Ligand-activated site-specific recombination in mice
(inducible gene targeting/Cre recombinase/estrogen receptor/tamoxifen/somatic mutations)

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ABSTRACT Current mouse gene targeting technology is unable to introduce somatic mutations at a chosen time and/or in a given tissue. We report here that conditional site-specific recombination can be achieved in mice using a new version of the Cre/lox system. The Cre recombinase has been fused to a mutated ligand-binding domain of the human estrogen receptor (ER) resulting in a tamoxifen-dependent Cre recombinase, Cre-ERT, which is activated by tamoxifen, but not by estradiol. Transgenic mice were generated expressing Cre-ERT under the control of a cytomegalovirus promoter. We show that excision of a chromosomally integrated gene flanked by loxP sites can be induced by administration of tamoxifen to these transgenic mice, whereas no excision could be detected in untreated animals. This conditional site-specific recombination system should allow the analysis of knockout phenotypes that cannot be addressed by conventional gene targeting.

The study of the genetic control of mammalian development and physiology has been revolutionized by the ability to inactivate (knockout) specific genes by homologous recombination in the mouse (1). However, using current gene targeting technology, interpretations of knockout phenotypes are often limited by several factors. First, the presence of a selection marker may influence the phenotype of the mutation (2, 3). Second, artefacts can arise due to the lack of a gene product for the whole lifetime of the animal. Third, the inactivation of a gene may result in intra-uterine lethality, precluding analysis of the possible function(s) of the gene at later stages of development and/or post-natally. A conditional gene targeting method based on the inducible activity of an engineered DNA recombinase could overcome these limitations by allowing the removal of the selection cassette and the timed and tissue-specific inactivation of target genes at will during development and in the adult mouse (4). Furthermore, such an inducible system could help in certain cases to distinguish between anomalies related to a mixed genetic background and those due to mutation of the targeted gene.

The bacteriophage P1 Cre recombinase efficiently excises DNA flanked by two directly repeated loxP recognition sites in mammalian cells (5, 6). We have previously reported that fusion of the ligand-binding domain (LBD) of the estrogen receptor (ER) to the Cre recombinase generates a chimeric recombinase whose activity in cultured cells is dependent on the presence of an estrogen (estradiol) or an anti-estrogen (tamoxifen) (7). To achieve conditional gene targeting in mice, where endogenous estradiol is present, we have subsequently fused Cre to a mutated LBD of the human ER (Gly 521 → Arg, G521R) resulting in the chimeric protein Cre-ERT. Indeed, the corresponding mouse ER LBD mutant (G525R) does not bind 17β-estradiol (E2), whereas it binds the synthetic ligands tamoxifen and 4-hydroxytamoxifen (OHT) (8). We report here that Cre-ERT is a functional tamoxifen-dependent recombinase in cultured cells and in transgenic mice.

MATERIALS AND METHODS

Construction of Plasmids and Generation of Transgenic Mice. pCMVCre-ERT was constructed by cloning the 2-kb EcoRI fragment isolated from pCre-ERT1 into the EcoRI site of the expression vector pMSGSV1. pCre-ERT1 was obtained by first replacing the 252-bp HindIII-BglII fragment of pCre-ER (7) with the corresponding fragment isolated from the expression vector HEG0 (9), coding for the human ER containing a glycine at amino acid 400. The amino acid corresponding to glycine 521 of the human ER was then mutated to an arginine by site-directed mutagenesis using the oligonucleotide 5'-CAC-ATGAGTAAACAAAAGAATGGGACATCTGTA-3'. To obtain pCMVCsv1, a 600-bp XbaI-HindIII restriction fragment containing the enhancer/promoter region of the major IE gene of the human cytomegalovirus (CMV), isolated from pCMVcat (10) was first cloned into BSM+ (Vector Laboratories cloning systems) digested with XbaI and HindIII, resulting in BSM-CMV. The simian virus 40 promoter region of pSG1 (11) was then replaced with the CMV promoter by cloning the 600-bp SacI-HindIII restriction fragment isolated from BSM-CMV into pSG1 digested with SacI and BamH1 after filling in the HindIII and BamHI restriction sites with T4 polymerase. The 4.6-kb PvuII DNA fragment of pCMVCre-ERT1 was injected into (C57BL/6 × SJL) F1 zygotes at a concentration of 4 ng/ml to generate transgenic mice according to established procedures (12).

PCR Conditions. PCR amplification was carried out in a buffer containing 10 mM Tris-HCl (pH 8.0), 50 mM KCl, 1.5 mM MgCl2, 0.2 mM dNTPs, 0.25 μM of each primer, and 2 units Taq polymerase using 1 μg of genomic DNA as template. After 35 cycles (30 sec at 94°C, 30 sec at 55°C) the products were analyzed on ethidium bromide-stained 2.5% agarose gels.

