Generation of inducible Cre systems for conditional gene inactivation in mice

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Frank Thomas Wunderlich

aus

Freiburg i. Br.

Berichterstatter: Prof. Dr. Klaus Rajewsky

Prof. Dr. Jens Brüning

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ABBREVIATIONS

aa	amino acid
AP	alkaline phosphatase
BAC	bacterial artifical chromosome
β-Gal	β-galactosidase
β-Geo	fusion protein between β-Gal and neo
bm	bone marrow cells
bp	base pair
bPA	bovine polyadenylation signal
BSA	bovine serum albumin
CAGGS	chicken β-actin promoter coupled to the CMV enhancer
CAT	chloramphenicol acetyltransferase gene
CCP	CAGGS Cre*PR
CMV	cytomegalovirus
cps	counts per second
Cre	causes recombination, recombinase from phage PI
CreLBD	fusion between Cre and the LBD of steroid hormone receptors
DMEM	Dulbecco's modified Eagle medium
dox	doxycycline
DTA	diphtheria toxin A
EF	mouse embryonic fibroblasts
EF1α	promoter of the elongation factor 1α gene
eGFP	enhanced green fluorescent protein
ER	estrogen receptor
ER-LBD	LBD of the ER
ERT1	LBD of the ER containing mutations G400V, L539A and L540A
ERT2	LBD of the ER containing mutations G400V, M543A and L544A
ES cells	embryonic stem cells
E_2	17-β-estradiol
FACS	fluorescence assisted cell sorting
FCS	fetal calf serum
floxed	loxP sites flanked
Flp	Flp recombinase from yeast
FRT	Flp recognition target site
GANC	gancyclovir
GLVP	Gal4 LBD VP16
HAT	hypoxanthine, aminopterine, thymidine
hCre	humanized Cre gene
hER	human ER
hPR	human PR

HPRT	hypoxanthine-phospho-ribosyl-transferase
HR	homologous recombination
HRP	horse-radish peroxidase
hsp	heat shock protein
HTNC	His-TAT-NLS-Cre
kb	kilo base pairs
kDa	kilo Dalton
lacZ	lacZ gene from *E. coli* encoding for β-Gal
LAH	long arm of homology
LBD	ligand binding domain
loxP	locus of X over in PI, recognition sequence of Cre
MCS	multiple cloning site
mER	murine estrogen receptor
neo	neomycin resistance gene
NLS	nuclear localisation signal
ORF	open reading frame
pA	polyadenylation signal
PBS	phosphate buffered saline
PR	progesterone receptor
PR891	LBD of the progesterone receptor ending with aa 891
PR914	LBD of the progesterone receptor ending with aa 914
PR-LBD	LBD of the PR
RRDR	ROSA26 double reporter
RRDR-DsRed	RRDR containing the DsRed-stop cassette
RRDR-eGFP	RRDR with excised DsRed-stop cassette
rtTA	reverse tet-transactivator
RU486	mifepristone
SA	splice acceptor
SAH	short arm of homology
SD	splice donor
SV40 pA	pA of simian virus 40
tetO	tet-operator sequences
tet-off	tet-controllable system using the tTA
tet-on	tet-controllable system using the rtTA
TK	thymidine kinase of HSV
tTA	tet-transactivator
V336A	mutation V336A in the hCre gene
4-OHT	4-hydroxy-tamoxifen
VP16	transactivation domain of VP16
WSS	Westphal stop sequence
wt	wild type

1. INTRODUCTION

The genomes of man (Lander *et al*., 2001), mouse (Waterston *et al*., 2002), *Drosophila* (Adams *et al*., 2000) and several other organisms have been completely sequenced in recent years. This progress tremendously facilitated the analysis of gene functions *in vivo*. Gene targeting by homologous recombination is a powerful technique to explore the function of genes in mice (for review, see e. g. Capecchi, 1989). First described in 1987 by Thomas and Capecchi, it became a standard technique during the last years. The gene of interest is modified in embryonic stem cells (ES cells) by inserting a selection marker into the open reading frame (ORF) of the gene via homologous recombination (Thomas & Capecchi, 1987). The targeted pluripotent ES cells are kept in culture under non-differentiating conditions and are subsequently injected into donor blastocysts, which then become implanted into pseudo-pregnant foster mice. The resulting chimeric animal, consisting of wild type and gene targeted cells, is then backcrossed to wild type mice. If the chimeras contain germ cells originating from the gene targeted cells the genetic alteration can be transmitted through the germ line. This method allows the production of animals which carry the mutation of the gene in all cells of the body (for review, see e. g. Müller, 1999; Cohen-Tannoudji & Babinet, 1998). However, this technique has several inherent limitations such as embryonic lethality, compensatory effects by redundant genes, and side effects due to the leftover of the selection marker in the genome. These problems can be circumvented by more sophisticated methods of gene manipulation. The technology of conditional gene targeting allows for modification of genes in defined cell types, in spatially and temporally controlled manners (for review, see e. g. Rajewsky *et al.,* 1996; Kühn & Schwenk, 1997). Thus, the function of a given target gene can be studied in individual cell types at different developmental stages or after administration of specific inducers.

1.1 Genetic engineering by sequence specific recombinases

The field of gene targeting was revolutionized through the application of sequence specific recombinases as tools for genomic engineering. A number of bacterial and yeast recombinase enzymes are able to rearrange DNA at specific target sequences. This simple, but elegant reaction results in a precisely defined recombination event between two appropriate DNA target sequences. The hitherto available site specific

recombinase systems belong to two major groups: The resolvase/invertase family and the λ-integrase family. Members of the resolvase/invertase family comprise phiC31 integrase, β-recombinase, HK022 integrase or γδ-resolvase, which catalyse site specific DNA rearrangements via a conserved serine residue (for review, see e. g. Smith & Thorpe, 2002). The two most famous λ-integrase members are the Flp and Cre recombinases which use a tyrosine located in the C-terminal part of the respective enzyme as catalytic residue. Some of these recombinase systems have been found to be suited for genetic engineering in eukaryotic cells (for review, see e. g. Kilby *et al.,* 1993).

The complexity of these recombination systems considerably varies both in their requirement for cofactors or accessory molecules and in the size of the DNA target sites involved. However, λ-integrases such as Cre from bacteriophage PI and Flp from yeast catalyse recombinations between specific target sites of 34 bp without further need for cofactors. Since the only other requirement for the reaction is the expression of the appropriate recombinase enzyme, the concomitant presence of target sites and appropriate recombinase is sufficient for specific gene modification in many different species.

The Cre/loxP system

Gene modification employing site specific recombinases was pioneered using the Cre/loxP recombination technology (for review, see e. g. Rajewsky *et al*., 1996). The site specific DNA recombinase Cre (**c**auses **re**combination) of the bacteriophage PI recognizes specifically 34 bp long sequences, called loxP (**l**ocus **o**f **X** over in **P**I) (Sternberg & Hamilton, 1981; Sternberg *et al*., 1981). Cre excises DNA segments flanked by directly repeated loxP sites as a circular molecule, leaving a single loxP site in the genome (Sauer *et al*., 1988; Gu *et al*., 1993). When the loxP sites are arranged in opposite directions, Cre catalyses inversion of the intervening DNA (Kano *et al*., 1998; Lam & Rajewsky, 1998). Also, intramolecular recombinations can be performed, when the loxP sites are on different strands of DNA (van Deursen *et al*., 1995). Insertion of a DNA segment into a loxP site is also possible, though the excision reaction is favoured over the integration event (Fukushige & Sauer 1992). The usage of mutant loxP sites was shown to be useful in insertion strategies (Kolb, 2001). Since Cre is one of the few recombinases which does not require cofactors or

accessory molecules in eukaryotic cells (Abremski & Hoess, 1984; Hoess *et al*., 1990), Cre is the first choice for introducing gene modifications into the mouse genome. Modification of the ES cell genome by the Cre/loxP system is a two step process. The desired genetic modification including the loxP sites is introduced into the genome of ES cells via homologous recombination. Subsequently, the targeted allele is generated by site specific recombination of the loxP sites through expression of Cre by transient transfections of Cre-containing plasmids or other means. The removal of positive selection markers is one important application of Cre-mediated recombination in ES cells, since the presence of the selection marker gene can interfere with expression of the targeted gene or adjacent genes (Pham *et al*., 1996). Therefore, the selection marker is flanked by two loxP sites in the same orientation, which allows its deletion by Cre-mediated recombination leaving a single loxP site in the genome. Gene targeting provides the possibility to introduce deletions, point mutations, and insertions in ES cells. For instance, it has been used to delete a small region of the coding region of the Igα gene (Torres *et al*., 1996), to introduce specific point mutations into the cytoplasmic tail of the Igα gene (Kraus *et al.,* 2001), and to insert rearranged variable regions into the Ig κ locus (Pelanda *et al*., 1996) and Ig λ locus (Oberdoerffer *et al*., 2003). Large genomic deletions or inversions can be introduced into ES cells using the Cre/loxP technology. This requires two independent successive gene targetings, but due to the lower frequency of Cre-mediated recombination at large distances between loxP sites, a positive selection strategy is recommended (Milstone *et al.,* 1999). This approach has been used to generate deletions of several centimorgans (Ramirez-Solis *et al.*, 1995).

The Flp/FRT system

Another system for the modification of genes was developed using the *Saccharomyces cerevisiae* derived 43 kDa Flp recombinase (for review, see e. g. Chen & Rice, 2003). The Flp recombinase is encoded by the 2µ circular plasmid of the budding yeast and mediates inversion between two 34 bp **F**lp **r**ecognition **t**arget (FRT) sites arranged in opposite direction, which are located on the same plasmid. Recombination between these FRT sites results in inversion of half of the 2µ plasmid with respect to the other and provides a mechanism for producing multiple plasmid copies from a single replication initiation by flipping the direction of the replication fork. Initial applications of the Flp/FRT recombination system at stably integrated chromosomal targets of eukaryotic cells displayed only low recombination

frequencies. Recently, the efficiency of this system has been considerably enhanced by specific modifications. The first improvement concerned the recognition sequence of Flp. The minimal 34 bp FRT site consists of two 13 bp inverted repeated binding sequences for Flp flanking the 8 bp asymmetric core region, which defines the directionality of the target site and which is the site of crossing over in the recombination event. In yeast, the 34 bp FRT recognition sequence is flanked by a third binding site for Flp in which a single nucleotide is exchanged resulting in a 48 bp wild type FRT site. Two 48 bp FRT sites can be much more efficiently recombined than two minimal 34 bp recognition sequences. The second improvement was achieved through the generation of novel Flp recombinase variants with enhanced thermostability isolated by protein evolution. Three versions of Flp recombinase are available: Flp wild type (wt), Flp-L and Flpe. In Flp-L, a point mutation changes the encoded amino acid 70 from phenylalanine to leucine, which causes an enhanced thermostability of Flp-L at 37°C, but, coincidently, there is a loss of approximately 80% activity in comparison to Flp-wt *in vitro* (Buchholz *et al*., 1996). Flpe harbours four amino acid exchanges (P2S, L33S, Y108N and S294P), which collectively improve the *in vitro* recombination activity 4-fold at 37°C and 10-fold at 40°C compared to Flp-wt (Buchholz *et al*., 1998). Mice expressing Flpe either in the germ line (Flp deleter) (Rodriguez *et al*., 2000) or constitutively in every organ (Flipper) (Farley *et al.,* 2000) were shown to efficiently excise DNA segments between two head to tail arranged FRT sites. These mouse strains can be used to delete FRT site flanked positive selection marker required for gene targeting *in vivo*.

The phiC31/att system

At present, the Cre/loxP and the Flp/FRT recombination systems are most commonly used to manipulate the genomes of eukaryotes in heterologous environments in a site specific manner. However, additional recombinase systems have to be established in order to improve the technique of gene targeting for more sophisticated strategies. Recently, the *Streptomyces* phage derived phiC31 integrase has been described to mediate recombination efficiently in eukaryotic cells when a nuclear import signal (NLS) was located at the C-terminus of the enzyme (Andreas *et al.*, 2002). The phiC31 system displayed deletion efficiencies of up to 50% at stably integrated chromosomal targets in a side by side comparison to the well defined Cre/loxP system. In contrast to the Cre and Flp systems, however, the phiC31 integrase catalyses both integration and excision events irreversibly, since the sites generated by the recombination cannot serve as substrates for the enzyme (Belteki *et al.*, 2003).

1.2 Conditional gene targeting

Conditional gene targeting is defined as a gene modification, which is restricted to specific cell types and/or developmental stages of the mouse. The usage of the Cre/loxP system in conditional gene targeting requires both a mouse strain containing a loxP-flanked modification of the target gene and an additional mouse strain, which expresses the Cre recombinase in specific cell types. Crossing these two strains results in the generation of a conditional mouse mutant, which carries the mutation restricted to those cell types, in which the Cre recombinase is expressed. Such a conditional mouse mutant can be used to study the function of ubiquitously expressed genes in individual defined cell types and to circumvent lethality at early embryonic stages due to inactivation of a putative essential gene.

Generation of loxP-flanked alleles

For the removal of a gene segment by conditional gene targeting, the region of interest has to be flanked by loxP sites of the same orientation. This can be achieved by the 'flox and delete' approach, which requires construction of a gene targeting vector with three loxP sites pointing in the same orientation, two of which flank the positive selection marker and the third loxP site is placed within a homology arm (Fig.1). Transient expression of Cre in targeted ES cell clones results in two types of clones which have lost the selection marker. Recombination between the two external loxP sites leads to deletion of the selection marker and the gene segment whereas recombination between the loxP sites flanking the selection marker generates the loxP-flanked version of the gene (Fig.1). ES cell clones harbouring the loxP-flanked, or conditional, allele can be used to generate mice used in conditional gene targeting experiments. The positioning of the two remaining loxP sites within the locus of the target gene is critical for conditional gene targeting. They have to be placed in non-coding sequences around critical sequences such as exons so that gene expression is not disturbed by their presence. The desired deletion of the gene segment is achieved upon Cre-mediated recombination of the loxP-flanked region. This 'flox and delete' strategy was first demonstrated to function in an approach to conditionally inactivate the DNA polymerase β (Gu *et al*. 1994). The promoter and the first exon of the DNA polymerase β gene were flanked by loxP sites and the generated pol-β flox mouse strain was crossed with the lck-cre mouse strain (Orban *et al*., 1992), in which Cre is expressed only in thymocytes. The DNA polymerase was thus inactivated exclusively in T lineage cells circumventing the embryonic lethality caused by ablation of pol-β in the entire mouse by conventional knock-out.

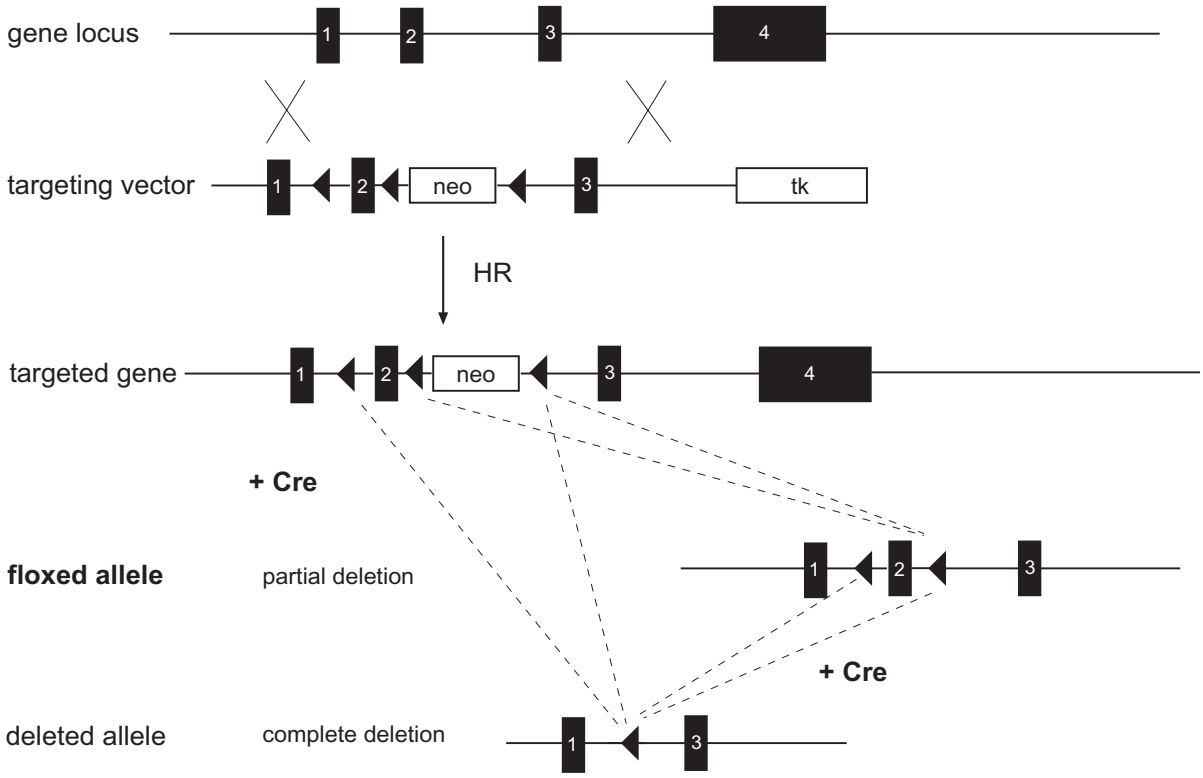

Fig. 1 Generation of a loxP-flanked allele.

The conditional targeting vector harbours a loxP (filled triangles) flanked neo cassette (neo) and a third loxP site which is inserted into intron 1. Homologous recombination (HR) in ES cells generates targeted clones which become subjected to transient Cre expression in a second transfection round. Two types of clones have lost the positive selection marker (neo) by this strategy. In one, recombination between the two external loxP sites leads to complete deletion of the selection marker and the gene segment, whereas the other recombination between the loxP sites flanking the selection marker generates the loxP-flanked allele (floxed allele).

Cre transgenic mice

At present, a large number of Cre transgenic mouse strains are available, enabling researchers to restrict conditional genetic modifications to B cells (Rickert *et al*., 1997), to T cells (Lee *et al*., 2001), to different areas of the brain (Tsien *et al*., 1996; Casanova *et al*., 2001) and to various other tissues of the mouse (Tonks *et al*., 2003; Wen *et al.*, 2003; Barlow *et al.*, 1997; more Cre-transgenic mouse strains in *Genesis* 2000, 26, 99-166 or under http://www.mshri.on.ca/nagy/cre-pub.html). Such Cre expressing mouse strains can be generated either by using conventional random transgenesis, or by targeted insertion into a gene (knock-in), or by using a bacterial artificial chromosome (BAC) strategy. In any case, the expression pattern of the promoter used to express Cre determines onset and cell type specificity of the Cre-mediated gene modification. The technique of conventional random transgenesis has several disadvantages: the limited understanding of promoter regions used to

express the transgene, the potential unpredictable effects of enhancer/silencer elements present at the integration site on transgene expression and the necessity to analyze many different founder lines which may contain multiple copies, tandem repeats, broken and differently integrated transgenic constructs. The method of targeted insertion of Cre into a gene can be applied, when the inactivation of one allele of the endogenous gene can be tolerated. This has been shown to work by Rickert and coworkers, who generated a knock-in mouse strain by targeted insertion of the Cre gene into the second exon of the CD19 locus (Rickert *et al*., 1995). In this CD19-cre mouse strain, the Cre recombinase is exclusively expressed in B lymphocytes and mediates 75-80% excision of loxP-flanked targets in B cells in the bone marrow, whereas 90-95% deletion is observed in splenic B cells due to continuous expression of Cre (Rickert *et al.*, 1997). Another technique, which is currently used to generate Cre expressing mouse strains, is the insertion of the Cre gene into bacterial artifical chromosomes (BAC) and the subsequent generation of BAC transgenic mice. BACs carry large genomic fragments from mouse or human of up to 400 kb and can be obtained commercially. The Cre gene can be introduced into defined genes encoded by the BAC via homologous recombination in bacteria (Testa *et al.*, 2003). The large size of the BAC renders it likely that most if not all of the transcriptional control elements of the gene, into which Cre has been introduced, are present. This should result in a Cre expression pattern closely related to the expression of the endogenous gene. This strategy was successfully used by M. Alimzhanov (unpublished data) to express Cre solely in mature B cells by inserting the Cre gene in the CD21 gene of a 250 kb BAC.

Indicators for Cre activity

The success of Cre-mediated conditional gene targeting critically depends on stringent regulation of Cre expressing mouse strains, which have to be intercrossed with mice containing loxP-flanked target genes. If expressed from a transgenic construct, Cre-mediated deletion may occur in a mosaic or leaky fashion rendering excision of the loxP-flanked target alleles incomplete or unspecific, respectively. A general complication of Cre transgenes is due to the irreversibility of Cre-mediated deletion. Any transient expression of Cre during mouse development caused by the control elements of the gene into which Cre has been inserted, or alternatively caused by artefacts of transgenesis, will lead to deletion of loxP-flanked targets in the cells expressing Cre. These cells and all cells arising from them through division will

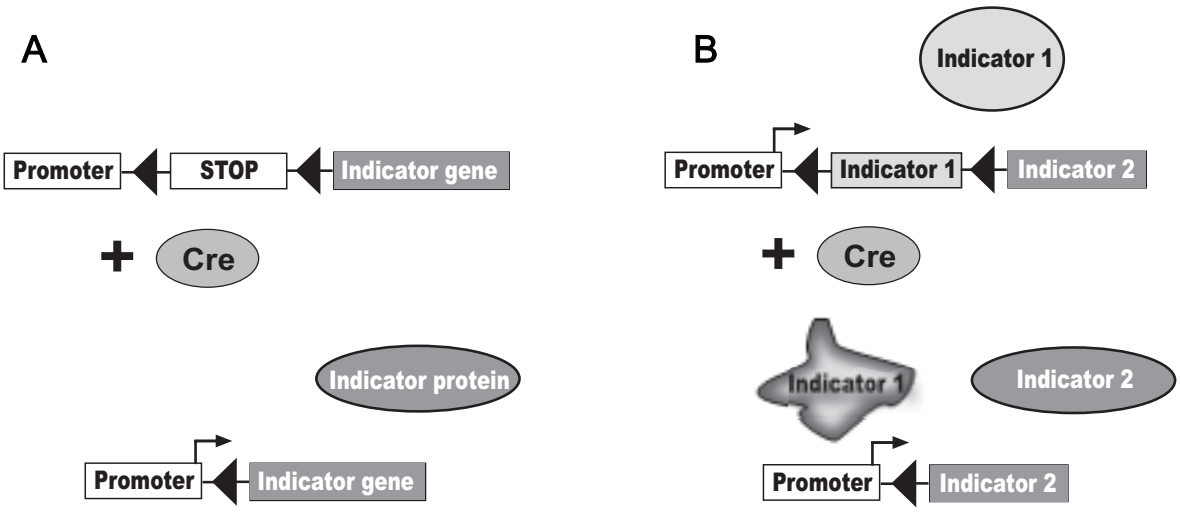

Fig. 2 Indicator constructs for Cre activity.

