering that the ratio of positives clones ranges from 0.5–30% (in our hands, when knocking out part of the *TRα* or *TRβ* loci), up to 250 colonies should be screened. In any case, a very large number of resistant clones does not predict high recombina-

nation efficiency, and most of the time this situation occurs when selection has been applied too late.

Interclone contamination can be a problem, especially if you handle numerous clones. Do not use the same pipet to aspirate the medium of the different clones. A convenient trick is to plug a small pipet tip on a Pasteur pipet, and to use a new tip for each well. As soon as you detect a contamination, eliminate this well and treat with bleach.

If you can perform the screening for homologous recombination immediately, you will avoid freezing the wells and, therefore, save time. Indeed the protocol timing allows to obtain the results of the PCR screening before the amplification culture has reached saturation. For this purpose, cell lysis is carried out overnight and PCR the day after. You can discard the negative clones and continue the amplification of the positive clones. You will later check for the correct structure of the recombined locus within the selected clones by PCR or Southern blot analysis.

5. Screening is one of the limiting step that needs particular attention. Many PCRs will be performed at the same time on crude lysates. The PCR should thus be very robust. One good way to settle the parameters of this PCR is to construct a "test plasmid" containing the positive selection cassette together with a genomic DNA fragment corresponding to the short arm extended by a few hundred base pairs. This test plasmid can be diluted (10^{-6}) in wild-type ES cell lysate to provide a control sample for the PCR. PCR conditions (choice of primer pairs, temperature, etc.) can be optimized on this plasmid. However, to be exactly in the right conditions, it is better to electroporate this vector into ES cells, to select and clone one or two resistant colonies, and to optimize the PCR on their DNA extracted in the true conditions. These cells can then be frozen in order to be thawed and cultured during the true screening and serve as a perfect control of every screening step from lysis to PCR. However this test plasmid is a source of PCR contaminations: a complete decontamination (bench and pipets) is required after plasmid amplification.

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