Analysis of F9 Cells. Mouse F9 embryonal carcinoma cells carrying a floxed tkneo marker integrated into exon 4 of one retinoid X receptor α (RXRa) allele [RXRa+/-(LNL), (13)] were transiently transfected with pCre-ER (7) or pCre-ERT1. After a 24-h incubation period, cells were grown in the presence of vehicle (ethanol) alone, 100 nM E2, or 1 μM OHT for 48 h. PCR amplification of a 175-bp fragment specific for the excised allele was carried out using 1 μg of genomic DNA as template. The 5' and 3' primers were 5'-GGCACAACATATGCG-3' and 5'-TTGCGTACTGCTCTTATT-3', respectively.

Genotyping of Mice. The Cre-ERT1 transgene and the RXRaΔAF2Δ(LNL) [target allele were detected in mouse tail DNA

Abbreviations: CMV, cytomegalovirus; Cre-ERT1, fusion protein between the Cre recombinase and a mutated ligand-binding domain of the human estrogen receptor (G521R); ER, estrogen receptor; LBD, ligand-binding domain; OHT, 4-hydroxytamoxifen; RT, reverse transcriptase; RXRa, retinoid X receptor alpha; tkneo, thymidine kinase/neomycin-resistance fusion gene; wt, wild type; HPRT, hypoxanthine phosphoribosyltransferase.

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both by PCR and Southern hybridization. For PCR detection of the Cre-ERT transgene, the 5′ primer for cre was 5′-ATCCGAAAAGCTTTGA-3′ and the 3′ primer was 5′-ATTCAGGTCTCGGATATG-3′. For Southern blot analysis, tail DNA (10 μg) was digested with EcoRI and a 0.7-kb BamHI–XhoI cre fragment isolated from pCre-ER (7) was used as a probe. For detection of the RXRαAF2(LNL) allele, the 5′ and 3′ PCR primers for tkneo were 5′-GGTTCTCCGGC-CCCTGGGT-3′ and 5′-GAAGGCCATGCCTGCGAAT-3′, respectively. For Southern blot analysis, tail DNA was digested with BamHI and probed with a 0.7-kb SacI fragment of the RXRa gene.

Detection of Cre-ERT mRNA Synthesis in Mice. The level of Cre-ERT mRNA was estimated by reverse transcriptase (RT)-PCR. Total RNA was isolated from mouse tissues by the LiCl/urea method (14). cDNA was synthesized for 20 min at 50°C using 50 units of Moloney murine leukemia virus RT and 1 μg of RNA and was then amplified by 35 cycles of PCR using primers 1 (5′-TTGACCTCCTAGAAGACAGC-3′) and 2 (5′-GCGGTACCTCCAGAATGTC-3′), resulting in a 254-bp fragment of the Cre-ERT cDNA. As an internal control, a 177-bp cDNA fragment of the hypoxanthon phosphoribosyltransferase (HPRT) mRNA was co-amplified in the same reaction using the 5′ and 3′ primers 5′-GTAATGTACGTTCAACGGGGGAC-3′ and 5′-CCAGCAAGCTTGCAACC-TTTAACC-3′, respectively.

Detection of Cre-ERT-Mediated DNA Excision in Mice. PCR and Southern hybridization was used to analyze Cre-ERT-mediated excision of the floxed tkneo marker from the RXRαAF2(LNL) target allele. PCR primers were primer 3 (5′-CAAGGAGCCTCCTTCTCTAATTTA-3′) and primer 4 (5′-CCCTGCTTACCTGCTTTT-3′). These primers amplify a 156-bp fragment of the RXRa wild type (wt) allele and a 190-bp fragment of the RXRαAF2(L) allele. Southern blot analysis was performed using a 2.0-kb KpnI–BamHI fragment or a 1.6-kb KpnI fragment of the RXRa gene as hybridization probes. Genomic DNA was digested with BamHI or NcoI.