(A) Expression of the indicator gene is prevented by a transcriptional stop sequence (STOP) which is flanked by loxP sites (closed triangles). Cre-mediated excision of the STOP results in expression of the indicator gene which can be monitored, for example, by enzymatic reactions.

(B) Shut down/activation indicator construct. The promoter expresses indicator 1, whereas expression of indicator 2 is interrupted by the presence of the indicator 1 gene. Cre-mediated excision of the indicator 1 gene results in shut down of indicator 1 expression and activation of indicator 2 expression.

contain the modified alleles, regardless whether Cre expression is continued or not. Cre expressing mouse strains can be characterized with the help of reporter mice that permit *in vivo* monitoring of Cre-mediated recombination events in diverse tissues and at different developmental stages (Fig. 2A). For instance, a reporter strain carrying a loxP-flanked stop sequence preceding the β-galactosidase (β-Gal) gene driven by the chicken β-actin promoter has been used to score excision events in mice (Tsien *et al*., 1996). Cre-mediated excision of the loxP-flanked stop sequence leads to β-Gal expression, which can be monitored by enzymatic reactions for β-Gal. However, a limitation of this Cre reporter mouse strain became apparent, when characterizing the expression pattern of the β-galactosidase gene upon Cre-mediated excision of the loxP-flanked stop sequence in all tissues. The indicator gene was expressed in a mosaic fashion in some organs such as brain. This mosaic expression pattern of the β-galactosidase gene may result from artefacts of the conventional random transgenesis, which was used to generate this mouse strain. Artefacts of transgenesis can be caused by integration of multiple copies, tandem repeats, breakage of the indicator constructs, unknown multiple integration sites, inactivation of the indicator gene by methylation, influences of enhancers/silencers

present at the integration sites and insufficiently characterized promoter fragments used to express the transgene.

A more convenient strategy for the generation of a Cre reporter strain would be to target a single reporter construct into a gene which is expressed ubiquitously throughout all developmental stages of the mouse. An excellent candidate for an appropriate locus is the ROSA26 locus, which was originally identified by gene trap (Zambrowicz *et al*., 1997). In the initially reported mouse line, retroviral sequences and a β-Gal neomycin resistance fusion gene (β-Geo) were integrated into the locus ROSA26. The wild type ROSA26 locus expresses two transcripts with no significant open reading frames (ORF) and a third in antisense orientation, which putatively encodes a protein, and which is not affected by the β-Geo insertion. Expression of the β-Geo gene is initiated from exon 1 of the ROSA26 allele and is ubiquitous during embryonic development and also in adult tissues. Mao and colleagues (1999) subsequently engineered the gene trapped proviral ROSA26 locus in ES cells by introducing a three loxP sites containing cassette, in which two loxP sites flanked the selection marker and one external loxP site flanking a transcriptional stop sequence to interrupt the expression of the β-Geo gene. This mouse strain expressed β-Gal only after Cre-mediated excision of the loxP-flanked region. This was verified by breeding the ROSA26 reporter strain to a Cre transgenic mouse strain which expresses Cre at early embryonic stages (Schwenk *et al.*, 1995), i.e. Cre mediated excision was disseminated to all tissues of the adult mouse. Enzymatic reactions for β-Gal with the substrates X-Gal and fluoresceine-di-β-D-galactopyranoside (FDG) were carried out to detect expression of β-Gal at the single cell level. The expression of β-Gal upon Cre-mediated excision of the three loxP-flanked cassette in this mouse strain was shown to be ubiquitous (Wunderlich & Rajewsky, unpublished results). Later on, however, several limitations of this reporter strain became apparent. For instance, recombination between two adjacent loxP sites by partial Cre-mediated deletion results in retention of either the selection marker or the stop fragment in the locus, which therefore still lacks β-Geo gene expression despite Cre-mediated recombination. The excision event remains undetectable by X-Gal staining. In addition, loxP-flanked targets, which are integrated into the ROSA26 locus, were shown to be excised with low efficiency by inducible Cre constructs for reasons not yet understood (Voojis *et al*., 2001; Seibler *et al*., 2003). Therefore, mouse strains

carrying a reporter construct for Cre in the ROSA26 locus are not suited for the characterization of inducible Cre mouse strains. Moreover, until recently, enzymatic reactions had to be performed to detect β–galactosidase expression in Cre reporter mouse strains. Such enzymatic reactions are time consuming and difficult to perform in some organs.

A more sophisticated reporter gene, which can be detected without any enzymatic reaction just by its presence, is the eGFP gene (**e**nhanced **G**reen **F**luorescent **P**rotein). The eGFP protein, originally derived from the jellyfish *Aequorea victoria,* can be visualized by its inherent fluorescence, even in living cells (for review, see e. g. Cubitt *et al*., 1995). EGFP owes its visible absorbance and fluorescence to a p-hydroxybenzylideneimidazolinone chromophore formed by cyclisation of Thr65, Tyr66 and Gly67 and 1, 2-dehydrogenation of the tyrosine. Site directed mutagenesis of the amino acids forming the chromophore leads to versions of the fluorescent protein whose emission spectrum are shifted to yellow or blue such as e**Y**FP (**Y**ellow) and e**C**FP (**C**yan). The only limitation of using these fluorescent proteins as reporter genes is the large amount of molecules ($8x10^5$), which have to be expressed in a single cell to detect fluorescence by fluorescence microscopy (Rizzuto *et al*., 1995). Therefore, strong promoters have to be used in order to generate a Cre reporter strain which expresses eGFP after Cre-mediated recombination. Another possibility is to use a loxP-flanked indicator 1 gene, which serves in a dual function to prevent expression of the indicator 2 gene before Cre-mediated deletion and, in addition, to control for expression from the reporter construct. In this case, Cre-mediated recombination leads to inactivation of indicator 1 gene and to expression of the indicator 2 gene. (Fig. 2B). This shut down/activation theme has been employed in the generation of the Z/EG and Z/AP Cre reporter strains, which switch their indicator genes either from lacZ to eGFP (Novak *et al.*, 2000) or from lacZ to alkaline phosphatase (Lobe *et al*., 1999) upon Cre recombinase activity.

1.3 Inducible gene targeting

One step forward in techniques of conditional gene targeting is the temporally controlled expression of Cre, by which the onset of the conditional genetic alteration can be chosen at specific developmental stages (for review, see e. g. Lewandowski, 2001). A pioneering breakthrough in this field was the generation of the Mx-Cre mouse strain (Kühn *et al.*, 1995). Here, the Cre gene was placed under the control of

the interferon α/β inducible promotor of the Mx-1 gene. Application of interferon α/β leads to Cre expression and, thus, to highly efficient deletion of the loxP-flanked target genes such as the DNA pol-β gene segment (Gu *et al*., 1994) in a wide variety of tissues in mice. Deletion of loxP-flanked sequences using the Mx-Cre system is not restricted to lymphocytes only, since many cell types in the mouse are responsive to interferon. At present, Cre activity is tried to be more tightly regulated at both the transcriptional and the posttranslational level by inducible systems.

1.3.1 Transcriptional control of Cre activity

Cell type specific transcriptional control of Cre can be achieved by systems, in which the activity of almost silent minimal promoters can be enhanced manifold through binding of certain inducer proteins. These inducer proteins are in turn regulated by small molecules such as tetracycline (Gossen & Bujard, 1992), RU486 (Wang *et al*., 1997a) or ecdysone (No *et al*., 1996). By placing the Cre expression under the control of a minimal promoter and directing the expression of the inducer protein (transactivator) to certain cell types, transcription of Cre in mice can be activated by administration of the above mentioned small molecules. A main disadvantage of these regulatory systems is the separate insertion of the gene encoding the transactivator and that of the controllable Cre into the genome leading to large numbers of transgenic mice, which have to be intercrossed and tested for the desired combination of the transgenes.

The GLVP system

In the GLVP system, the inducer protein consists of the **G**al4 DNA binding motif, the **L**igand binding domain (LBD) of the progesterone receptor and the **VP**16 transactivation domain. This chimeric transactivating unit specifically binds to the 17-mer Gal4 target sequence fused to a minimal promoter upon administration of RU486 and activates expression of genes of interest placed under control of the 17-mer Gal4 target sequence/minimal promoter hybrid (Wang *et al.*, 1997a). Thereby, DNA specific binding to the 17-mer sequences is mediated by the GAL4 DNA binding motif, which in turn enables the transactivation domain of VP16 (Triezenberg *et al*., 1988) to activate transcription from the minimal promoter only in the presence of RU486, since the LBD of the progesterone receptor regulates the temporal inducer dependent activity of the synthetic molecule. Tight regulation of gene expression by this system has been reported in tissue cultures (Burcin *et al*., 1998), but rare and

ineffective *in vivo* applications minimize the value for the temporal control of the Cre recombinase (Pierson *et al*., 2000; Chaisson *et al*., 2002). The main obstacle of this system seems to be the inefficient activation of the transactivator molecule by the steroid compound, since stably expressing cell types in mice mediate only a low extent of transcription upon induction from the minimal promoter.

The ecdysone inducible system

In an alternative approach for the inducible regulation of Cre, Cre could be placed under control of a system involving the insect molting hormone ecdysone as an inducer. The effect of ecdysone is mediated through the functional ecdysone receptor, which is a heterodimer of the ecdysone receptor and the gene product of the ultraspiracle gene. The functional ecdysone receptor binds to specific promoter elements of the target gene in order to activate transcription. Mutagenesis of the transactivation domain and the DNA binding domain of the natural occurring receptors resulted in a tightly controllable system in mice (No *et al.*, 1996). However, the binary character of the heterodimeric receptor complicates the usage *in vivo*, since both partners must be present at equal amounts. Otherwise, homodimeric complexes are formed, which cannot activate the promoter and may function in a competitive way. Furthermore, the inducer ecdysone or its synthetic counterpart muristerone have to be available at non-physiological micromolar concentrations to sufficiently activate the ecdysone heterodimeric receptor complex (Saez *et al*., 2000).

The tet-system

Tetracycline controlled expression systems are widely used to regulate genes in cell lines (Resnitzky *et al*., 1994), plants (Weinmann *et al*., 1994), yeast (Nagahashi *et al*., 1997), *Drosophila* (Bello *et al*., 1998), and mice (Hasan *et al*., 2001; Schönig *et al*., 2003). Fig. 3 schematically outlines the function of the original tet-off system and the modified tet-on system. The tet-off system uses a synthetic transcription factor tTA (tetracycline controlled transcriptional activator), which is a fusion between a bacterial tet-repressor (tetR) and the transcriptional activating domain of VP16. tTA activates a minimal promoter which is fused downstream to an array of tet-operator (tetO) sequences (Gossen & Bujard, 1992), the cognate binding sites of tetR and, thus, of tTA. In the tet-off system, transcription is abrogated by administration of doxycycline, which upon binding to the tTA prevents binding of tTA to the tet-responsive promoter. Despite of its extensive use, however, the tet-off system has

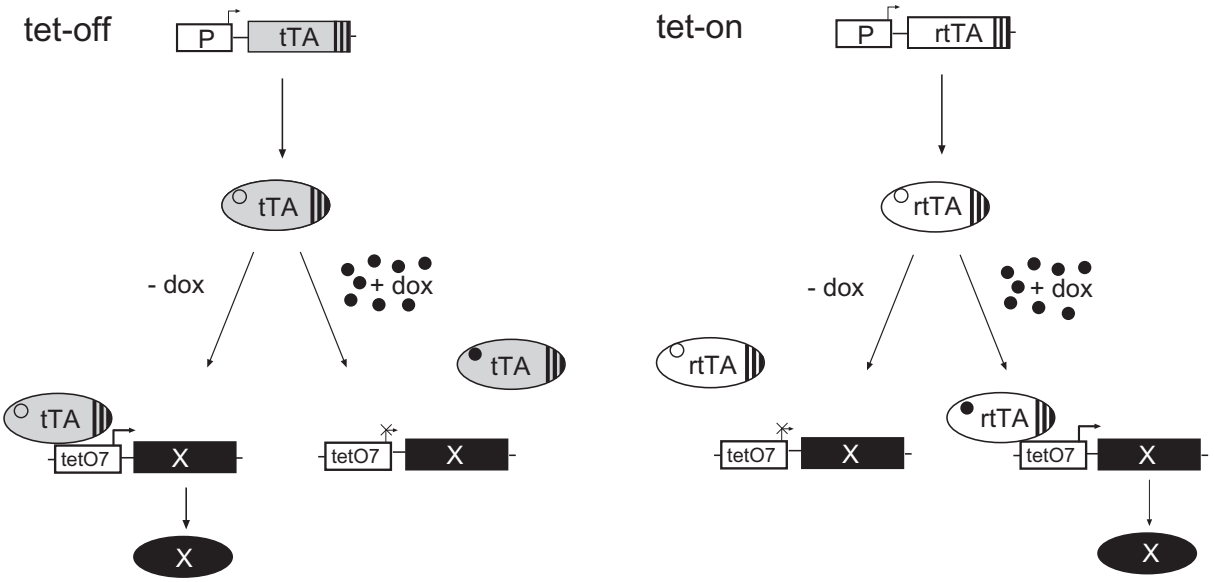

Fig. 3 Transcriptional regulation of gene expression by the tet-systems.

The tet-off system. The promoter (P) drives the expression of the tet-transactivator (tTA), which binds in the absence of doxycycline (dox) to the tet-responsive promoter (tetO7) and activates expression of the gene of interest (X). Administration of dox causes dissocation of tTA from tetO7 and transcription is terminated.

The tet-on system. Only in the presence of dox, the reverse tet-transactivator (rtTA) can bind to its promoter and activate expression of the gene of interest.

some disadvantages for applications in mice. Doxycycline has to be continuously fed to prevent transcription of the target gene, until expression of the target gene can be initiated by removal of doxycyline (Kistner *et al*., 1996). Such long periods of doxycycline application results in storage of the antibiotic into cartilage and bones of mice (Bocker *et al*., 1984; Sande & Mandell, 1990). This may result in the slow release of doxycycline from its storage places after discontinuation of doxycycline administration and therefore may interfere with activation of the target genes by tTA. In the complementary tet-on system, transcription of the target gene will only be activated in the presence of doxycycline. This system is based on rtTA (reverse tetracycline controlled activator), which contains mutations in the tetR part resulting in requirement of doxycycline binding in order to bind to tetO (Gossen *et al*., 1995). However, the affinity of rtTA to doxycycline is three magnitudes lower than that of the tTA and, therefore, rtTA requires more inducer to become activated (Baron *et al*., 1997). A recent report addressing this problem generated new rtTA mutants termed rtTA-M2 and rtTA-S2, which could already be activated by the low dose of 2 ng/ml doxycycline in tissue culture resulting in a 50-fold improved affinity of these new mutants to doxycycline (Urlinger *et al*., 2000). In both the tet-off and tet-on system, the genomic integration site of the tetO promoter driving the transgene of interest is critical. When endogenous enhancers are located in close environment to the tetO

promoter, the minimal promoter though silent in principle is activated, thus resulting in leaky expression of the transgene. A recently described integration site for the tetO promoter offers an alternative approach. A bidirectional tetO promoter (Baron *et al*., 1995) in transgenic LC-1 mice (Schönig *et al*., 2003) drives the expression of Cre and luciferase, respectively, and can be tightly regulated by transactivating units of both the tet-on and tet-off system. Hitherto, a general disadvantage of both tet-systems is their binary character with the consequence of large breeding effort to obtain the desired combination of the transgenes, which is time consuming and expensive. Recently, however, this problem was overcome by Utomo *et al*. (1999) who placed both the rtTA with its own promoter and the tetO driving expression of Cre on the same fragment of DNA.

The lac system

The lac system from *E.coli* can also be applied to regulate gene expression in an inducible manner in mice. The principle of the lac system constitutes of a tetrameric lac repressor complex, which binds to operator sequences in the lac promoter thus blocking the docking sites for the RNA polymerase in the absence of inducer. Administration of allolactose or isopropyl-beta-D-thiogalactopyranoside (IPTG) induces conformational changes of the lac repressor proteins, which subsequently dissociate from the promoter thus allowing for initiation of transcription of the desired gene. However, also the lac system has its problems. The major limitation is the positioning of the operator sequences into the eukaryotic promoter which should be regulated without disturbing its activity. Another limitation is the occurrence of CpG islands in the lac repressor gene which causes its silencing in mice. However, this limitation was recently overcome by eliminating the naturally occurring CpG islands and the mutation of cryptic splice sites in the lac repressor gene (Cronin *et al*., 2001). This modified lac gene was indeed suited for heterologous expression in mammals.

1.3.2 Posttranslational control of Cre activity

Steroid hormones such as estrogen, progesterone, androgen and glucocorticoid regulate gene expression directly through classical intracellular steroid hormone receptors and possibly indirectly through novel unconventional membrane bound receptors belonging to the G protein coupled receptor superfamily (for review, see e. g. Wehling, 1997; Picard, 1998). The intracellular steroid hormone receptors possess a well characterized ligand binding domain (LBD), which can be used for

posttranslational control of Cre activity. A fusion protein between Cre and the LBD can be expressed from any given cell type specific promoter. In the absence of steroid hormone, the LBDs are bound by heat shock proteins, which inactivate recombinase activity presumably by sterical hindrance. Only after addition of the steroid hormones as inducers, the fusion protein becomes activated and can mediate recombination of loxP sites flanked sequences in the nucleus (Fig. 4). This posttranslational inducible system was extensively used in exploring the regulation of transcription factors (Littlewood *et al*., 1995; Jackson *et al*., 1993).

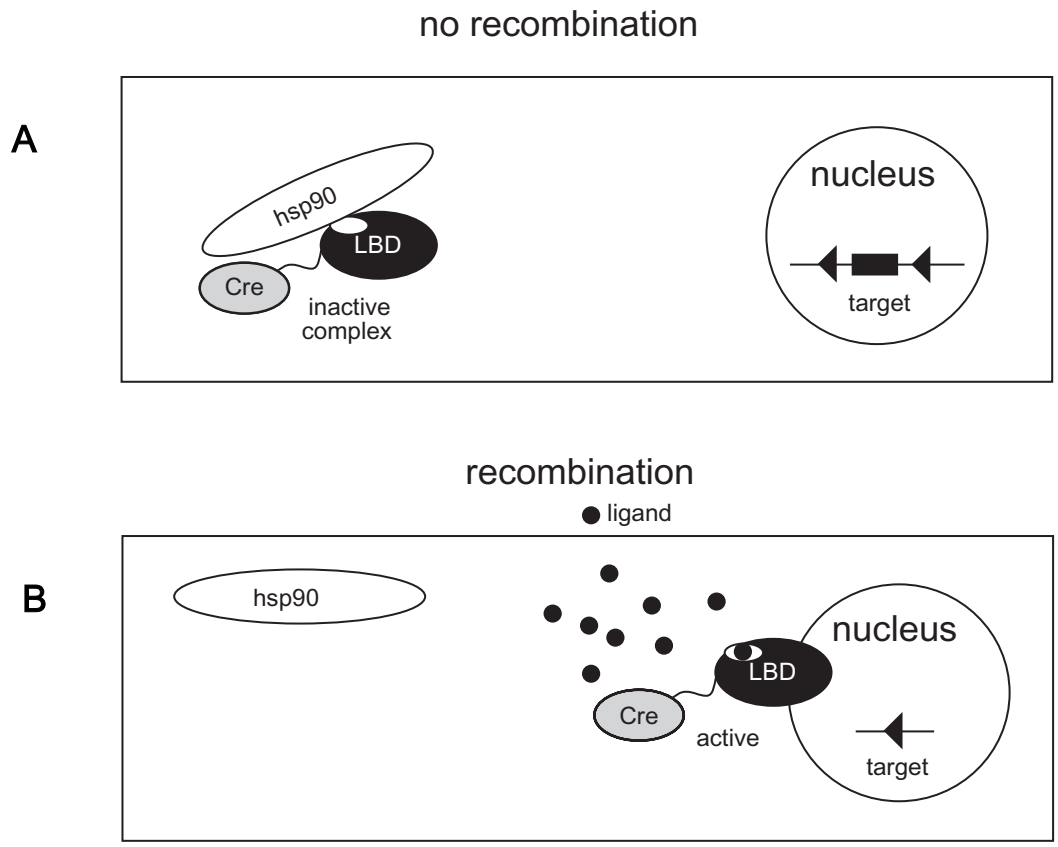

Fig. 4 Posttranslational control of Cre activity.

(A) Cre is fused to the ligand binding domain (LBD) of a steroid hormone receptor and kept as an inactive complex in the cytoplasm by bound heat shock proteins (hsp90).

(B) The complex dissociates upon ligand binding and CreLBD translocates to the nucleus and mediates recombination.

The family of intracellular steroid receptors share similarities in their structure, function and signal transduction capacity (reviewed in Beato *et al*., 1995). After binding the hormone, the receptors translocate from the cytoplasm to nucleus and activate or repress transcription of their target genes, which is mediated through binding to special palindromic HRE sequences (hormone responsive element) in the

promoter. Fig. 5 shows the functional domains of steroid hormone receptors: the non-conserved transactivation domain (A/B), the DNA binding domain (C), the hinge domain (D) and the ligand binding domain (E/F) (reviewed in Evans, 1988).

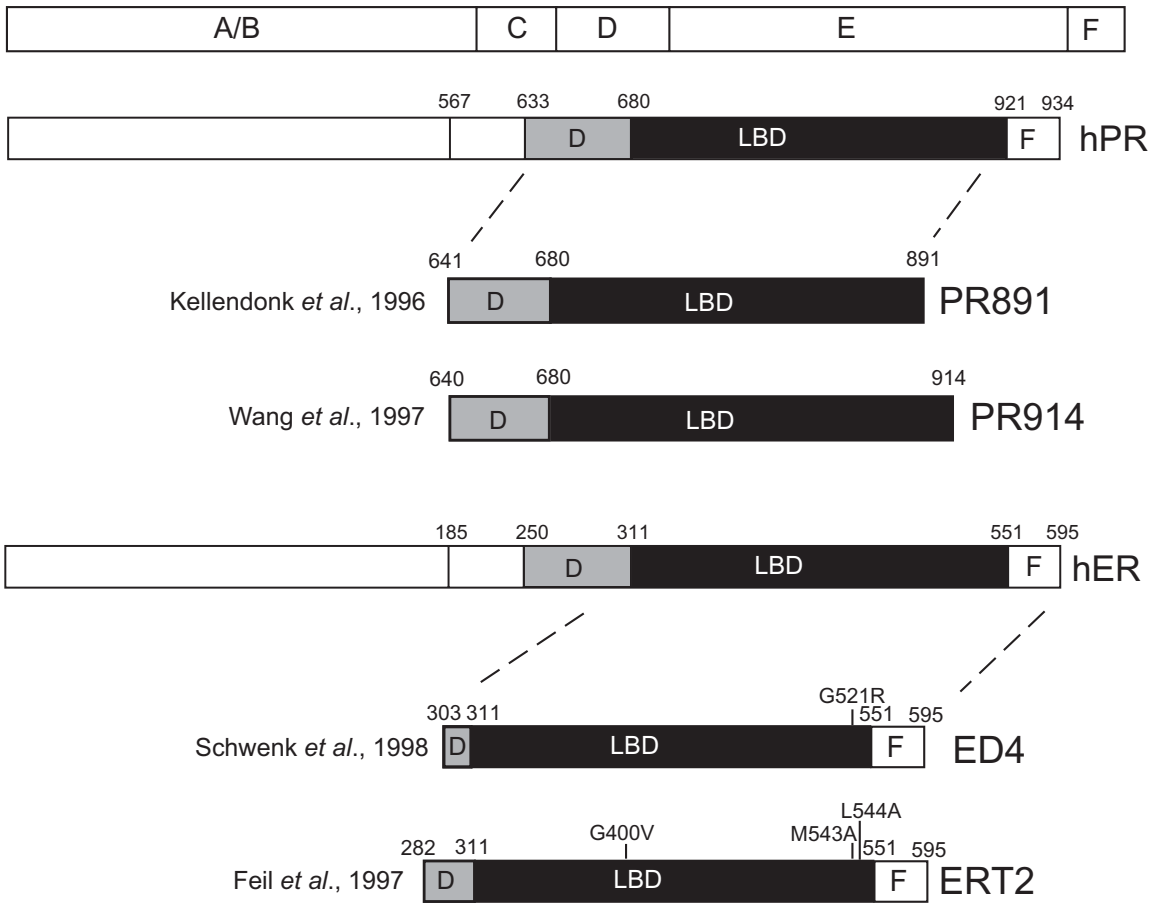

Fig. 5 Modular structure of steroid hormone receptors.

All steroid hormone receptors consist of a non-conserved transactivation domain (A/B), a DNA binding domain (C), a hinge domain (D) and a ligand binding domain (E/F). Numbers indicate the position of amino acids in the human progesterone receptor (PR) and the human estrogen receptor (ER), respectively. Ligand binding domains (LBD) which are marked were used by the cited groups to inducible regulate fusion proteins at the posttranslational level.

In the absence of hormone, complexes of heatshock proteins retain the receptors in the cytoplasm (Passinen *et al*., 1999). Binding of hsp90 and other factors like the immunophilins hsp56 and hsp70 to the LBD maintain the receptors in an inactive but ligand-friendly conformation. Hormone binding causes dissociation of this large multiprotein complex and subsequent import of the receptors into the nucleus. The nuclear import is achieved by specific amino acid sequences termed NLS (nuclear localisation signal) which are masked by the binding of the hsp chaperones in the cytoplasm (Guiochon-Mantel *et al*., 1989; Tyagi *et al*., 1998). Once in the nucleus, the steroid receptors dimerize, bind as homodimers to specific HRE in the promoter

of their target genes and change thereby expression levels of the latter by either activating or repressing transcription.

The LBDs of steroid hormone receptors contain (i) the binding pocket for the hormone, (ii) the internal NLS, (iii) the hsp90-binding sites and, (iv) the domains necessary for dimerization of the monomeric receptors (for review, see e. g. Moras & Gronemeyer, 1998). The LBDs of all steroid hormone receptors exhibit a common structural feature. They consist of 12 α-helices which form an antiparallel sandwich (Williams & Sigler, 1998; Brzozowski *et al*., 1997). Ligand binding causes sequential conformational changes of helices H11, H10, H3 and H12, whereas H12 functions as a lid of the binding core. These conformational changes lead to dissocation of hsp from the receptor and to its translocation into the nucleus (Allan *et al*., 1992a).

Until recently, posttranslationally inducible recombinase systems used wild type LBDs of steroid hormone receptors fused to Flp or Cre (Logie & Stewart, 1995; Metzger *et al.*, 1995). However, these chimeric recombinases are activated by endogenous steroids of the LBD and are therefore of limited use for applications in mice. Consequently, mutations were introduced into the LBDs of chimeric Cre recombinases, that abolish the activation by natural ligands while retaining the ability to be activated by synthetic hormone antagonists (Fig. 5). Mutated LBDs of the glucocorticoid receptor (Brocard *et al*., 1998), the androgen receptor (Kaczmarczyk & Green, 2003), the progesterone receptor (Kellendonk *et al*., 1999) and the estrogen receptor (Schwenk *et al*., 1998) have been used to regulate Cre activity at the posttranslational level. Fig. 4 shows a general scheme for the regulation of Cre through LBDs. The fusion protein between Cre and the LBD is kept as an inactive complex in the cytoplasm by the bound hsp90 proteins. Ligand binding causes dissociation of this complex and the CreLBD fusion protein enters the nucleus and mediates recombination of loxP-flanked targets (for review, see e. g. Picard, 1994). Several limitations of this system became evident in initial experiments, and the system has to be further optimized to control Cre activity. For instance, there is still significant background recombination in the absence of inducer. Several reasons may contribute to the background activity of the fusion protein. First, the addition of strong NLS domains as that from the SV40 large T antigen to the fusion protein results in a higher background activity. Second, the hinge domain between Cre and the LBD could be cleaved by intracellular proteases resulting in uncontrolled Cre

recombinase activity. Third, unexpected processing of CreLBD mRNAs, as e.g. by cryptic splicing, could also result in background activity.

An additional limitation of the system is the lower deletion efficiencies obtained with CreLBD fusion proteins in comparison to Cre (Kellendonk *et al*., 1996, Zhang *et al*., 1996). This results in only partial deletion of loxP-flanked targets after ligand administration in most of the available CreLBD transgenic mouse strains (Schwenk *et al*., 1998). The lower efficiencies can be explained by the insufficient availability of the inducer, i.e. the inducer concentration is not sufficient in certain organs to activate CreLBD. Alternatively, Cre-mediated catalysis could be impaired by sterical hindrance of the fused LBD. In addition, recombinase activity could be impeded by unfavourable chromatin configurations of the loxP-flanked DNA segment at the time period of nuclear localisation of the fusion protein.

Fusions between Cre and the LBD of the estrogen receptor

In order to generate a posttranslational system of Cre fused to the LBD of the estrogen receptor (ER), single amino acid substitutions have to be introduced in the ER-LBD to prevent its activation by the endogenous hormone 17 β–estradiol (E_2) but, coincidently, to maintain its binding capacities for synthetic steroids. In 1989, a natural ER mutant was isolated from chicken, which exhibited diminished affinity for E_2 due to an amino acid exchange from glycine to valine at position 400 (G400V) (Tora *et al*., 1989). Another ER mutant was described for the murine ER (mER) with a glycine for arginine substitution at position 525 (G525R), which displayed only 1/1000 of the affinity for E_2 of the WT-ER (Danielian *et al.,* 1993). However, this G525R mutant could be still activated by the antiestrogen 4-hydroxy-tamoxifen (4-OHT) at the physiological concentration of 200 nM as well as by the ER-antagonists raloxifene and ICI 182,780 (Van Den Bemd *et al*., 1999). These ER-antagonists are known to bind at the same binding pocket of the ER as E_2, but they induce different conformational changes in the LBD (Brzozowski *et al.*, 1997). However, these conformational changes still induce translocation of the ER to the nucleus, but once inside the nucleus, the antagonist-ER complex induces a completely different activation and suppression pattern of genes in comparison to the E_2-ER complex.

Besides the LBD of the mutant mER, LBDs of the mutant human ER (hER) have also been used in posttranslational inducible systems. In particular, the mutant human ER (G521R) (Feil *et al*., 1996), harbouring a homologous mutation to the mER (G525R),

was fused to Cre and expressed under a ubiquitously active promoter in transgenic mice. This mouse strain was shown to regulate Cre activity in every organ, except the thymus, in a tamoxifen dependent manner (Brocard *et al*., 1997; Metzger & Chambon, 2001). Tamoxifen injections led to heterogenous excision efficiencies of loxP-flanked target genes with highest efficiencies of approximately 40-50% in tail, skin, kidney and spleen. Schwenk *et al*. (1998) have described a similar CreER construct expressed exclusively in B cells since the transgene was controlled by the heavy chain enhancer combined with the SV40 minimal promoter. *Intra-peritoneal* injections of 8 mg 4-OHT in mice carrying both the B lymphocyte restricted CreER allele and the loxP-flanked region of the DNA pol-β gene caused up to 65% excision of the loxP-flanked gene segment solely in B cells.

The homologous mutations G521R (hER) and G525R (mER) possess not only reduced affinity to E_2, but also to 4-OHT. In consequence, nearly lethal doses of 4-OHT have to be injected to achieve activation of the CreER fusion protein (Danielian *et al*., 1998).

Feil *et al*. (1997) significantly improved the affinity of the hER LBD to synthetic ligands. They developed two different mutant hER LBDs, termed ERT1 and ERT2, each containing three different amino acid substitutions, which result in unresponsiveness to E_2 but in a 10-fold higher affinity to synthetic ligands as compared to the former single mutants of the ER-LBD (mERG525R and hERG521R, respectively). A fusion protein between Cre and the triple mutant ERT1 with the mutations G400V, L539A and L540A is activated at nanomolar concentrations of ICI 182,780 in tissue culture. Also, a chimeric molecule between Cre and the triple mutant ERT2 (G400V, M543A, L544A) can be activated by 10 nM 4-OHT. The activation characteristics of the CreERT2 *in vivo* were confirmed by recent publications. A transgenic mouse strain with CreERT2 expression restricted to keratinocytes (K14 CreERT2) requires 10-times lower doses of tamoxifen in order to activate Cre activity in comparison with a mouse strain, which expresses the CreERT (G525R) single mutant controlled by the same promoter (Indra *et al*., 1999; Vasioukhin *et al*., 1999). During the last years, numerous CreERT2 transgenic mouse strains were generated, which express the fusion protein ubiquitously (Seibler *et al*., 2003), or restricted to muscle (Kühbandner *et al*., 2002), to brain (Casanova *et al*., 2002), and to several other tissues (Forde *et al.*, 2002; Leone *et al*., 2003). In most of these strains, highly efficient deletion of loxP-flanked targets can be induced by oral application or *i. p.* injection of tamoxifen in the range of 2-5 mg daily for 5

days. The reduced doses of tamoxifen, which have to be injected and subsequently block endogenous estrogen receptor mediated signalling, do not interfere with most experimental applications. Coincidently, the background activity of the CreERT2 fusion protein in the absence of inducer is ranging at tolerable levels between 3-10% in mice. A disadvantage of tamoxifen inducible systems is that they cannot be applied in the brain, since the hormone does not pass the blood brain barrier at sufficient concentrations to activate CreERT2 fusion proteins. Seibler *et al.* (2003) generated a ubiquitously expressed CreERT2 mouse strain by knock-in into the ROSA26 locus, which yielded almost complete deletion of a loxP-flanked gene segment of the ect2 gene in all tissues upon tamoxifen injection, except in the brain, though the CreERT2 protein was expressed in the brain as revealed by Western blotting.

Fusions between Cre and the LBD of the progesterone receptor

Posttranslational control of Cre activity has also been achieved by chimeric molecules between Cre and the LBD of the progesterone receptor (PR), which are expressed under a constitutive active cell type specific promoter. Mutants of the LBD of the CrePR fusion protein result in a CrePR that is unresponsive to progesterone, but can still be activated by synthetic derivatives of progesterone. These mutations are located at the C-terminus of the PR, which is known to undergo conformational changes upon binding of progesterone (Allan *et al*., 1992b). Indeed, C-terminal truncations of the PR-LBD are sufficient to mediate unresponsiveness to progesterone, but not to the antiprogesterone RU486 (Vegeto *et al*., 1992; Xu *et al*., 1996). Two carboxy-terminal truncations are presently in usage, a 42 amino acid truncation (PR891) and a 19 amino acid truncation (PR914). The latter keeps a higher affinity to RU486 and is activated at nanomolar concentrations of RU486 (Wang *et al*., 1997b). Kellendonk *et al*. (1996) demonstrated for the first time that Cre activity of CrePR fusion proteins is dependent on the presence of RU486 in tissue culture. These authors reported that expansion of the hinge domain between Cre and PR enhanced the maximal activity of CrePR in the presence of RU486. However, expansion of the hinge domain is also accompanied with a progressive increase of background activity. A fusion between Cre and the PR-LBD ranging from amino acid 641-891 (CrePR1) displayed the best properties in their assay and had therefore been tested in mice by either targeted or random transgenesis. Usage of cell type specific promoters restricted CrePR1 expression to different areas of the brain

(Kellendonk *et al*., 1999; Kitayama *et al*., 2001; Tsujita *et al*., 1999), to the heart (Minamino *et al*., 2001), and to the skin (Zhou *et al*., 2002; Cao *et al*., 2001). A common feature of all these CrePR1 mouse strains was the appearance of the background activity with prolonged age of the mice, whereas RU486 administration led to high extents of inducible Cre mediated recombination.

1.5 Objectives

The aim of this study is to generate novel mouse strains allowing the inducible expression of Cre and, therefore, the inducible modification of genes by the Cre/loxP-technology. I chose two methods to achieve this aim, an inducible CrePR system and a tet-on system for control of Cre-expression. These systems were first tested *in vitro* and the most promising constructs were subsequently transmitted into the mouse germ line to investigate their performance *in vivo*. As a first step, a reporter construct allowing for quantitative monitoring of Cre activity was introduced into the ROSA26 locus by homologous recombination in HM-1 ES cells. This Cre reporter consists of a loxP-flanked DsRed gene followed downstream by an eGFP gene. Cre-mediated excision of DsRed turns on the eGFP gene which can be visualized by a change of fluorescence from red to green. Second, various inducible Cre constructs were tested in these Cre reporter containing ES cells. In order to avoid differences caused by genomic integration site and variations in copy numbers of the transgene, these constructs were targeted by knock-in into two defined genomic loci: the HPRT locus and the ROSA26 locus. ES cell clones displaying favourable characteristics, i.e. high inducibility of Cre paired with low background activity can be immediately injected into blastocysts for the generation of chimeras. The *in vivo* characteristics of the respective inducible Cre system can immediately be tested *in vivo* in chimeras, since ES cell derived cells of the chimeras contain both the Cre reporter and the inducible Cre construct. This test can yield quantitative results due to the nature of the Cre reporter, which allows to distinguish between ES cell derived tissues in which Cre-mediated recombination occurs (eGFP positive), and those in which no recombination occurred (DsRed positive). This experimental set-up allows for the rapid screening of inducible Cre systems in ES cells and in chimeras and subsequently for the immediate generation of mouse strains from constructs displaying favourable characteristics. In addition, I generated a posttranslational inducible CrePR system restricted to B cells by targeted insertion into the CD19 locus.

2. MATERIALS AND METHODS

2.1 Chemicals and antibodies

All chemicals were obtained from Sigma (Steinheim, Germany) or Merck (Darmstadt, Germany), if not otherwise stated. Enzymes were delivered from the following companies: Boehringer (Mannheim, Germany), Gibco (Karlsruhe, Germany), NEB (Schwalbach, Germany), Stratagene (Heidelberg, Germany), and Takara (over Boehringer, Ingelheim, Germany). Size markers for agarose gel electrophoresis were provided from Gibco (1 kb marker, λ-HindIII Marker, 100 bp ladder).

Table1. List of antibodies

Specificity	Clone	Reference	Supplier
B220/CD45R	RA3-6B2	Coffman *et al*., 1982	Home made/ Pharmingen
CD4	GK.1.5/4	Dialynas *et al*., 1983	Pharmingen
CD5	53-7.3	Ledbetter & Herzenberg, 1979	Pharmingen
CD8	53-6.7	Ledbetter & Herzenberg, 1979	Pharmingen
CD19	1D3	Krop *et al*., 1996	Pharmingen
Cre	Polyclonal serum	Kellendonk *et al*., 1996	Babco
Cre	Polyclonal serum		Novagen
Actin	AC-15		Sigma
GFP	Polyclonal serum		Clontech
TCR-β	H57-597	Kubo *et al.*, 1989	Pharmingen

2.2 Molecular biology

Standard methods of molecular biology were performed - if not otherwise stated - according to protocols described in Sambrook *et al*. (1989).

2.2.1 Competent *E.coli* and isolation of plasmid DNA

Competent *Escherichia coli* DH5α cells or Stbl-2 cells were prepared according to the protocol of Inoue *et al*. (1990) and used in heat shock transformations of plasmid

DNA. Ligation was performed with NEB T4-ligase according to the manufacturer's instructions.

Plasmid DNA was isolated from transformed *E. coli* DH5α with an alkaline lysis method (QIAGEN, Hilden, Germany) according to the protocol of Zhou *et al*. (1990). Plasmid DNA of a higher purity was obtained using QIAGEN columns (QIAGEN, Hilden, Germany) following the supplier's instructions.

2.2.2 Constructions of expression plasmids and targeting vectors

Primers for the amplification of cDNAs used in plasmid constructions are depicted in Table 2. Schematic overviews of the generated expression plasmids and targeting vectors together with major restriction sites are shown in Fig. 6-9. All constructs used for plasmid generation were confirmed by sequencing. Sequences of the generated plasmids and targeting vectors are available in chapter 10 supplementary data as pdf formats.

Table 2. Primers used for cloning

Sequences of oligonucleotides are shown in 5' to 3' direction. Explanation indicates the usage of the primer in a PCR reaction.

Name of primer	Sequence	Explanation
5PR650	TTTAAGGATCCACCATGGGCGCCCTGGATGCTGTTGCTC	Used for amplification of coding regions of PR650-914 and PR650-891 with 3PR914 and 3PR891.
5PR676	TTTAAGGATCCACCATGGGCGCCTCACCAGGTCAAGACATACA	Used for amplification of coding regions of PR676-914 and PR676-891 with 3PR914 and 3PR891.
5PR678	TTTAAGGCGCCGGCGGCGGTCAAGACATACAGTTGATT	Used for amplification of coding region of PR678-914 with 3PR914.
3PR891	AAATTGATATCTATCCGTACGCATGCATAGCAGTACAGATGAAGTTGTTT	Used for amplification of coding region of PR650-914 and PR676-914 with 5PR650 and 5PR676.
3PR914	AAATTGATATCTATCCGTACGCATGCATAGCAATAACTTCAGACATCATTT	Used for amplification of coding region of PR678-914, PR650-914 and PR676-914 with 5PR678, 5PR650 and 5PR676.
5Cre19	TTTAAGGCGCCACGAGTGATGAGGTTCGCA	Used for amplification of coding region of phage Cre19 with 3Cre343.
3Cre343	AAATTGGCGCCATCGCCATCTTCCAGCAGG	Used for amplification of coding region of phage Cre19 with 5Cre19.
5hCre2	GGGGGATCCACCATGGGTGCCTCCAACCTGCTGACTGTG	Used for amplification of coding region of hCre2 with 3hCre343.