Quantification of DNA Excision and mRNA Levels. Semi-quantitative PCR was used to estimate the relative level of Cre-ERT-mediated gene excision and semi-quantitative RT-PCR was used to estimate Cre-ERT mRNA expression. For each sample the appropriate cycle number for remaining within the exponential phase was determined by running different cycle numbers (27, 30, 33, and 35). The ratio between the intensity of the bands derived from the excised allele and from the wt allele, as well as the ratio between the intensity of the bands derived from the Cre-ERT mRNA and from the HPRT mRNA, was determined by laser densitometry of Polaroid photographs of ethidium bromide-stained agarose gels after subtracting the gel background. Only amplification reactions for which these ratios remained constant for at least two consecutive cycle numbers were used for quantitation. The percentage of Cre-ERT-mediated deletion in a given organ was calculated from the intensities of the bands derived from the RXRαAF2(L) and wt RXRa alleles using the formula RXRαAF2(L)/RXRa × 100. To estimate relative levels of Cre-ERT mRNA expression, the Cre-ERT signal obtained in a given tissue was normalized by division of the signal obtained for the HPRT standard mRNA. The value calculated for the skin was taken as 100%.

RESULTS AND DISCUSSION

To express a tamoxifen-dependent Cre recombinase, we constructed an expression vector, pCre-ERT, encoding the fusion protein Cre-ERT consisting of Cre fused to a mutated LBD of the human ER (G521R). The functionality of Cre-ERT was tested by transient transfection using a mouse F9 embryonal carcinoma “reporter” cell line, which carries a chromosomally integrated “floxed” tkneo gene, i.e., a tkneo gene that is flanked by two directly repeated loxp sites. In these cells, Cre-ERT excised the tkneo gene in the presence of 1 μM of OHT, but not in the presence of 100 nM of E2 or in the absence of ligand, whereas, as reported (7), Cre-ER targeted the gene both in the presence of E2 and OHT (Fig. 1). While the present study was in progress, a similar conditional Cre-ERT recombinase was described (15).

Transgenic mice expressing Cre-ERT under the control of a CMV promoter were generated (Fig. 2a). Out of three transgenic mouse lines, one showed stable maintenance and mRNA expression of the Cre-ERT transgene in the tail (data not shown) and was used in all subsequent experiments. Mice expressing Cre-ERT appeared phenotypically normal.

To analyze the efficiency of DNA excision by Cre-ERT, we crossed Cre-ERT mice with mice harboring a floxed target gene. This “reporter” line contains one wt allele of the RXRα gene and one modified RXRα allele carrying a floxed tkneo selection marker integrated by homologous recombination into the intron located between exon 8 and exon 9 [RXRαAF2(LNL)]. The wt RXRa allele and the excised RXRαAF2(L) allele can be simultaneously detected by polymerase chain reaction (PCR) using one set of primers (Fig. 2b). The relative efficiency of excision was estimated by comparing the intensity of the band amplified from the deleted RXRαAF2(L) allele with that of the band amplified from the wt RXRa allele, which differs in sequence only by the absence of the loxp site.

Offspring generated by crossing Cre-ERT and RXRa reporter mice, which harbored both the Cre-ERT transgene and the RXRαAF2(LNL) allele were identified by genotyping of tail biopsies (data not shown). These mice were treated with OHT at the age of 4 weeks and analyzed for Cre-ERT-mediated DNA excision (Fig. 3a). Excision of the floxed marker gene was undetectable in oil-treated control animals (Fig. 3a Upper and data not shown), whereas mice injected intraperitoneally (i.p.) with OHT reproducibly showed excision of the floxed target gene in all organs tested except in the thymus (Fig. 3a Lower and data not shown). Importantly, in the tail the deleted RXRαAF2(L) allele was absent before OHT administration to the animal, whereas its presence was detected following OHT treatment [Fig. 3a Lower, compare tail(a) with tail(b)]. The excision pattern and the absence of recombination background in control animals was confirmed using different routes of ligand administration (i.p., subcutaneous, orally) and Southern

![Fig. 1. Ligand-dependent activation of Cre-ERT in mouse F9 embryonal carcinoma cells. Excision of the tkneo gene in RXRαAF2(LNL) cells transfected with a control plasmid encoding an E2-activated recombinase (pCre-ER) or with a plasmid encoding Cre-ERT (pCre-ERT) was analyzed by PCR. Cells were treated with vehicle ( ) 100 nM E2 or 1 μM OHT. A control reaction without DNA template is also shown. The position of the product amplified from the deleted allele (Δ) is indicated.](image-url)
FIG. 2. (a) Structure of the Cre-ERT transgene and strategy for detection of Cre-ERT mRNA by RT-PCR. The DNA fragment used to generate transgenic mice contained the enhancer/promoter region of the major IE gene of the human CMV promoter, a rabbit β-globin intron, the Cre-ERT consisting of the Cre recombinase gene fused to the cDNA coding for the G521R mutant of the human ER LBD, and a simian virus 40 polyadenylation signal [poly(A)]. The positions of the RNA startsite (arrow) and of the primers used for RT-PCR (primers 1 and 2) are indicated. (b) Genomic structure of the RXRa wt allele, the RXRaΔAF2(LNL) target allele, and the deleted RXRaΔAF2(L) allele, and PCR strategy (primers 3 and 4) to analyze Cre-ERT-mediated excision of the floxed tkneo marker. Restriction sites are indicated.

blot analysis (data not shown). These results indicate that Cre-ERT is a tightly regulated recombinase that displays undetectable activity in the absence of its cognate ligand and can be activated in mice by OHT treatment. We did not observe any deleterious effects of OHT treatment during this study, in agreement with reports indicating that tamoxifen has a very low acute toxicity and causes no severe anomalies in mice (16).