5hCre19	TTTAAGGATCCACCATGGGTGCCACGAGTGATGAG GTTCGCA	Used for amplification of coding region of hCre19 and Cre* with 3hCre343 and 3hCreV336A.
3hCre343	CCCTTGGCGCCGTCCCCATCCTCGAGCAG	Used for amplification of coding region of hCre2 and hCre19 with 5hCre2 and 5hCre19.
3hCreV336A	AAATTGGCGCCGTCCCCATCCTCGAGCAGCCTCGC CATGGCCCC	Used for amplification of coding region of Cre* with 5hCre19.
3PacloxBNco	CACCATGGTGGATCCATAACTTCGTATAATGTATGC TATACGAAGTTATTTAATTAACCACTGGAAAGACCG CGAA	Cloning of RRDR
3loxAscEVElNheMsc	TTTTGGCCAGCTAGCGAATTCGATATCGGCGCGCC GATAACTTCGTATAATGTATGCTATACGAAGTTATAA GCTTACTTACCATGTCAGAT	Cloning of RRDR
3BstEIIRFP	TTTGGTCACCTTCAGCTTCACGG	Cloning of RRDR
3EGFP	TTTGATATCTAAGATACATTGATGAGTTTGGA	Cloning of RRDR
3NotBambPA	TTTGCGGCCGCGGATCCCCCCAGCTGGTTCTTTC	Cloning of RRDR
3XhoFRTneo	TTTCTCGAGGAAGTTCCTATACTTTCTAGAGAATAG GAACTTCGGAATAGGAACTTCCCCCAGCTGGTTCTT TC	Cloning of RRDR
3RedStu	TTTTAGGCCTCCCAGCCCATCGTCTTCTTCTGCATT ACGGGGCCGTCGGAGGGGAAGTTCACGCCGATGA ACTTCACCTTGTAGATGAAGCAGCCGTCTTGCAGTG ACGAGTCTTGGGTCACAGTCACGAC	Cloning of RRDR
3SacIIROSA	TTTCCGCGGCCGCCGGATCCCCGCAAACGCACCAA GC	Cloning of pROSA26w/oATG
5BstEIIROSA	AAAGGTGACCCTTCTTTCCCCC	Cloning of pROSA26w/oATG
5RedBstEII	AAAAGGTGACCAAGGGCGGCCCCCTGCCCTTCGC CTGGGACATACTGTCGCCGCAATTCCAATAC	Cloning of RRDR
5PacloxRFP	AAATTAATTAAATAACTTCGTATAGCATACATTATAC GAAGTTATCTGCAGGGATCCACCATGGGCAAGAAG AAGAGGAAGGTGGCCTCCTCCGAGAACGTCATCAC C	Cloning of RRDR
5SalWSS	AAAGTCGACTCGGGGACACCAAATATGGC	Cloning of RRDR
5EGFP	AAAGATATCTCCACCATGGTGAGCAAGGGCGAGG	Cloning of RRDR
5NotbPA	AAAGCGGCCGCGGGGATCAATTCTCTAGAGC	Cloning of RRDR
5SalFRTneo	AAAGTCGACGAAGTTCCTATTCCGAAGTTCCTATTC TCTAGAAAGTATAGGAACTTCTACCGGGTAGGGGA GGCG	Cloning of RRDR
XbaNheSA	AAATCTAGAGCCTCGCTAGCATCTGTAGGGCGCAG TAGTC	Cloning of RRDR
3AscWSS	AAAGCGGCCGCGGCGCGCCGATATCCTGCAGACTT ACCTTCCATGTCAGATCCAGACATGATAAG	Cloning of RRDR
3BamCAGGS	TTTGGATCCGAATTCACGCGTGTTTAAACTTGCCCA GGAGCTGTAGGAA	Cloning of pExChange
3BamSV40	TTTGGATCCTACCACATTTGTAGAGGTTTTAC	Cloning of pExChange
3EFPme	TTTGTTTAAACCACGACACCTGAAATGGAAGAAAAA AAC	Cloning of pExChange
5BamWSSmod	AAAGGATCCTCGGGGACACCAAATATGGC	Cloning of pExChange
5BglIISV40	AAAAGATCTCAGACATGATAAGATACATTGA	Cloning of pExChange

5PmeEF	AAAGTTTAAACAAAGCTAACTGTAGGACTGAGT	Cloning of pExChange
5SpeCAGGS	AAAACTAGTGGCGCGCCGTTTAAACTATTAATAGTA ATCAATTACGGG	Cloning of pExChange
3BamEx1	TTTGGATCCGGTAGCCAGGCTCCCTGGG	Cloning of pCD19
3PacEx11	TTTTTAATTAACAGCTGTGGAAGAGAGAGC G	Cloning of pCD19
3probeB	GGATCCCTCTCATATCTTCATA	Amplification of probe B for CD19
3XbaEx2	TTTTCTAGATTAATTAAGCTAGCACTAGTGGGGAGC CCCAGCCCCTCT	Cloning of pCD19
5NheLA	AAAGCTAGCCCTCCCGGACTCCTCACCT	Cloning of pCD19
5NotEX1e	AAAGCGGCCGCATTTAAATCTAAGCTAGAATGAAAC CTA	Cloning of pCD19
5BamEx1	AAAGGATCCATCTCCTCTCCCTGTCTC	Cloning of pCD19
5probeB	CAGCTGAGTCTTATGAAAATGCA	Amplification of probe B for CD19

2.2.2.1 CrePR expression plasmids

PCR was applied to amplify the coding regions of the LBD of the progesterone receptor PR650-891 and 650-914 (Misrahi *et al*., 1987) from the template pPAPGLVP (Wang *et al*., 1997b) using the 5'-primer 5PR650 with the 3'-primers 3PR891 and 3PR914, respectively. The PR coding regions were cloned into the BamHI- and EcoRV-sites of the pNN265E^{-}bpA vector containing a CMV promoter, a synthetic intron and a bovine polyadenylation signal (R. Kühn, Artemis Pharmaceuticals, Cologne, Germany). Fig. 6 shows pNN265E^{-}bpA and constructs cloned into this plasmid. For amplification of PR676-891 and PR676-914, the primer 5PR676 was used in combination with 3PR891 and 3PR914. In these new PR-LBD containing vectors, the phage Cre gene starting from amino acid 19 was cloned into the SfoI site after amplification using pPGK-Cre-bpA (Fellenberg, 1997) as template and 5cre19 and 3cre343 as primers. Constructs containing the humanized version of Cre, hCre2 and hCre19 were amplified from pBluehCre (Shimshek *et al*., 2002) by using the primers 5hCre2 and 5hCre19 together with 3hCre343 and then cloned into the BamHI/SfoI-sites of the pNNPR676-914. The mutant humanized CreV336A (Cre*) was amplified using the primers 5hCre19 and 3hCreV336A and then cloned as described above into pNNPR676-914 and pNNPR650-914. Coding region of PR678-914 was amplified by PCR from template pPAPGLVP (Wang *et al*., 1997b) using the 5'-primer 5PR678 with 3'-primer 3PR914 and cloned in pNNCre19PR650-914, which was BsiWI digested and then partially digested with SfoI to generate pNNCre19PR678-914. This vector was digested with BamHI/SfoI to cut out the phage Cre, which was subsequently replaced by BamHI/SfoI digested Cre* from pNNCre*PR650. The resulting vector was termed pNNCre*PR.

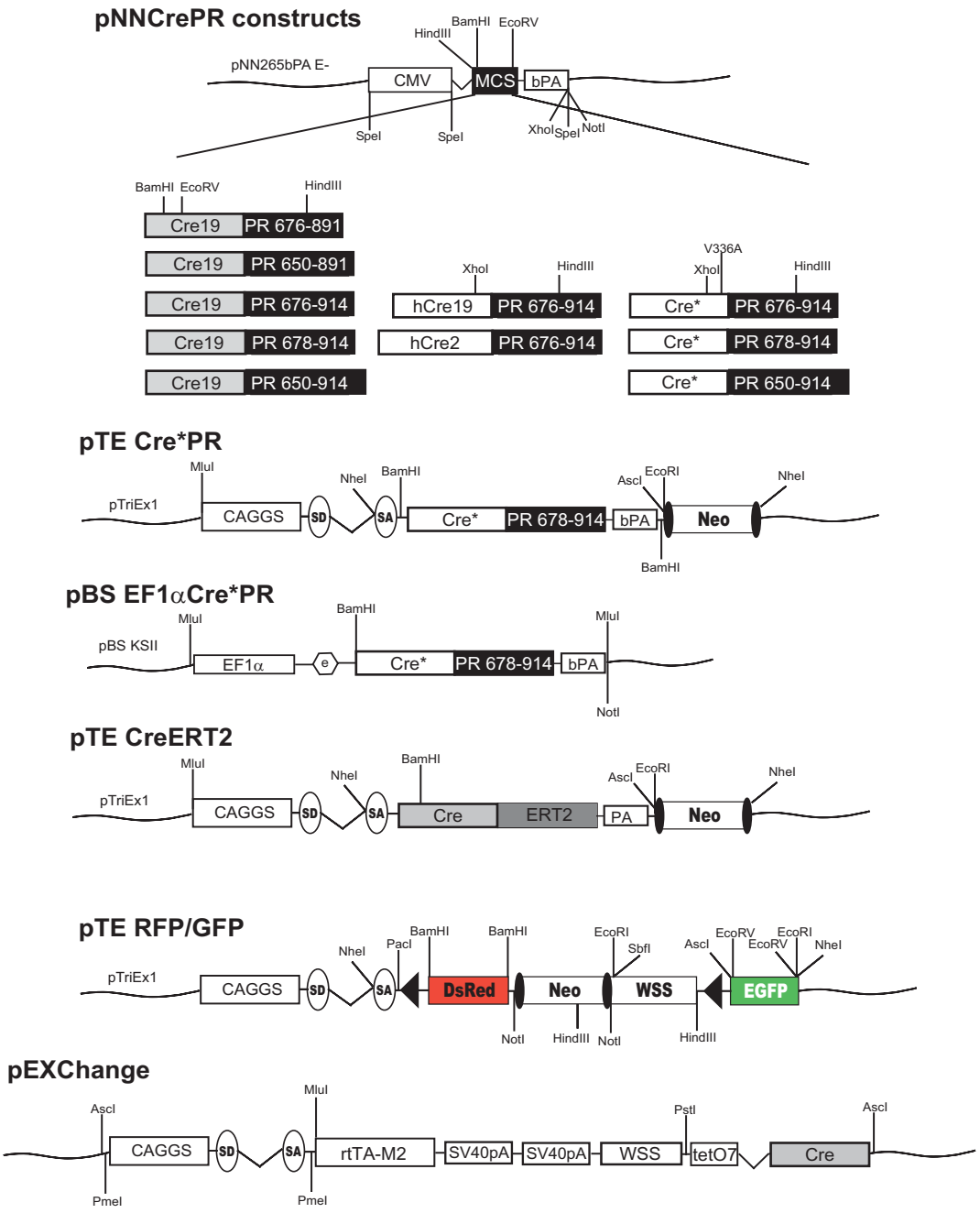

Fig. 6 Maps of expression vectors.

Parts of the plasmids with major restriction sites constructed for expression in mammalian cells. pNN265bPAE- was kindly provided by R. Kühn and contains a CMV enhancer/promoter (CMV), a multiple cloning site (MCS) and a bovine pA (bPA). In the BamHI/EcoRV sites, I have cloned the Cre19PR constructs consisting of a 18 aa truncated Cre gene from PI fused to different LBDs of the PR with the indicated aa, the hCrePR constructs derived from a humanized Cre gene and the Cre*PR constructs with the humanized Cre* gene containing mutation V336A. pTriEX1 was obtained from Novagen and contains the CAGGS promoter (CMV enhancer; chicken β–actin promoter) and a synthetic intron ranging from SD (splice donor) to SA (splice acceptor). The neomycin resistance gene is driven by the pGK promoter (neo) and is flanked by FRT sites (closed ellipses). pBS KSII was obtained from Stratagene. The EF1α promoter was amplified as described in Materials and Methods under 2.2.2.5. The CreERT2 from pTECreERT2 contains the full length NLS Cre gene fused to the ERT2 LBD from aa 282 to 594 containing aa substitutions G400V, M543A and L544A. The construction of pTE RFP/GFP is described in detail in 2.2.2.2. The plasmid contains two loxP sites (filled triangles), the DsRed gene, a FRT site flanked neo, the Westphal stop sequence (WSS) and the eGFP gene. pEXChange contains the rtTA-M2, two polyadenylation signals from SV40 (SV40pA), the WSS and the tetO7 (CMV minimal promoter fused to 7 x tet responsive elements) Cre cassette.

2.2.2.2 ROSA26 Cre indicator targeting vector

In order to generate a new indicator mouse for Cre, the pRRDR targeting vector was constructed to introduce the Cre indicator into the ROSA26 locus of embryonic stem cells. The RRDR targeting vector is shown in Fig. 7 and consists of a 2.5 kb SAH to ROSA26, a strong adenoviral splice acceptor, a loxP site, a slightly modified DsRed gene, a FRT site flanked neo gene, the WSS, a loxP site, an eGFP gene, a 4.2 kb LAH to ROSA26 and the diphtheria toxin gene. The pRRDR was constructed in nine cloning steps. An 800 bp NcoI/ NotI fragment from pDsRed1 (Clontech, Germany) containing the ORF of DsRed was cloned into pTriEx1 (Novagen, Darmstadt, Germany). This vector was digested with XbaI/NcoI and ligated to an 170 bp PCR fragment generated with the primers XbaNheSA and 3PacloxBNco from pSAβ-Geo (Friedrich & Soriano, 1991). The PCR product consists of a splice acceptor from adenovirus and a loxP site from phage PI. Silent mutation of two cryptic splice acceptors in DsRed ORF (Baird *et al*., 2000) was performed by PCR directed mutagenesis with the primers 5RedBstEII and 3RedStu. The resulting PCR fragment was digested with BstEII/StuI and inserted into the vector described above. The neo cassette flanked by FRT sites was amplified from pgkSSneo (Christ *et al.*, 2002) with the primers 5SalFRTNeo and 3XhoFRTNeo and cloned into XhoI of the plasmid generated before. The PCR fragment of the WSS (Lakso *et al*., 1992) generated from pBS302 (Gibco, Karlsruhe, Germany) with the primers 5SalWSS and 3loxAscEVEINheMsc was ligated into the XhoI/SwaI opened vector. It contains the WSS and the second loxP site. To produce a functional DsRed gene, the bPA PCR product generated with primers 5NotbPA and 3NotBambPA was cloned into the NotI site. The NLS sequence and the two mutations K5E and K9T were inserted at the N-terminus of DsRed to prevent aggregation in transfected cells (Bevis *et al*., 2002). PCR was performed with the primers 5PacloxRFP and 3BstEIIRFP and the resulting PCR fragment was digested with PacI/BstEII and ligated with PacI/BstEII digested vector. The pTE RFP/GFP expression vector was generated by ligating the ORF of eGFP (Yang *et al*., 1996; Baird *et al*., 1996) into the EcoRV site (Fig. 6). The eGFP was amplified with the primers 5EGFP and 3EGFP from a MCS lacking pEGFP-CI plasmid (Clontech, Germany). Removal of the MCS of pEGFP-CI was performed by BglII/BamHI digestion and self ligation.

The Cre indicator construct was cut out with NheI and cloned into pROSA26w/oATG opened by XbaI to produce the final targeting vector pRRDR. pROSA26w/oATG was

generated by inserting an SacI/SacII 1.6 kb fragment from A-04 (Mao *et al*., 1999). The 1.6 kb fragment was modified before by replacing the BstEII/SacII fragment of ROSA26 genomic DNA by a PCR product generated with the primers 5BstEIIROSA and 3SacIIROSA, in which the endogenous start codon was mutated by inserting a BamHI site (Fig. 7).

2.2.2.3 ROSA26 Cre*PR targeting vector

Ubiquitous expression of the posttranslational inducible Cre*PR can be achieved by targeted insertion in the ROSA26 locus (Fig. 7). The targeting vector pRRCP generated consists of 1 kb SAH to ROSA26, a strong adenoviral splice acceptor, the Cre*PR gene, a FRT site flanked neo gene, 4.2 kb LAH and the diphtheria toxin gene as negative selection marker. A schematically map of pRRCP is represented in Fig. 7. The targeting vector was constructed by NheI digestion of a transition plasmid pTECre*PR (Fig. 6), which results in a fragment of the splice acceptor, the Cre*PR gene and the FRT flanked neo, which was subsequently ligated to the pROSA26-1 targeting vector, which was digested with XbaI. To produce pTECre*PR (Fig. 6), the Cre*PR678-914 fusion protein ORF was digested with BglII, blunted and then digested with NotI from pNNCre*PR (Fig. 6). This fragment was cloned into a modified version of pTE RFP/GFP (Fig. 6) lacking loxP sites, DsRed, WSS and eGFP.

The SA Cre*PR FRT neo cassette was cut out from the pTECre*PR plasmid with NheI (Fig. 6) and cloned into XbaI of pROSA26-1 (Soriano, 1999) to generate pRRCP (Fig. 7).

2.2.2.4 HPRT CAGGS Cre*PR targeting vector

In order to generate ubiquitously expressed Cre*PR in mice, a CAGGS promoter driven Cre*PR gene was inserted into the HPRT targeting vector pMP10 (Fig. 8). A MluI/AscI fragment from pTECre*PR (Fig. 6), containing the sequences of the CAGGS promoter (Niwa *et al*., 1991), a synthetic intron (Choi *et al*., 1991), the Cre*PR678-914 and the bPA, was cloned into AscI digested pMP10 (S. Casola, unpublished) to generate pMP10 CCP (Fig. 8). pMP10 is a derivative from pMP8 (Reid *et al*., 1991) and contains an AscI and PmeI site to insert the transgene of interest upstream of the HPRT promoter. In addition, pMP10 contains SbfI, SwaI and SgrAI sites to linearize the targeting vector (Fig. 8).

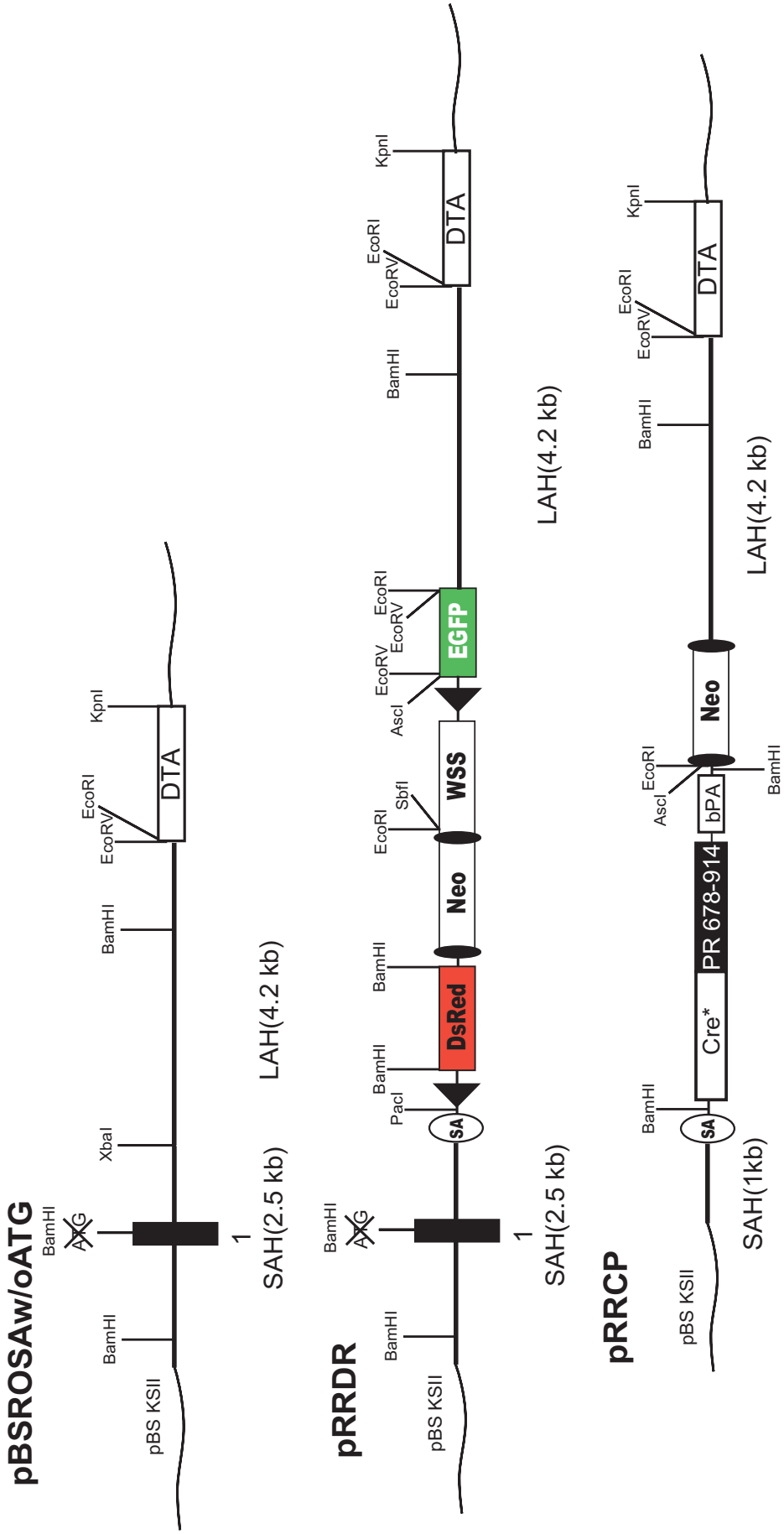

Fig. 7 Maps of ROSA26 targeting vectors.

Schematical drawings of the generated ROSA26 targeting vectors with major restriction sites. ATG destroyed start codon; DTA, diphtheria toxin A gene driven by the pGK promoter; SAH, short arm of homology; LAH, long arm of homology; SA, adenoviral splice acceptor; filled triangles, loxP sites; DsRed, DsRed gene; closed ellipses, FRT sites; Neo, neomycine resistance gene driven by the pGK promoter; WSS, Westphal stop sequence; eGFP, eGFP gene; Cre*, a 18 aa truncated humanized Cre gene containing mutation V336A; PR678-914, LBD of the progesterone receptor ranging from aa 678-914; bPA, bovine polyadenylation signal.

2.2.2.5 HPRT EF1αCre*PR targeting vector

The human EF1α promoter (Kim *et al*., 1990; Wakabayashi-ito *et al*., 1994) was amplified from BAC RP11-206H23 (a gift from J. Seibler, Artemis Pharmaceuticals, Cologne, Germany) with the primers 5PmeEF and 3PmeEF and cloned into the EcoRV of pBS KSII (Stratagene, Heidelberg, Germany). This plasmid was digested with BamHI/ NotI and ligated with the BamHI/NotI 1.8 kb fragment from pNNCre*PR containing the Cre*PR and the bPA to generate pBS EF1αCre*PR (Fig. 6). The 4.3 kb MluI fragment of pBS EF1αCre*PR was cloned into pMP10 (S. Casola, unpublished) to produce pMP10 EF1αCre*PR (Fig. 8).

2.2.2.6 HPRT CAGGS tet-on targeting vector

In order to establish a tet-on inducible system for Cre, the pEXchange plasmid was constructed in which the rtTA-M2 transactivator (Urlinger *et al*., 2000) with its own promoter and the tetO7 Cre cassette are located on one DNA fragment (Fig. 6). Cell type specific expression of the rtTA-M2 can be achieved by exchanging the promoter region. AscI digestion releases the tet-on cassette consisting of the promoter, the rtTA-M2, two SV40 pAs and a WSS (Lakso *et al.*, 1992), followed by the tet-responsive cassette which consists of the tetO7 promoter, a synthetic intron and the Cre ORF. The AscI fragment was cloned into the HPRT targeting vector pMP10 (Fig. 8). The 1.1 kb WSS fragment produced by PCR with the primers 5BamWSSmod and 3AscWSS from pBS302 was ligated into BamHI/AscI digested pTriEx1 (Novagen, Darmstadt, Germany). In the resulting vector, a non-functional CAGGS promoter fragment was cloned in XbaI/BamHI amplified from pCAGGS Flpe (Schaft *et al*., 2001) with the primers 5SpeCAGGS and 3BamCAGGS. Between the promoter and the WSS, the rtTA-M2 followed by two SV40pA from pUHrT 62-1+SV40 (a derivative of pUHrT 62-1, in which an additional SV40pA was inserted) was cloned with EcoRI/BamHI. This plasmid was digested with PstI/AscI and a 2.1 kb PstI/AscI fragment from ptetO7-I-Cre-pA was inserted, which contained the tetO7 promoter, a synthetic intron, the Cre ORF and a pA (gift from F. Schwenk, Artemis Pharmaceuticals, Cologne, Germany). In the final pEXchange, the promoter can be exchanged by a single digest (PmeI) to get different cell type specificities (Fig. 6). For strong ubiquitous expression of the rtTA-M2, a HincII CAGGS fragment from pTECre*PR was inserted into PmeI. The tet-inducible cassette was cut out with AscI and ligated to pMP10 (S. Casola, unpublished) to generate pMP10 Ctet-on (Fig. 8).

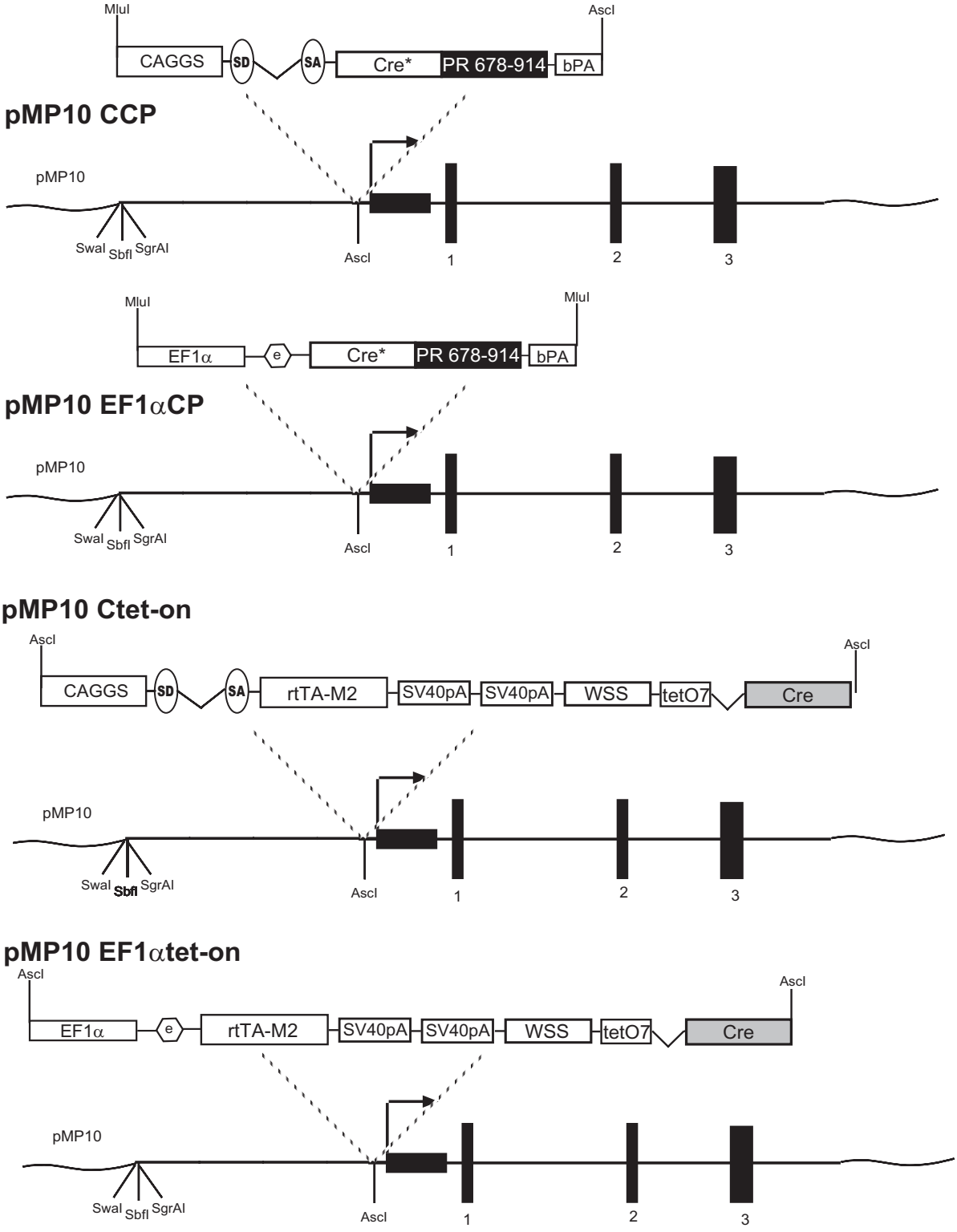

Fig. 8 Maps of HPRT targeting vectors.

Schematical drawings of the generated HPRT targeting vectors. Abbreviations used are as in Fig. 7. CAGGS, CMV enhancer combined with the chicken β–actin promoter; SD, splice donor; EF1α, promoter of the EF1α gene; rtTA-M2, reverse tetracycline controlled transactivator M2; SV40pA, polyadenylation signal from SV40; tetO7, CMV minimal promoter fused to 7 x tet-responsive elements.

2.2.2.7 HPRT EF1αtet-on targeting vector

The pEXchange plasmid described in Materials and Methods under 2.2.2.6 was digested with PmeI to release the CAGGS promoter region and ligated to the PCR fragment containing the EF1α promoter, which was described in Materials and Methods under 2.2.2.5. The same cloning strategy was used as for construction of pMP10 Ctet-on described in Materials and Methods under 2.2.2.6 to produce pMP10 EF1αtet-on (Fig. 8).

2.2.2.8 CD19 Cre*PR targeting vector

In order to achieve B cell specific expression of transgenes, a targeting vector for CD19 was constructed (pCD19), in which the transgene of interest can be inserted into the NheI site in the second exon of the CD19 gene (Fig. 9). The SAH of the mCD19 gene was generated in two steps. First, a 900 bp fragment, amplified with the primers 5NotEx1e and 3BamEx1 from genomic 129/ola DNA containing part of the promoter and part of exon 1, was used for ligation with pBS TK, which contains the TK gene for negative selection with GANC. Second, a BamHI/XbaI digested 420 bp PCR product generated with the primers 5BamEx1 and 3XbaEx2 consisting of the destroyed, endogenous start AUG of CD19, which was replaced by a BamHI site and the first intron, was introduced into the plasmid pBS TK containing the 900 bp fragment described above. The LAH containing exons 2-11 of mCD19 was amplified from genomic 129/ola DNA with the primers 5NheLA and 3PacEx11 and ligated into the plasmid with the SAH. The single NheI site in exon 2 can be used to introduce the transgene of interest.

The SA Cre*PR FRT neo cassette was cut out from pTECre*PR (Fig. 6) with NheI and was cloned into the NheI site to generate the CD19 Cre*PR targeting vector pCD19Cre*PR (Fig. 9).

2.2.2.9 CD19 CreERT2 targeting vector

The transitional vector pTECreERT2 (Fig. 6) was generated, which consists of the CAGGS promotor, a synthetic intron, the CreERT2 and a FRT flanked neo cassette. It was produced by a 2 kb HincII/AscI fragment of pCAGGSCreER (Seibler *et al*., 2003) consisting of the CreERT2 ORF cloned into BamHI blunted/AscI digested pTECre*PR. From the resulting plasmid, the CreERT2 FRT flanked neo cassette was cut out with NheI and inserted into pCD19, which results in pCD19CreERT2 (Fig. 9).

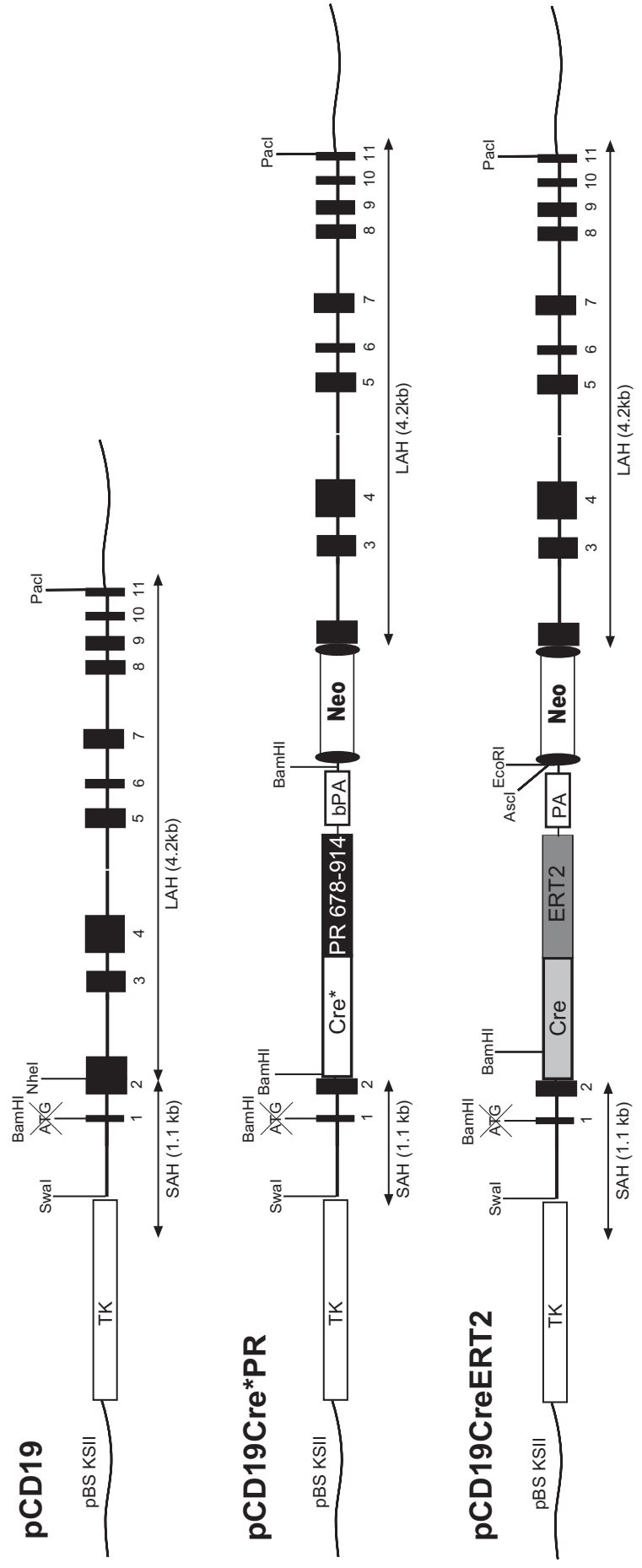

Fig. 9 Maps of CD19 targeting vectors.

Schematical drawings of the generated CD19 targeting vectors with major restriction sites. Abbreviations used are as in Fig. 7 and 8. TK, thymidine kinase gene driven by the pGK promoter; ERT2, LBD of the human estrogen receptor from aa 282-594 containing aa substitutions G400V, M543A and L544A.

2.2.3 Isolation of genomic DNA

Cells were incubated overnight at 56°C in lysis buffer (10 mM Tris-HCl, pH 8; 10 mM EDTA; 150 mM NaCl; 0.2% SDS; 400 mg/ml proteinase K). DNA was precipitated by adding an equal volume of isopropanol, pelleted by centrifugation, washed in 70% EtOH, and resuspended in TE-buffer (10 mM Tris-HCl, pH 8; 1 mM EDTA) plus RNAseI (50μg/ml). DNA from ES cell clones grown in 96-well tissue culture dishes was extracted and prepared according to the protocol of Pasparakis and Kollias (1995). For the preparation of DNA from mouse tissue, the latter was incubated overnight at 56°C in tissue lysis buffer (0.1 M Tris-HCl, pH 8.5; 5 mM EDTA; 0.2% SDS; 0.2 M NaCl; 600 mg/ml proteinase K). Debris were pelleted and the supernatant was mixed with an equal volume of isopropanol and the precipitated DNA was washed in 70% EtOH, dried and resuspended in TE-buffer.

2.2.4 RT-PCR

The mRNA from mouse organs was prepared using the RNeasy mini kit (QIAGEN, Hilden, Germany) according to the manufacture's protocol. DNA was removed by DNaseI digestion (Promega, Mannheim, Germany). cDNA synthesis of 5 µg total RNA was done as described in superscript II protocol (Gibco, Karlsruhe, Germany).

Table 3. Oligonucleotides used for amplification of specific cDNAs from ROSA26 and CD19 locus.

Name of primer	Sequence	Explanation
ROSAG	CGCCGTAGGTCAGGGTGGT	Used with ROSA512 to amplify GFP cDNA from RRDR.
ROSA512	GCCGTTCTGTGAGACAG	Used in combination with ROSAG, ROSAR1 and ROSA31 for amplification of cDNAs from ROSA locus.
ROSAR1	CGCCCTCGATCTCGAACT C	Used with ROSA512 to amplify DsRed cDNA from RRDR.
ROSA31	AAATGTTCTGGACAAACACTTC	Used with ROSA512 to amplify transcript 1 from ROSA locus.
5EGFP	AAAGATATCTCCACCATGGTGAGC AAGGGCGAGG	Used with ROSAG for amplification of GFP cDNA.
5BamEx1	AAAGGATCCATCTCCTCTCCCTGTC TC	Used to amplify Cre*PR transcript from CD19 locus of CD19CP with hCreintrev.
hCreintrev	ACCAGGGACACAGCATTGG	Used to amplify Cre*PR transcript from CD19 locus of CD19CP mice with 5BamEx1.

2.2.5 DNA electrophoresis

Separation of DNA fragments by size was achieved by electrophoresis in agarose gels (0.7% - 2%; 1 x TAE; 0.5 mg/ml ethidiumbromide (Sambrook *et al.*, 1989)). DNA fragments were extracted from agarose gel slices with either the QIAEX II or the QIAquick Gel Extraction Kit (QIAGEN, Hilden, Germany) following the manufacturer's protocols.

2.2.6 DNA sequencing

DNA was sequenced using the 'Big Dye termination Cycle Sequencing Kit' (Applied Biosystems, Foster City, USA), which is a PCR-based modification of the original Sanger protocol (Sanger *et al*., 1977). The fluorescently labelled DNA fragments were separated and analysed with the ABI373A and ABI377 systems (Applied Biosystems, Foster City, USA).

2.2.7 Quantification of DNA and RNA

The concentration of nucleic acids was determined by measuring the absorption of the sample at 260 nm and 280 nm in a spectrophotometer. An OD_{260} of 1 corresponds to approximately 50 μg/ml for double stranded DNA or 40 μg/ml for RNA and single stranded DNA. Purity of nucleic acids can be estimated by the ratio OD_{260}/OD_{280} with 1.8 and 2.0 for DNA and RNA, respectively. Alternatively, the DNA was electrophoresed in an agarose gel, and the concentration was approximated from the band intensity in comparison with a standard.

2.2.8 PCR

PCR (Mullis and Faloona, 1987; Saiki *et al*., 1985) was used to screen mice and ES cells for the presence of targeted alleles or transgenes and to amplify fragments for sequencing (primers shown in Table 4). Reactions were performed in either Hybaid machines (MWG-Biotech, Ebersberg, Germany) or Triothermocyclers (Biometra, Göttingen, Germany). Genotyping of mice and ES cells was generally performed in a total volume of 50 μl in the following reaction mix: 50 pmol of each primer, 1.5 U of *Thermus aquaticus* (*Taq*) DNA polymerase (homemade), 250 μM dNTPs, 10 mM Tris-HCl pH 8.3, 50 mM KCl, 2.5 mM $MgCl_2$, 100 ng template DNA. Amplification started with denaturation for 4 min at 94 °C followed by 30-35 cycles of 94 °C for 30 sec, 52-60 °C for 30 sec, 72 °C for 30 sec and a final extension step at 72 °C for 10

min. To amplify cDNA fragments for cloning or sequencing, the High Fidelity Kit with proofreading activity was used (Roche, Mannheim, Germany) with the primers shown in Table 2.

Table 4. Mouse typing by PCR

Sequences of oligonucleaotides are shown in 5'-3' direction. Explanation indicates the usage of the primer in standard PCR reactions. PCR of transgenic Cre lines was performed as published.

Name of primer	Sequence	Explanation
ROSA1	CAGGGTTTCCTTGATGATGTCA	Typing of RRDR-DsRed and RRDR-eGFP with ROSAG and ROSAR; Typing of RCP with hcreintrev.
ROSAG	CGCCGTAGGTCAGGGTGGT	Typing of RRDR-eGFP with ROSA1.
ROSAR	AGCTTCACGGTGTTGTGGCC	Typing of RRDR-DsRed with ROSA1.
hCreintrev	ACCAGGGACACAGCATTGG	Typing of RCP with ROSA1; CCP with 5Cre*; CD19CP with 5BamEx1.
5Cre*	TCCACCATGGGTGCCACCTC	Typing of CCP with hcreintrev.
5BamEx1	AAAGGATCCATCTCCTCTCCCTGTCTC	Typing of CD19CP with hcreintrev.

2.2.9 Southern blot analysis

Digestion of 5-15 μg DNA was performed overnight with 50 - 100 U of the appropriate restriction enzyme. Subsequently, the DNA fragments were separated by agarose gel electrophoresis and transferred onto Hybond™-N+ (Amersham, Illinois, USA) or GeneScreenPlus (Dupont, Wilmington, USA) nylon membranes by an alkaline capillary transfer according to the method of Chomczynski and Qasba (1984). Membranes were baked at 80°C for 20 min to fix the DNA equilibrated in 2x SSC (Sambrook *et al*., 1989) and then prehybridized at 65°C for 4 h in hybridization solution (1M NaCl, 1% SDS, 10% dextran sulfate, 50 mM Tris-HCl pH 7.5, 250 μg/ml sonicated salmon sperm DNA). The radioactively labelled probe was added to the hybridization solution and allowed to hybridize at 65°C for 10 h in a rotating cylinder.

The following probes were used:

(i) For the ROSA26 locus:
800 bp EcoRI/PacI fragment from A-04 (Mao *et al*., 1999).
1000 bp SacII/XbaI fragment from pROSA26-1 (Soriano, 1999).

(ii) For the HPRT locus:
300 bp RsaI probe (Reid *et al*., 1991).
1000 bp Cre* probe digested with BamHI/XhoI from pNNCre*PR650-914 (Wunderlich *et al*., 2001).
1000 bp Cre probe digested with XhoI/SpeI from pGKCrebPA (Fellenberg, 1997).

(iii) For mCD19 locus:
900 bp PCR fragment amplified with 5NotEx1e and 3BamEx2 and subcloned into pGemTeasy (Promega, Mannheim, Germany).
1000 bp PCR fragment amplified with 5ProbeB and 3ProbeB and subcloned into pGemTeasy (Promega, Mannheim, Germany).

(iv) For the neomycin resistance gene:
400 bp BamHI fragment from pBSNeoprobe (N.Uyttersprot, unpublished)

(v) For the eGFP gene:
800 bp GFP ORF from an EcoRV digest of pTE RFP/GFP.

(vi) For the pol-β locus:
900 bp fragment of BamHI/HindIII digested pPolbeta (Gu *et al*., 1994).

Aliquots of 50 ng DNA of the above probes were radioactively labeled with 2.5 mCi [α^{32}P]dATP (Amersham, Braunschweig) using the Ladderman™ Labeling Kit (Takara, Japan) based on the principle of random primed oligolabeling (Feinberg and Vogelstein, 1984). Unincorporated radiolabeled nucleotides were removed with MicroSpin™ S-200HR columns to reduce background during hybridization (Pharmacia, Freiburg, Germany). The probe was denatured at 100°C for 5 min and then cooled on ice, before it was added to the hybridization solution. After hybridization, stringent washes were initially performed twice in 2 x SSC/0.1 % SDS and then followed by washes in 1 x SSC/0.1 % SDS and 0.5 x SSC/0.1 % SDS, if necessary. All washes were performed at 65°C under gentle shaking for 30 min. After each wash, the filter was monitored with a Geiger-counter and the washes were stopped when specific signals reached 20 to 100 cps. Then, the filter was sealed in a plastic bag and exposed to X-ray film (Kodak XAR-5 or BioMAX MS; Eastman

Kodak) at -80°C. Films were developed in an automatic developer or, alternatively, filters were exposed to a phosphoimager screen (Fuji, Fuji, Japan) and analyzed on a Bio-Imaging Analyser (Fuji Bias 1000; Fuji, Japan).