Cre-ERT mRNA was detected in all organs analyzed except in the thymus (Figs. 3b and 4), suggesting that the protein is expressed in most tissues. Interestingly, the relative level of Cre-ERT mRNA correlated well with the level of DNA excision in the various organs examined (Figs. 3 and 4). Excision was most efficient in tail, skin, kidney, and spleen where it occurred for 40–50% of the reporter allele. The level of excision was approximately 30% in the liver and stomach and less than 15% in other organs. In the tail, the excision remained at the same level (~50%) after three or five injections of OHT (Fig. 4). Assuming that in the tail and possibly also in other tissues Cre-ERT expression might be restricted to a subset of specific cells, the actual level of excision in these cells could in fact be higher. Note in this respect that CMV-driven transgene expression is known to vary considerably between different cell types in a given organ (17).

Cre-ERT-mediated gene excision using the present Cre-ERT expressing transgenic mouse line generates mosaic animals in which cells containing excised and nonexcised target DNA are mixed. Similar mosaics generated with mice homozygous for a targeted gene will allow the analysis of the function of this gene, provided its mutation results in a phenotype that does not depend on the inactivation of the gene in all cells of a given tissue. Furthermore, genetic mosaics can reveal several aspects of the mutant phenotype not necessarily apparent in knockout mutants (18), and also allow the analysis of mutations that result in lethality during embryogenesis or early postnatal development (19). Note that creating Cre-ERT mouse lines expressing the conditional recombinase under cell/tissue-specific promoters will allow the selective mutation of a given gene at a given time in a given tissue. Note also that using an efficient inducible promoter system (e.g., one based on tetracycline inducibility (20)) that expresses the Cre-ERT recombinase may be required to ensure that the recombinase levels are high enough to result in 100% excision of the targeted DNA.

Kuenz et al. (21) recently reported a method for conditional gene targeting in mice based on regulating the expression of a constitutively active Cre recombinase using an interferon-responsive promoter. The interferon-inducible system resulted in some tissues in higher rates of activated excision than those observed with the present Cre-ERT mice, but was not as tightly controlled since background recombination was observed in interferon-un-treated animals. We believe that further improvement of ligand-dependent Cre recombinase systems sim-

FIG. 3. (a) PCR detection of Cre-ERT-mediated DNA excision in mice. Deletion of the floxed tkneo gene in various organs of untreated mice (Lower) and OHT-treated mice (Lower) was analyzed. PCR was also performed with tail DNA isolated before OHT administration (tail a). Four-week-old Cre-ERT/RXRaΔAF2(LNL) positive littermates were injected i.p. once per day with vehicle (oil) or with 1 mg OHT for 5 consecutive days. One day before the first injection DNA was prepared from tail biopsies of the animals. Two days after the last injection mice were killed and genomic DNA was isolated from various organs. The positions of the PCR products amplified from the RXRa allele and from the deleted RXRaΔAF2(L) allele are indicated. (b) RT-PCR analysis of Cre-ERT mRNA expression. RNA was isolated from the same organs of an untreated littermate transgenic for Cre-ERT, sacrificed on the same day (similar results were obtained when RNA was isolated from OHT-treated animals). PCR products corresponding to the Cre-ERT mRNA and to the HPRT mRNA used as an internal control are indicated. Reactions run without DNA template are also shown. RT-PCR assays performed without RT did not result in any of the products (data not shown).

FIG. 4. Pattern of Cre-ERT-mediated DNA excision and Cre-ERT mRNA expression in various organs. The level of DNA excision after five i.p. injections of OHT in the indicated organs (shaded bars), the level of DNA excision in the tail 1 day after the third injection (hatched bar), and the corresponding levels of Cre-ERT mRNA (open bars) are shown. Mice were treated as described in the legend to Fig. 3. No excision could be detected in untreated animals.
iliar to the present one will allow the manipulation of mouse genes to create somatic mutations in a spatio-temporally controlled manner.

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