2.3 Cell biology

2.3.1 Fibroblast cell culture

CV1-5B cells originally obtained from Kellendonk *et al*. (1996) or murine embryonic fibroblasts (MEF) were cultured in DMEM supplemented with 10% FCS, 100U/ml penicillin and 0.1 mg/ml streptomycin. Aliquots of 8 $x10^4$ cells were plated on a 6 well-plate, grown for 24h and then transfected with CrePR plasmid DNAs using Fugene6 according to manufacturer's protocol (Roche, Mannheim, Germany). To determine transfection efficiencies, the plasmid pHD2-AP encoding the alkaline phosphatase was cotransfected. Twelve hours after transfection, cells were trypsinized and split into 2 wells of a 12 well-plate containing medium with or without RU486 and, 60 hours later, cells were fixed in 4% formaldehyde in PBS and stained overnight with X-Gal staining solution as described (Kellendonk *et al*., 1996). After heat inactivation of endogenous alkaline phosphatases (AP) at 65°C for 30 min, cells were stained for the cotransfected AP activity using fast red tablets (Roche, Mannheim, Germany). In each well, blue and red cells in three different areas were counted. The ratios of blue to red cells were determined and the value obtained with authentic Cre was set to 100%. Each experiment was performed at least in triplicate.
In dose response assays, $8x10^5$ CV1-5B cells were plated on 9 cm dishes, grown for 24 h and then transfected with 5 µg of the test plasmids. Twelve hours after transfection, cells were trypsinized and split into seven wells of 6 well-plates containing increasing concentrations of RU486. Seventy two hours after transfection, cells were fixed and stained for β-galactosidase activity as described above. Relative β-galactosidase activity was expressed as the percentage of the maximal activity after substraction of the activity obtained in the absence of ligand. Each experiment was performed in duplicate.

2.3.2 Embryonic stem cell culture

All targetings were performed in HM-1 ES cells (Magin *et al*., 1992). Culturing and transfection of ES cells was performed according to published protocols (Pasparakis

and Kollias, 1995; Torres and Kühn, 1997). To maintain the pluripotency of the ES cells, the latter were cultured in the presence of leukaemia inhibiting factor (LIF) containing ES cell medium on a layer of embryonic feeder (EF) cells. The ES cell medium (DMEM, 15 % FCS, 1 mM sodium pyruvate, 2 mM L-glutamine, 1 x non essential amino acids, 1:1000 diluted LIF supernatant, 0.1 mM 2-β-mercaptoethanol) contained FCS, which had been priorly tested to promote ES cell growth and to prevent *in vitro* differentiation (Gibco, Karlsruhe, Germany). ES and EF cells were grown in tissue culture dishes (Falcon, Bedford, USA) and kept at 37°C under humid atmosphere with 10% CO_2. EF cells in DMEM supplemented with 10% FCS, 1 mM sodium pyruvate, 2 mM L-glutamine were never passaged more than three times and mitotically inactivated by mitomycin-C treatment (10 μg/ml for 2 h) before seeding with ES cells. ES cell colony growth was stopped before they became confluent. Colonies were washed twice with PBS and then treated shortly with trypsin (0.05 % trypsin, 0.02 % EDTA in PBS; Gibco, Karlsruhe, Germany) at 37°C, until the cells detached from the dish. The cell suspension was then used for passaging, transfection, or freezing. ES cells were frozen in 90% FCS, 10 % DMSO at -80°C and later transferred into liquid nitrogen for long term storage. For transfection, 1 x 10^7 ES cells were mixed with 30 to 40 μg DNA in 800 μl transfection buffer (RPMI w/o phenol red, Gibco, Karlsruhe, Germany) and electroporated at 23°C (500mF, 240V). After 5 min. incubation, ES cells were transferred onto an embryonic feeder layer and 48 hours later placed under G418 (300 μg/ml, 71% active) or HAT selection (120 μM hypoxanthine, 0.4 μM aminopterin, 20 μM thymidine). If necessary, selection against HSV-*tk* containing random integrants started at day five after transfection by supplementing the medium with 2 mM gancyclovir (Cymeven, Syntex). On approximately day 9 after transfection, double resistant colonies were picked and split into EF-containing 96-well tissue culture dishes for expansion. In HPRT targetings, picked HAT resistant clones were maintained in HT medium (120 μM hypoxanthine, 20 μM thymidine) for five days to dilute out the toxin aminoptherin (A) and then switched to normal ES medium.

2.3.3 Culture of *ex vivo* splenocytes

The spleens were aseptically removed from mice and then pressed through a sterile sieve. Erythrocytes were lysed for 2 min by NH_4Cl (140 mM NH_4Cl, 17 mM Tris-HCl pH 7. 65). Splenocytes were kept in DMEM supplemented with 10 % FCS, 1 mM

sodium pyruvate, 2 mM L-glutamine, 1x non essential amino acids, 0.1 mM 2-β-mercaptoethanol supplemented with various activation compounds (LPS; ConA; PMA; BAFF) for not longer than three days. In experiments, in which Cre activity should be induced, the cells were treated once with various amounts of inducer (RU486, Org31710, 4-OHT, 1mM stocks in ethanol).

2.3.4 HTNC treatment

Various cell types were treated by His-TAT-NLS-Cre (HTNC) (Peitz *et al*., 2002) to delete loxP-flanked gene segments as follows.

ES cells:

$2x10^5$ ES cells were plated in a well of a 6 well culture dish with feeders, 5 h prior to HTNC treatment. The medium was removed and replaced with DMEM w/o FCS/PBS, 1:1, 4-0.25 µM HTNC from a 50% glycerol stock solution of 180 µM HTNC for 20 h (Peitz *et al*., 2002; M. Peitz, unpublished). Thereafter, the cells were cultured as described before. The deletion efficiency at 2 µM HTNC was usually 97% (T. Wunderlich, N. Hövelmeyer, C. Merkwirth, unpublished results).

Ex vivo splenocytes:

$3x10^6$ *ex vivo* splenocytes were treated with 2-0.5 µM HTNC in DMEM w/o FCS/PBS, 1:1 or 10 µM HTNC in medium for 20 h. Subsequently, cells were centrifuged and cultured in the presence of LPS (10 µg/ml). After three days, cells were surface stained and FACS analysed or lysed for preparation of DNA and/or RNA.

Fibroblasts:

$3x10^5$ CV1-5B or RRDR/RRDR or RRDRΔneo/RRDRΔneo fibroblasts were treated for 20 h with 2-0.25 µM HTNC in DMEM w/o FCS/PBS, 1:1.

2.3.5 Induction of Cre activity by ligand addition

Induction by hormone:

$2x10^5$ double targeted ES cells (RRDR-DsRed/+; CCP or EF1αCP) were treated with various amounts of either RU486 or Org31710 (Organon, Oss, The Netherlands) for 4-21 days. The medium was changed every day and ligand was added freshly.

Induction by doxycycline:

$2x10^5$ double targeted ES cells (RRDR/+; Ctet-on or EF1αtet-on) were treated for 4 - 10 days with doxycycline 1µg/ml in medium. Medium was freshly prepared and exchanged every day.

2.3.6 Preparation of cells from lymphoid organs

Thymus, spleen and lymph nodes were squashed between two frosted sides of a microscope slide to obtain single cell suspensions. Bones were flushed with medium (DMEM, 10% FCS, 2 mM L-glutamine) to extract bone marrow cells. Erythrocytes were lysed from spleen and bone marrow preparations in 140 mM NH_4Cl, 17 mM Tris-HCl pH 7.65 for 2 min. Blood from the tail vein was collected in a tube with heparin (Liquemin, Roche, Mannheim, Germany) and then layered on top of 7% Ficoll 400 (Pharmacia, Freiburg, Germany). After 1350 g centrifugation at 23°C for 15 min., lymphocytes were recovered from the interphase of the gradients and resuspended in DMEM, 10% FCS, 2 mM L-glutamine and kept on ice.

2.3.7 Flow cytometry

Cells (10^6 per sample) were surface stained in 30 µl PBS, 0.1 % BSA, 0.01 % NaN_3 with combinations of fluoresceine isothiocyanate (FITC), phycoerythrine (PE) and Cy-Chrome™ (Cyc) conjugated mAbs for 20 min on ice. After staining, the samples were washed and resuspended in the above medium. Stained cells were analyzed on a FACSCalibur and data were evaluated using CellQuest software (Becton Dickinson, Mountain View, USA). Dead cells were labelled with Topro-3 for exclusion from the analysis. Monoclonal antibodies, listed in Table 1, were either homemade (C.Uthoff-Hachenberg, B. Hampel) or purchased from Pharmingen (San Diego, USA).

2.4 Biochemistry

2.4.1 Protein extracts preparations

Lysates from mouse organs were prepared either freshly or from frozen tissue. Tissue was diced into small pieces using a clean razor blade. Then, RIPA buffer (1xPBS, 1% NP-40) was added at 3 ml/g tissue supplemented with various protease inhibitors. Samples were centrifuged at 13,000 rpm for 5 min to separate supernatants from debris. Supernatants were transferred to a fresh tube and protein concentrations were determined in a photometer according to the Warburg formula. Protein extracts were diluted with RIPA buffer to 15 mg/ml before adding running buffer (3xSDS sample buffer, NEB, Schwalbach, Germany) and DTT (30xDTT, NEB, Schwalbach, Germany) to 200 µg of protein extracts. To separate the cytoplasmic protein fractions from the nuclear extracts, MEFs were resuspended at 10^6/15 µl cells

in hypotonic solution (10 mM Hepes [pH 7.9], 10 mM KCl, 2 mM MgCl2, 0.5 mM DTT, 0.1 mM EDTA, supplemented with various protein inhibitors) and incubated at 4°C for 10 min. Then NP-40 was added to 1 % and the cells were centrifuged at 13,000 rpm for 1 min. The supernatant containing the cytoplasmic fraction was recovered from the nuclear pellet and the nuclear pellet was resuspended in 10^6/10 µl cells of high salt buffer (20 mM Hepes [pH 7.9], 420 mM NaCl, 1.5 mM MgCl2, 0.5 mM DTT, 0.2 mM EDTA and 10 % glycerol) and incubated on ice for 30 min. Nuclear extracts were recovered after centrifugation at 10,000 rpm for 10 min at 4°C and stored at – 80 °C.

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2.4.2 Western blot

Protein extracts were electrophoresed by SDS-PAGE (10-15 %) (Laemmli, 1970) and transferred to immobilon-NC membranes (Millipore, Bedford, USA). The membranes were blocked with 5 % NF-milk/PBS or 5 % BSA/PBS for 1 h and probed using either one of two polyclonal antisera against Cre or anti-actin as primary antibodies. Membranes were then incubated with goat anti-rabbit IgG-horseradish peroxidase (HRP) conjugates (Vector, Burlingame, USA) as secondary antibody and developed using the ECL or ECL$^+$ kit.

2.5 Mouse experiments

Vasectomy of male mice, tail bleeding as well as breeding of foster mothers and the general handling of mice were performed according to Hogan (Hogan *et al*., 1987) and Silver (Silver, 1995).

2.5.1 Mice

C57BL/6, C57BL/6 x Balb/c, 129/Sv and CD1 mice were obtained from Bomholtgard, Charles River, Harlan Winkelmann or Jackson Laboratories. CB20 mice were taken from breedings in our animal facility. CD19-cre mice (Rickert *et al*., 1997), *deleter* mice (Schwenk *et al.*, 1995), RAGE-EG mice (Constien *et al*., 2001), Flp deleter (Rodriguez *et al*., 2000) and pol-β flox/flox (Gu *et al*., 1994) mice were intercrossed with the newly generated RRDR strain, the ROSACre*PR strain, the CAGGS Cre*PR strain and the CD19Cre*PR strain in conventional facilities.

2.5.2 Activation of CrePR

100 mg RU486 were dissolved in 50 µl DMSO and vortexed. Then, 1.95 ml of either

trioctanoin (Acros, Geel, Belgium) or sesame oil was added and sonicated for 1-2 min. Aliquots of the emulsions were frozen at -20°C and sonicated again after thawing. The emulsions were either fed or *i.p*. injected into mice. Feeding and *i.p*. procedures were done as described (Seibler *et al*., 2003). The Org31710 was obtained at a 400 mg/ml EtOH solution from Organon (Oss, The Netherlands). Seven ml of trioctanoin were added to 1 ml of the solution and vortexed. Administration and further handling of Org31710 was performed as described above.

3. RESULTS

3.1 A strategy for rapid screening of inducible Cre constructs

In order to test efficiency and potential background activity of newly generated inducible Cre constructs first in ES cells and then in chimeric mice, I followed the strategy shown in Fig. 10. Knock-in procedures were preferred over the generation of classical transgenic mice to produce defined conditions and to circumvent tandem repeats, multiple integration sites and breakage of the construct that can affect expression of the transgene. The karyotypic male HM-1 ES cell line (Magin *et al*., 1992) was used, which harbours a spontaneous deletion of the promoter and the first two exons of the single X-linked hypoxanthine-phospho-ribosyl-transferase (HPRT) gene. The HPRT gene was chosen, since it is a housekeeping gene, so that any promoter inserted is not restricted in its expression by unfavourable chromatin configurations (Bronson *et al.,* 1996; Vivian *et al*., 1999; Hatada *et al*., 1999; Cvetkovic *et al*., 2000; Evans *et al*., 2000; Guillot *et al*., 2000). It is also of advantage that the targeting vector pMP10 is available, which mediates a highly efficient, directly selectable homologous recombination event at the HPRT locus in HM-1 ES cells (Reid *et al*., 1991). This targeting vector pMP10 is arranged such that recombination between the targeting vector and the homologous genomic sequences inserts the missing HPRT promoter and exons into the HM-1 genome. Upstream of the HPRT promoter in the pMP10 targeting vector, the inducible Cre transgene is inserted, which is driven by its own promoter (Fig. 10A). A successful gene targeting creates an intact HPRT gene and restores HPRT activity. Homologous recombined ES cell clones can be efficiently enriched by HAT selection for the presence of the HPRT gene product.

To have a quantifiable readout of Cre action, a special indicator consisting of DsRed and eGFP was inserted into the ubiquitously expressed ROSA26 locus of HM-1 ES cells (Fig. 10B). This locus was originally identified by Friedrich & Soriano (1991) in a gene trap in embryonic stem cells and was shown to be expressed in all cells of the adult mouse. The introduced Cre reporter expresses the DsRed gene flanked by loxP sites. Upon Cre-mediated excision of the DsRed gene, the eGFP gene is turned on, which should be visualized by a change of cell fluorescence from red to green. The inducible Cre construct can therefore be analyzed in doubly targeted ES cells only

when the promoter of the Cre transgene is active in ES cells. The presence of the DsRed/eGFP Cre indicator in the ROSA26 locus allows quantitative analysis of the inducible Cre construct, which is inserted into the HPRT locus in chimeras, since DsRed expression identifies ES cell-derived cells and tissues within the chimeras. All ES cell-derived cells should be red before Cre-mediated recombination and green thereafter.

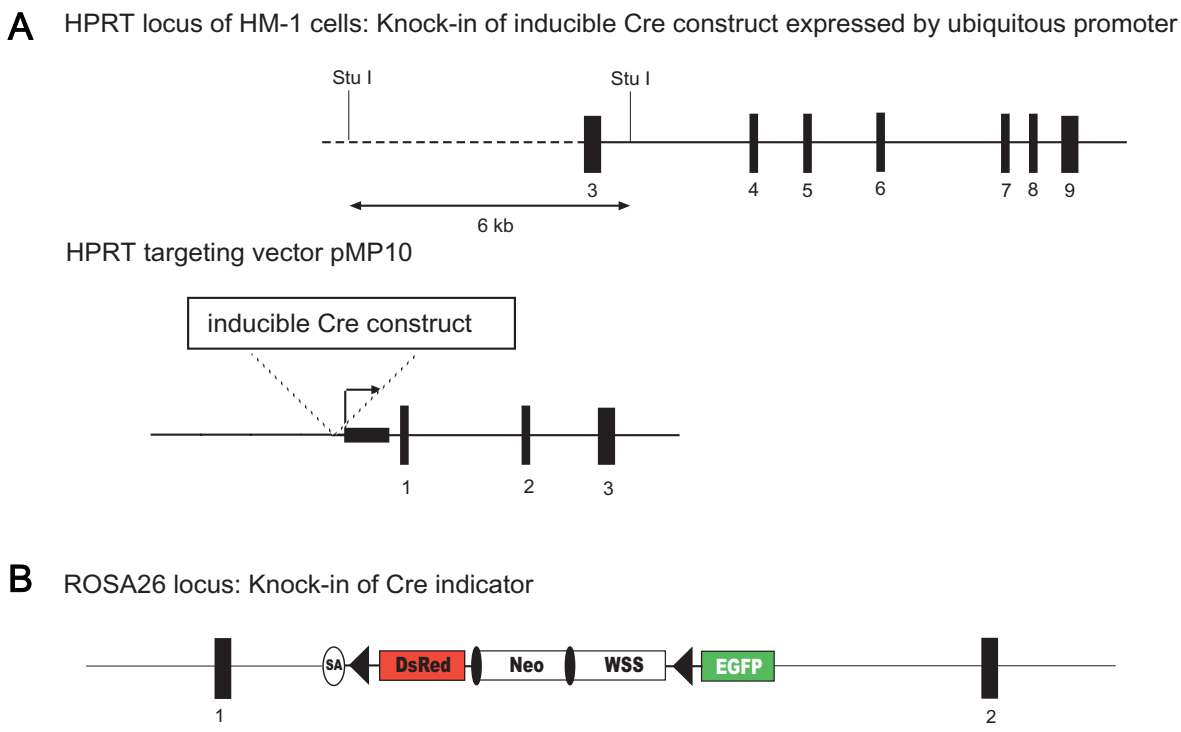


Fig. 10 Rapid screening for inducible Cre constructs.

(A) The X-linked HPRT locus of HM-1 ES cells harbours a spontaneous deletion of 50 kb including the promoter and the first two exons. This deletion of the HPRT locus is complemented by the HPRT targeting vector, together with the inducible Cre construct driven by its own promoter. The inducible Cre construct is inserted 5' of the HPRT promoter. Selection procedure for homologous recombination will be done in HAT medium, for the presence of the HPRT gene product. Southern blot is performed for determination of homologous recombinant clones by a StuI digest.

(B) A special Cre indicator (RRDR) was inserted before the HPRT targeting into the ubiquitously expressed ROSA26 locus. Before Cre-mediated recombination, the loxP-flanked DsRed gene is expressed. After deletion, the eGFP gene is turned on and the cell switches its fluorescence from red to green.

3.2 An attempt to generate an improved Cre indicator mouse strain

In order to test the DsRed/eGFP Cre indicator RRDR (**RR**osa **D**ouble **R**eporter) in transient transfection experiments, it was cloned into the expression vector pTriEX1 containing the CAGGS promoter and a downstream splice donor as shown in Fig. 11. The resulting plasmid was termed pTE RFP/GFP and consists of a strong adenoviral splice acceptor inserted in front of a loxP site, followed by a DsRed gene which was

modified by introducing silent mutations and thereby inactivating two strong cryptic splice acceptors and by fusing a NLS to the N-terminus. Downstream is a neo gene flanked by two FRT sites, followed by the Westphal stop sequence (WSS) (Lakso *et al*., 1992), a second loxP site, and the eGFP gene. The FRT sites flanking the positive selection marker neo provide the possibility to remove neo by Flp transfection, since it was shown to negatively influence the expression of neighbouring genes (Pham *et al*., 1996). The WSS was introduced to prevent transcriptional read-through of the pA of the DsRed, which would lead to eGFP expression simultaneously to DsRed expression. For further reference, RRDR containing the loxP-flanked DsRed-stop cassette is termed RRDR-DsRed and the RRDR, from which this cassette has been excised, is termed RRDR-eGFP.

Transient transfections of the RRDR into fibroblasts

Transiently transfected fibroblasts of pTE RFP/GFP revealed strong fluorescence of the DsRed protein restricted to the nuclei of transfected cells (Fig. 11B). When a Cre coding plasmid was cotransfected, nearly all transfected cells exhibited green fluorescence in the cytoplasm (Fig. 11C), demonstrating that the DsRed/eGFP Cre indicator system works well in transient transfections.

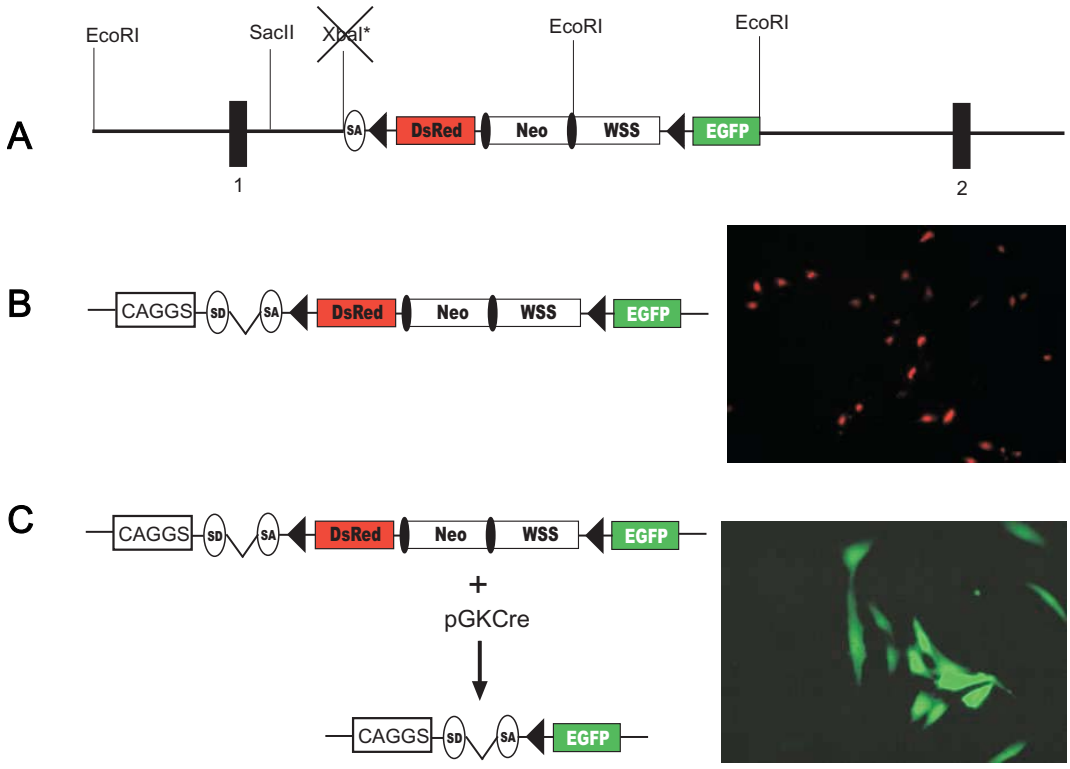


Fig. 11 Transient transfection of the Cre indicator into fibroblasts.

(A) Location of the Cre indicator after targeting into the ROSA26 locus.

(B) $8x10^4$ CV1-5B cells were transiently transfected with 1 µg pTE RFP/GFP or (C) with 1 µg pTE RFP/GFP together with 1 µg pGKCre in a 6 well-plate. After two days, DsRed is expressed in the nucleus of CV1-5B fibroblasts and eGFP in nucleus and cytoplasm.

Knock-in of the RRDR indicator into the ROSA26 locus of HM-1 ES cells

When the project was started, the ROSA26 locus was considered to be the best locus for the insertion of a Cre indicator construct. This locus was identified in a gene trap by Friedrich & Soriano (1991). Fig. 12 shows that the ROSA26 locus expresses two transcripts (transcripts 1 and 2) with no significant ORF and a third transcript in antisense orientation (transcript AS) which putatively encodes a protein. In the ROSA26 gene trap line, the adenoviral β–Geo gene trap construct was inserted into the first intron of ROSA26, disrupting transcripts 1 and 2 (Fig. 12). The transcript AS in antisense orientation was not affected by the gene trap insertion (Zambrowicz *et al*., 1997). The strong adenoviral splice acceptor (SA) in front of the β–Geo gene directed splicing from exon 1 of transcript 1 and 2 of ROSA26 to the SA of the β–Geo gene, which was then expressed by the endogenous ROSA26 promoter preceding exon 1. Transgenic mice harbouring the ROSA26 β–Geo insertion express β-galactosidase in all cells at early developmental stages of mouse embryos and in all tissues of young mice. The targeting strategy used here resembles the integration of the adenoviral β–Geo vector into the first intron of the ROSA26 locus (Soriano, 1999). Since it is now known that the expression level of the gene is relatively low (Mao *et al*., 2001), I modified the original strategy, i.e. the putative endogenous start

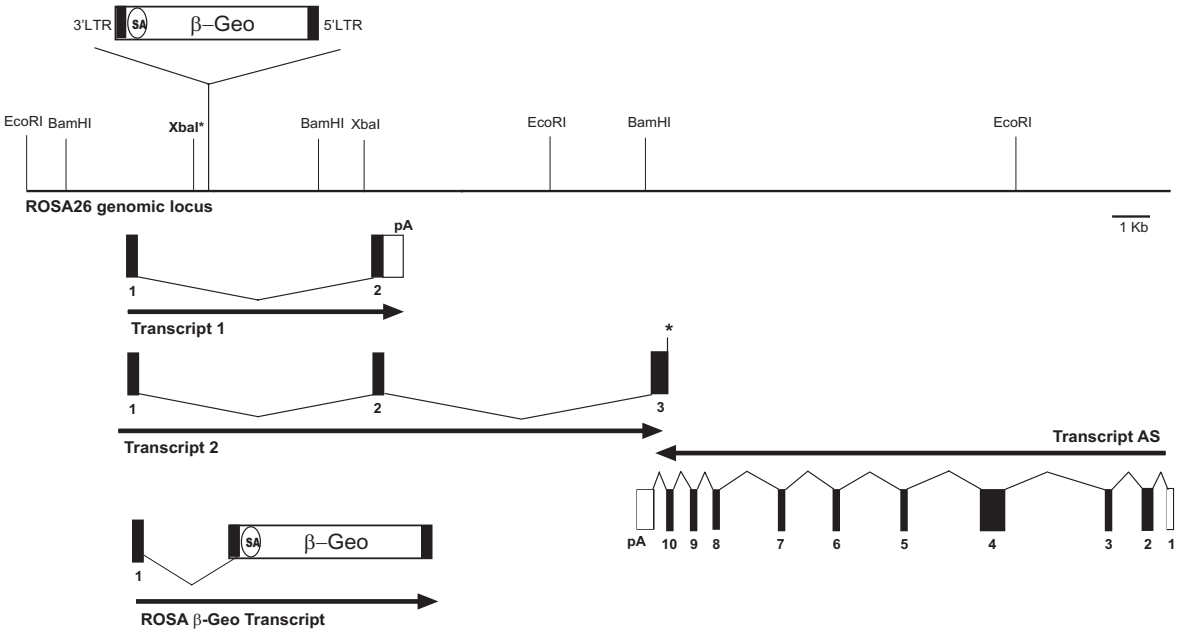


Fig. 12 Transcripts of the ROSA26 locus.

Major restriction sites of the genomic ROSA26 region are indicated together with the integration site of the β-Geo gene trap construct and the endogenous ROSA26 transcripts. Note that transcript 2 and transcript AS share homology. The asteriks at the end of transcript 2 indicates that its 3' end is not yet determined. The figure is adapted from Zambrowicz *et al*., 1997.

codon in the first exon of transcripts 1 and 2 was replaced by a BamHI site in order to achieve higher expression levels of transgenes which are inserted into this locus. Independent targeting experiments with the ROSA26 targeting vector pRRDR containing the DsRed/eGFP Cre indicator (Fig. 13A) in HM-1 ES cells resulted in homologous recombination frequencies ranging from 1 in 5 to 1 in 10 as described previously (Soriano, 1999). ES cell clones harbouring homologous recombination events were identified by Southern blot: Homologous recombination leads to the appearance of a novel 7.2 kb band in addition to the 16 kb wild type band (Fig. 13B) in EcoRI digested DNA using Eco/Pac probe A (Fig. 13C). The cointegration of the BamHI site replacing the start codon (AUG) was detected by probing BamHI digested DNA with Xba/Sac probe B using Southern blot analysis (Fig. 13D). A band of 1.5 kb identifies clones in which the endogenous start codon was replaced by the BamHI site of the targeting vector, whereas clones retaining the start codon were identified by a 2.5 kb band. None of the homologous recombinant ES cell clones revealed DsRed expression using fluorescence microscopy (data not shown). Nevertheless clone D2 contained the correct integration of the Cre indicator, as verified by Southern blot and by sequencing the DNA of the Cre indicator from targeted ES cells. Subsequently, D2 ES cells were injected into blastocysts of C57BL/6 or CB20 mice. This resulted in five chimeric mice, all of which transmitted the Cre indicator transgene through the germ line after backcrosses to C57BL/6 or CB20 mice. Germ line transmission was first identified by coat colour and then verified by Southern blotting and/or PCR using the primers ROSA-1 with ROSA-R and ROSA-G.

Deletion of the DsRed-stop cassette in ES cells by HTNC treatment

His-**T**AT-**N**LS-**C**re (HTNC) can be used to efficiently mediate recombination of loxP-flanked alleles in tissue culture (Peitz *et al*., 2002). HTNC is a highly purified Cre protein which is modified by fusing the TAT domain and a NLS sequence to the N-terminus of Cre. These two domains enable the Cre protein to cross cell membranes and to mediate recombination. In order to test whether excision of the DsRed-stop cassette of the RRDR indicator leads to eGFP expression in D2 ES cells carrying the RRDR indicator insertion in the ROSA26 locus (Fig. 14A), $2x10^5$ of these ES cells were plated on 6 well-plates for 5 h, 1 day or 3 days prior to HTNC treatment (Fig. 14B). Subsequently, the cells were incubated with HTNC for 20 h, and the extent of excision of the loxP-flanked DsRed-stop cassette and eGFP expression was

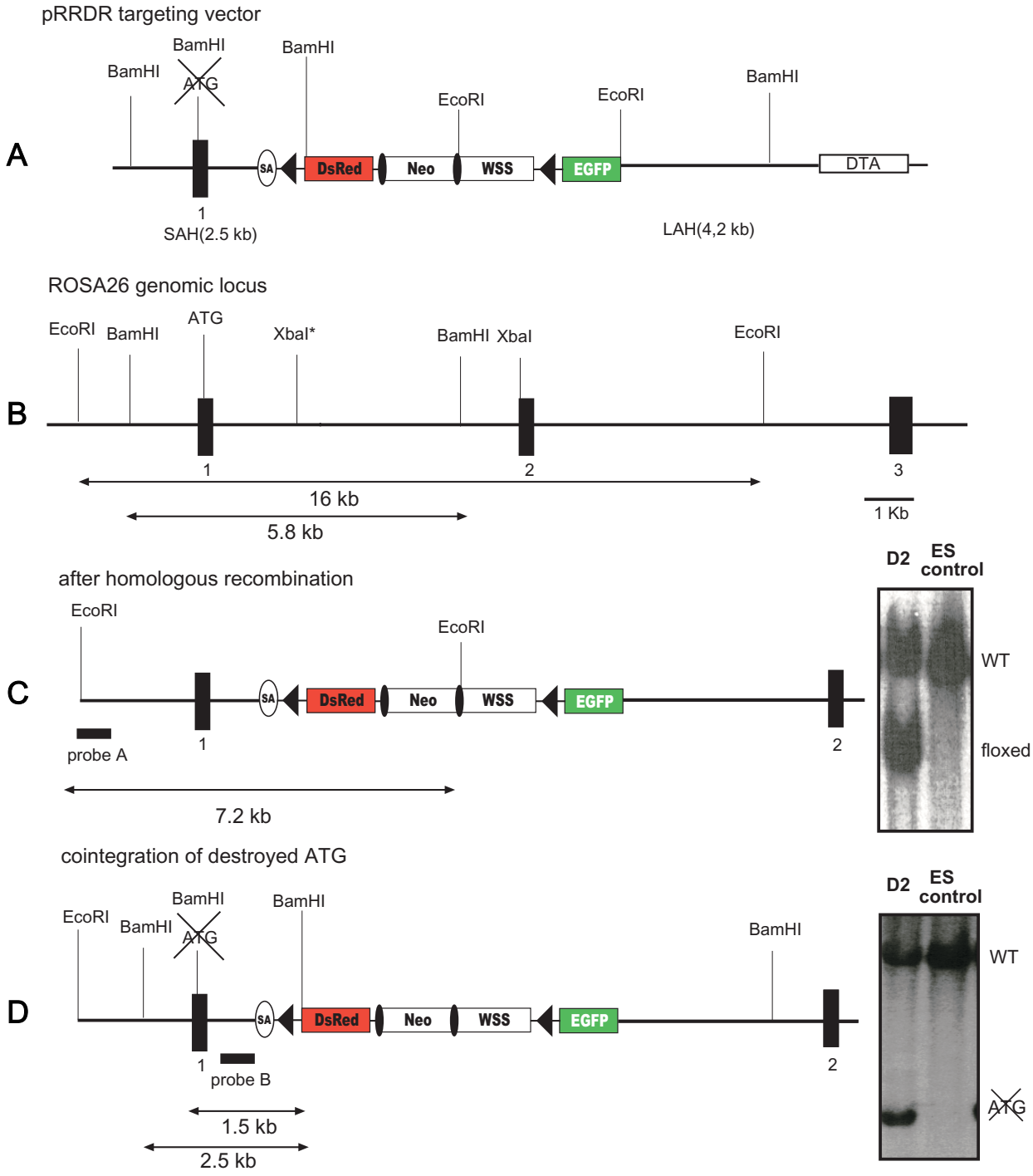


Fig. 13 Targeting of the Cre indicator RRDR into ROSA26.

(A) RRDR targeting vector with major restriction sites. Short arm of homology (SAH) is elongated to 2.5 kb in comparison to the original pROSA26-1 targeting vector. The endogenous ROSA26 start codon is replaced by a BamHI site. The long arm of homology (LAH) is 4.2 kb with downstream negative selection marker diphtheria toxin A (DTA). The Cre indicator was inserted into the original XbaI* site resulting in its disappearance.

(B) The ROSA26 genomic locus diplays bands in EcoRI and BamHI digested DNA of 16 kb and 5.8 kb, respectively.

(C) After homologous recombination, Southern blot analysis of EcoRI digested genomic DNA with external probe A resulted in a 7.2 kb band (floxed) besides the 16 kb wild type band (WT).

(D) In Southern blot analysis using internal probe B on BamHI digested genomic DNA, clones with the destroyed start codon displayed a 1.5 kb band (~~ATG~~), besides the 5.8 kb wild type band (WT).

determined by Southern blot and by fluorescence microscopy, respectively. Probing EcoRI digested DNA with probe A in Southern blot analysis showed 97% deletion in samples treated with 10 µM HTNC in medium containing FCS and 2 µM HTNC in medium without FCS for 5 h after plating, respectively. Excision of the DsRed-stop cassette became progressively less efficient the later HTNC was administered after plating of the ES cells (Fig. 14C). Therefore, it seems that HTNC works best on small ES cell colonies. Fig. 14D shows ES cell colonies with 50% deletion from sample 5, which were incubated with 2 µM HTNC w/o FCS at 3 days old colonies (Fig. 14C). The DsRed-stop cassette of the colony on the right of Fig. 14D was excised by HTNC as indicated by its green fluorescence, whereas HTNC failed to excise the loxP-flanked target in the colony on the left.

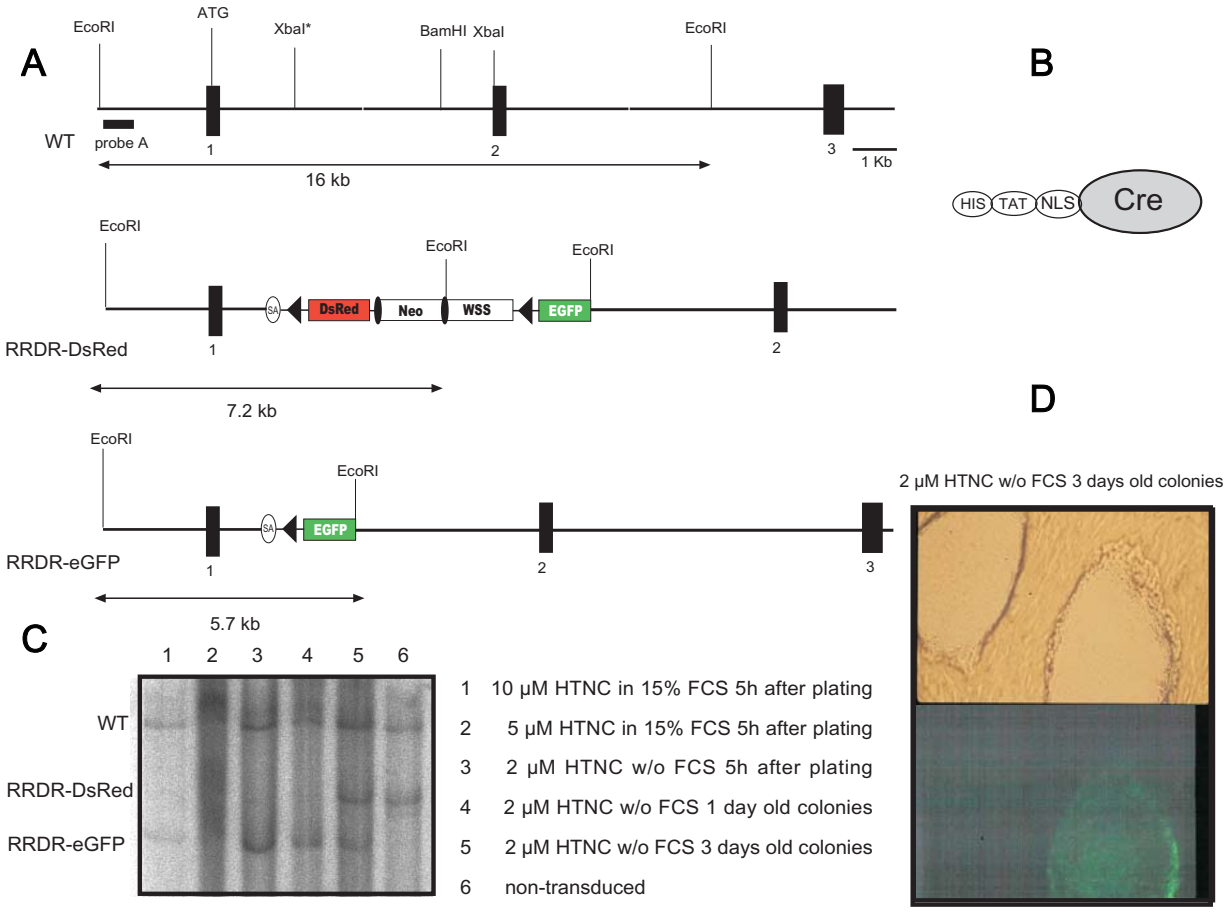


Fig. 14 Deletion of the DsRed-stop cassette in RRDR ES cells by HTNC treatment.

(A) Southern blot screening strategy for deletion of the DsRed-stop cassette. EcoRI digested DNA reveals a 16 kb wild type band (WT), a 7.2 kb RRDR-DsRed band and a 5.7 kb RRDR-eGFP band using probe A.

(B) The **H**is-**T**AT-**N**LS-**C**re protein (HTNC) was highly purified by Ni-NTA column chromatography. The minimal TAT domain and the NLS sequence enable the native protein to cross membranes and to mediate recombination of floxed targets to a high extent.

(C) Southern blot of ES cells incubated for 20 h with HTNC at indicated concentrations with or without (w/o) FCS.

(D) RRDR ES cells (3 days old colonies) were incubated with 2 µM HTNC for 20 h resulting in 50% deletion. Upper part reveals two ES cell colonies and lower part the corresponding fluorescence photograph.

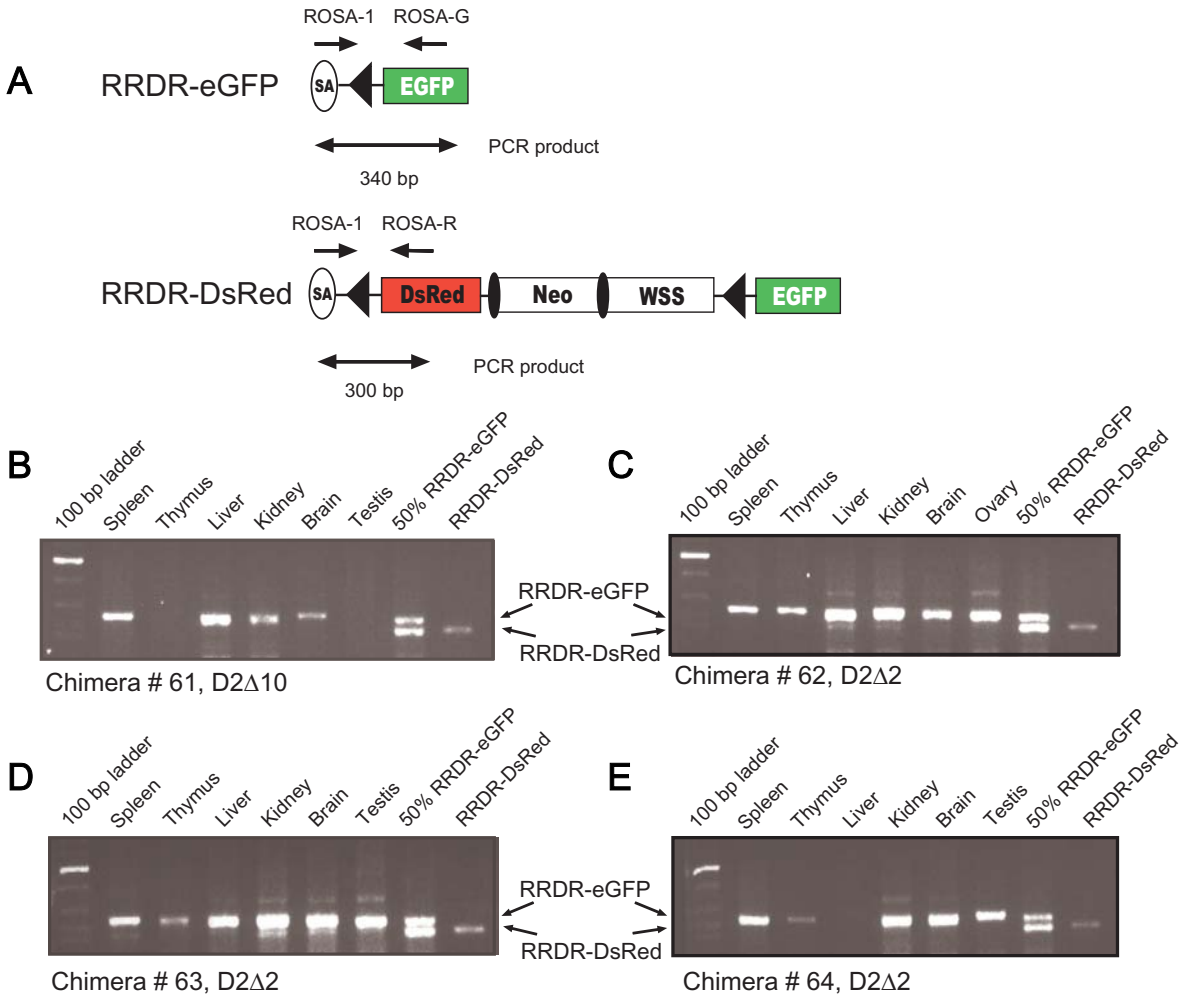


Fig. 15 PCR analysis of organs of chimeric mice generated from HTNC treated ES cells.

(A) PCR screening to distinguish between RRDR-DsRed and RRDR-eGFP. Using a three primer approach, RRDR-DsRed is indicated by a 300 bp band generated by ROSA-1 and ROSA-R, whereas the RRDR-eGFP appears as a 340 bp band generated by ROSA-1 and ROSA-G.

PCR analysis of genomic DNA from different organs removed from (B) chimera # 61, D2Δ10 generated from RRDR-DsRed ES cells treated with 10 µM HTNC in 15% FCS and (C) chimera # 62, D2Δ2, or (D) chimera # 63, D2Δ2, or (E) chimera # 64, D2Δ2, all generated from RRDR-DsRed ES cells treated with 2 µM HTNC w/o FCS.

HTNC treatment of ES cells could be a fast and useful technique for removing loxP-flanked target sequences *in vitro*. Every chimera generated from the parental clone D2 transmitted the inserted transgene through the germ line. Therefore, ES cells from samples 1 (D2Δ10) and 3 (D2Δ2) of the previous experiment were injected into blastocysts to evaluate whether HTNC treatment affects the pluripotency of ES cells, i.e. the ability to give rise to germ cells in chimeric animals. Three chimeras were generated from both D2Δ10 (#59, #60, #61) and D2Δ2 (#62, #63, #64).Two chimeras generated from the former died before reaching breeding age. The remaining 4 chimeras were bred to CB20. One animal of D2Δ2 (#63) was sterile; the other three

did not transmit the transgene through the germ line in 10 litters. To determine whether parts of the organs of the chimeric mice were derived from ES cells, chimeras were sacrificed, DNA was prepared and analyzed by PCR. Since the ES cell preparations D2Δ10 and D2Δ2 both contained ES cells, in which HTNC treatment resulted in excision of the loxP-flanked DsRed-stop cassette (RRDR-eGFP), and ES cells where this did not occur (RRDR-DsRed), a PCR was designed to detect both versions of the RRDR indicator. Fig. 15A shows the PCR strategy used to distinguish between RRDR-DsRed and RRDR-eGFP. In this PCR, three primers were used in a single reaction. Amplification by the primers ROSA-1 and ROSA-R results in a product of 300 bp and indicates the presence of RRDR-DsRed. In the case of the RRDR-eGFP allele only the ROSA-1 and ROSA-G primers can anneal to DNA and will amplify a 340 bp PCR product. Fig. 15B, C, D and E display the results of PCR reactions performed on DNA isolated from the indicated organs of chimeras #61, #62, #63 and #64. All organs of chimera #61 showed the band amplified from RRDR-eGFP except in thymus and testis. All organs of chimera #62 and #63 were positive for the RRDR-eGFP by PCR depicted in Fig. 15C and D. Fig. 15E displays PCR reactions performed on DNA isolated from organs of chimera #64. Except the liver, all organs of chimera #64 were partly derived from ES cells.

In toto, this experiment indicates that ES cells which have been transduced by HTNC are able to give rise to all tissues of the mouse including testis and ovary. However, the chimeric mice consisting of wild type and transgenic cells failed to transmit the RRDR-eGFP allele to the offspring. This might be due to the fact that the germ cells were not derived from the transduced ES cells or that germ cells derived from HTNC-treated ES cells were not able to give rise to fertility.

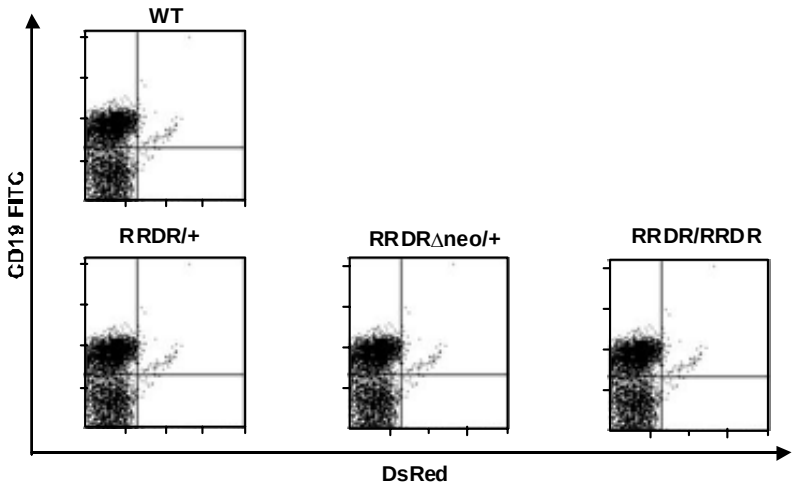


Fig. 16 Flow cytometry of RRDR splenocytes to detect DsRed.

Splenocytes isolated from wild type (WT), RRDR/+, RRDRΔneo/+ and RRDR/RRDR homozygote mice were labelled with CD19 FITC. RRDRΔneo/+ are mice in which the neo gene of the DsRed-stop cassette was deleted by the Flpe deleter. Dead cells were excluded from analysis by TOPRO-3.

Detection of DsRed in mice with the RRDR indicator

To explore DsRed expression in RRDR/+ mice, in RRDR/RRDR mice and in RRDRΔneo/+ mice, all major organs were removed and analyzed by fluorescence microscopy. None of the organs revealed any red fluorescence (data not shown).

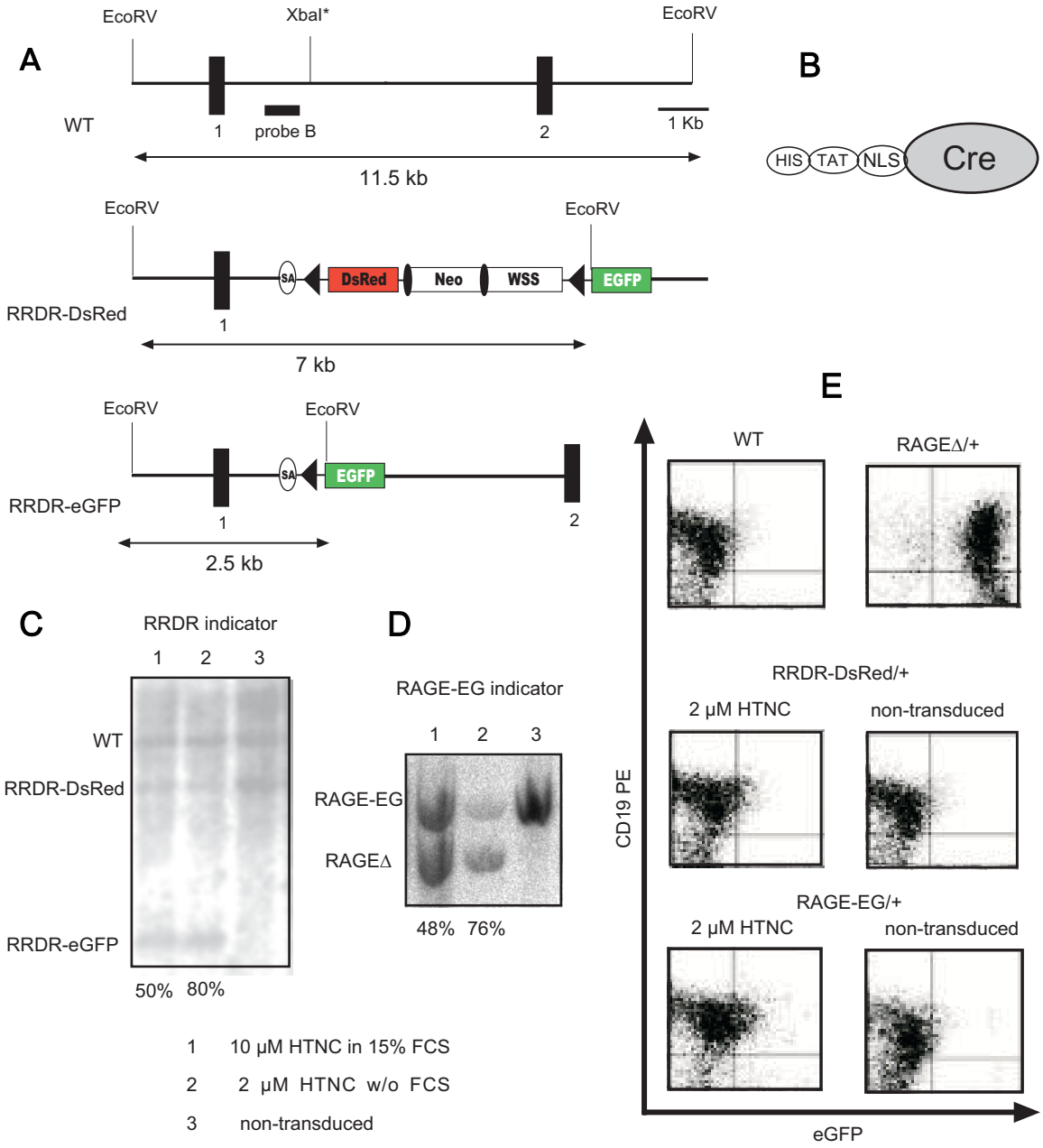


Fig. 17 Transduction of HTNC into RRDR splenocytes activated *ex vivo* by LPS.

(A) Southern blot screening strategy for deletion of the DsRed-stop cassette. Probe B detects the 11.5 kb WT band of EcoRV digested DNA, the RRDR-DsRed band of 7 kb and the RRDR-eGFP band of 2.5 kb, respectively.

(B) The HTNC protein was highly purified by Ni-NTA column chromatography. The minimal TAT domain and the NLS sequence make HTNC permeable to membranes.

(C) Southern blots of *ex vivo* RRDR and (D) RAGE-EG splenocytes treated for 20 h with 10 µM HTNC in 15% FCS (lane 1), 2 µM HTNC w/o FCS (lane 2) and control (lane 3). Deletion efficiencies are indicated as percent. Southern blot screening strategy for Cre-mediated excision of the RAGE-EG indicator is shown in Fig.24A.

(E) LPS activated splenocytes were labelled with CD19PE and eGFP expression was analyzed by flow cytometry.

Splenocytes isolated from these mice were stained with CD19 FITC and CD5 Cyc and analyzed by FACS. TOPRO-3 was used to exclude dead cells from the analysis. No DsRed expression was observed in either T cells (CD5 positive, data not shown) or B cells (CD19 positive, (Fig. 16)). Fig. 16 shows the FACS analysis of the splenocytes isolated from the above mentioned mice. Cells shifted in the red channel of the dot blot are either contaminating erythrocytes or dead cells.

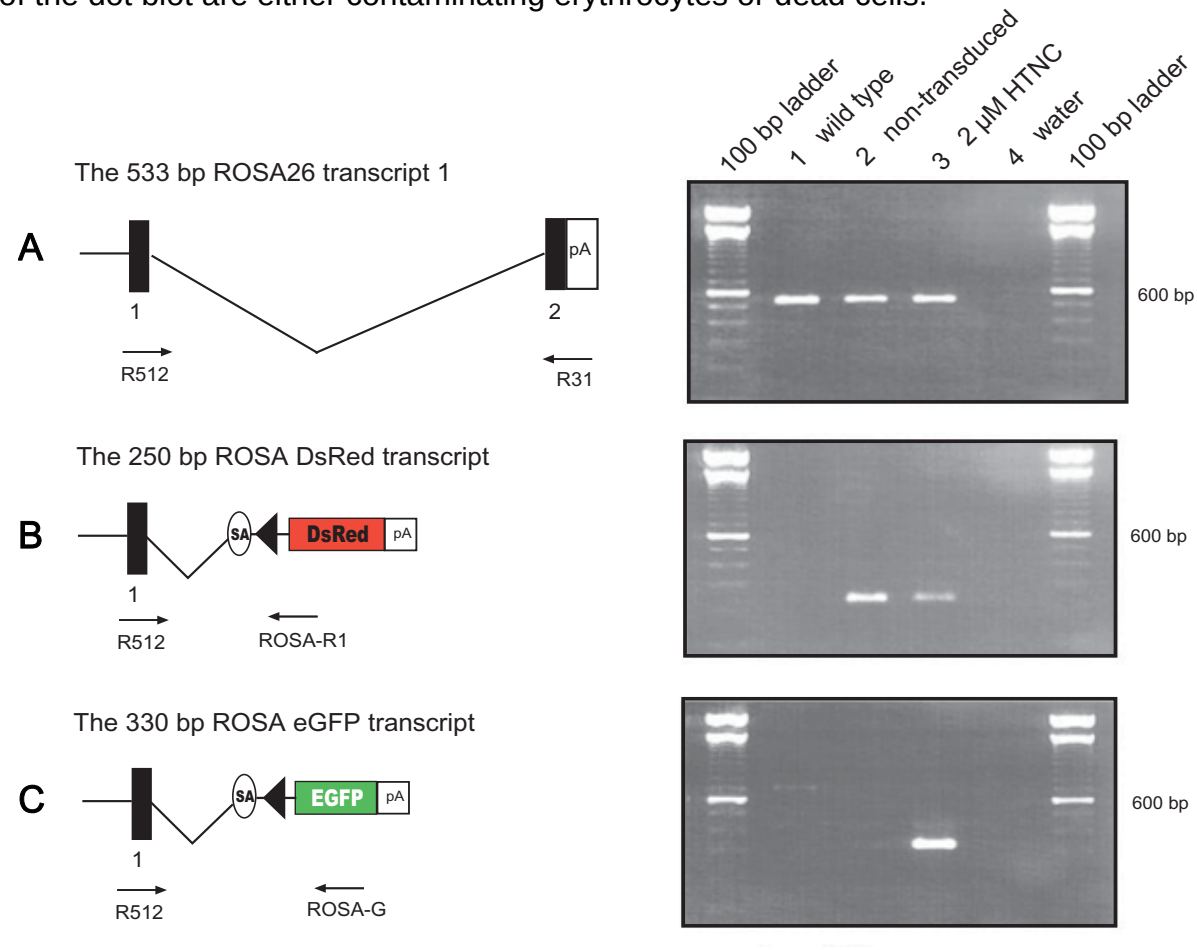


Fig. 18 RT-PCR of HTNC transduced RRDR/+ splenocytes.

(A) Detection of endogenous ROSA26 transcript 1. cDNA from wild type (lane 1), non-transduced (lane 2) and 2 µM HTNC treated (lane 3) RRDR/+ splenocytes is used in PCR with primers R512 and R31. The correctly spliced transcript 1 appears as a 533 bp band in all samples.

(B) Detection of the DsRed transcript. PCR is carried out using primers R512 and ROSA-R1. The correctly spliced DsRed transcript is 250 bp.

(C) Detection of the eGFP transcript. PCR reaction was performed using primers R512 and ROSA-G. The correctly spliced eGFP transcript PCR product has a size of 330 bp.

All bands were isolated from the gel, cloned in pGEM Teasy and confirmed by sequencing.

Deletion of the DsRed-stop cassette by HTNC in LPS activated splenocytes

In order to test whether eGFP is expressed from the ROSA26 locus, I isolated splenocytes from either RRDR/+ mice (Fig. 17A) or RAGE-EG/+ mice (Constien *et al*., 2001) and incubated them with 2 µM HTNC (Fig. 17B) in DMEM w/o FCS in order

to delete the loxP-flanked DsRed-stop cassette. After 20h, an excess of B cell medium, supplemented with 20 µg/ml LPS was added and cells were kept in culture for further three days. Deletion efficiencies of the loxP-flanked regions of RRDR/+ and RAGE-EG/+ were determined by Southern blotting and were about 80% in both cases (Fig. 17C and D). No difference could be observed between HTNC transduced and non-transduced splenocytes in terms of cell number and expression of eGFP determined by FACS (Fig. 17E). From these cells, mRNA was isolated and subjected to RT-PCR using primers for amplification of transcripts of the endogenous transcript 1 of ROSA26, DsRed and eGFP (Fig. 18). All samples contained the correctly spliced transcript 1 as indicated by a band of 533 bp (Fig. 18A). Non-transduced splenocytes of RRDR/+ mice only show the DsRed transcript detected with the primers R512 and ROSA-R1 by PCR (Fig. 18B), whereas the eGFP transcript is only present in splenocytes transduced by HTNC, indicating the presence of a functional RRDR-eGFP allele (Fig. 18C). In addition, transduced splenocytes contained less DsRed mRNA compared to non-transduced splenocytes.

The residual DsRed mRNA present in RNA preparations of HTNC-treated RRDR splenocytes is presumably due to the remaining 20% cells retaining the RRDR-DsRed allele, in which Cre-mediated excision did not take place (Fig. 17C). All PCR products had the expected sizes and subsequent sequencing of the fragments confirmed their identity as properly spliced transcripts. In DsRed and eGFP transcripts, the start AUGs on the mRNA were those, which had been introduced.

Deletion of the DsRed-stop cassette *in vivo* by the Cre expressing mouse strains CD19-cre and *deleter*

RRDR-DsRed/+ mice were crossed to CD19-cre mice (Rickert *et al*., 1997) and *deleter* mice (Schwenk *et al*., 1995) in order to delete the loxP-flanked DsRed-stop cassette from RRDR-DsRed specifically in B lymphocytes or ubiquitously in all cells of the mouse, respectively. FACS analysis of splenocytes and fluorescence microscopy of all major organs revealed no difference between CD19-cre/+; RRDR/+ or *deleter*; RRDR-eGFP/+ and control mice (Fig. 19A and data not shown). Western blot analysis of lysates from all major organs using antibodies against eGFP did not reveal any significant eGFP expression (Fig. 19B). Only in lysates prepared from offspring of RAGE-EG/+ mice intercrossed with the *deleter* (RAGEΔ/+), which were

shown to express eGFP at high levels (Constien *et al*., 2001) and which were used as positive controls, the eGFP protein was detected by FACS (Fig. 19A) and Western blot (Fig. 19B), respectively. Nevertheless, eGFP mRNA could be detected by RT-PCR performed with RNA isolated from spleens of CD19-cre/+; RRDR/+ mice, *deleter;* RRDR-eGFP/+ mice and *deleter;* RAGE∆/+ mice (Fig. 20).

The fact that eGFP mRNA, but not protein could be detected in spleens of CD19-cre/+; RRDR/+ and *deleter-cre;* RRDR-eGFP/+ could mean that the mRNA expressed from the RRDR-eGFP allele is not translated efficiently. Alternatively, eGFP protein is not expressed by the RRDR-eGFP allele at levels sufficient for detection in FACS and Western blot. Ultimately, this suggests that, although the DsRed/eGFP indicator construct is functional in principle, fluorescent proteins are not expressed, or not at sufficient levels to be used as a convenient indicator for Cre-mediated recombination.

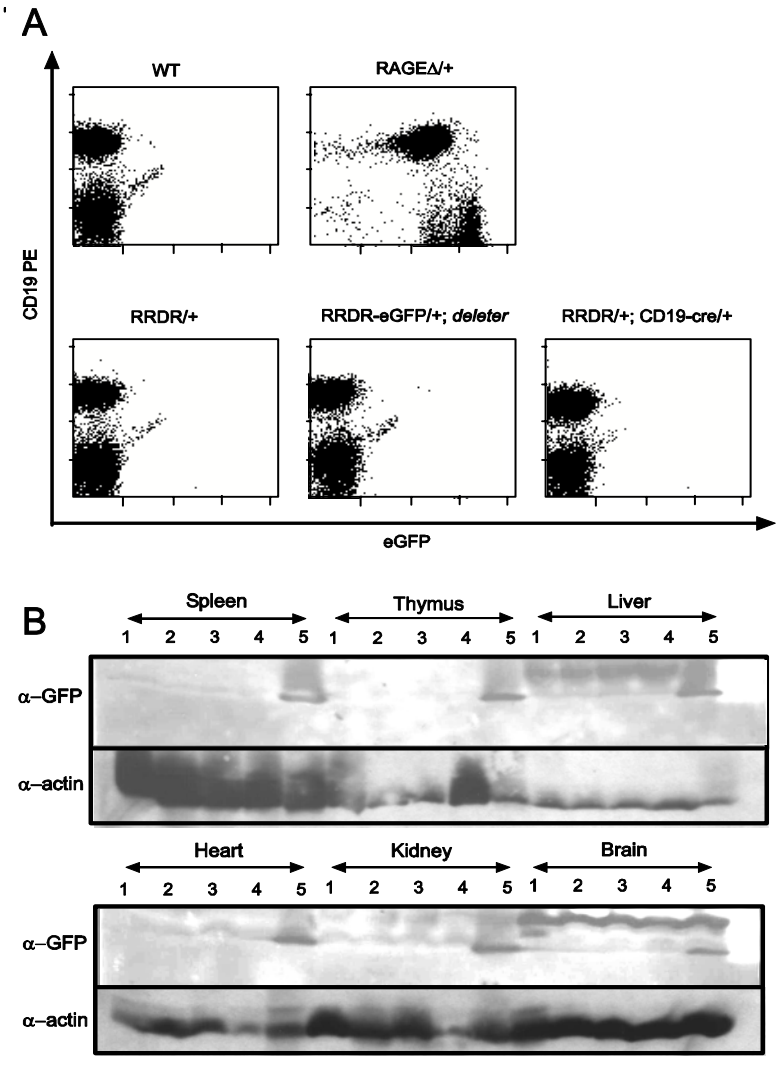


Fig. 19 Detection of eGFP in mice.

(A) FACS analysis of splenocytes isolated from the indicated mice. Cells were labelled with CD19 PE.

(B) Western blots of various organs of the indicated mice using α-eGFP antibody.

1 wild type
2 RRDR/+
3 RRDR-eGFP/+; *deleter*
4 RRDR/+; CD19-cre/+
5 RAGE∆/+; *deleter*

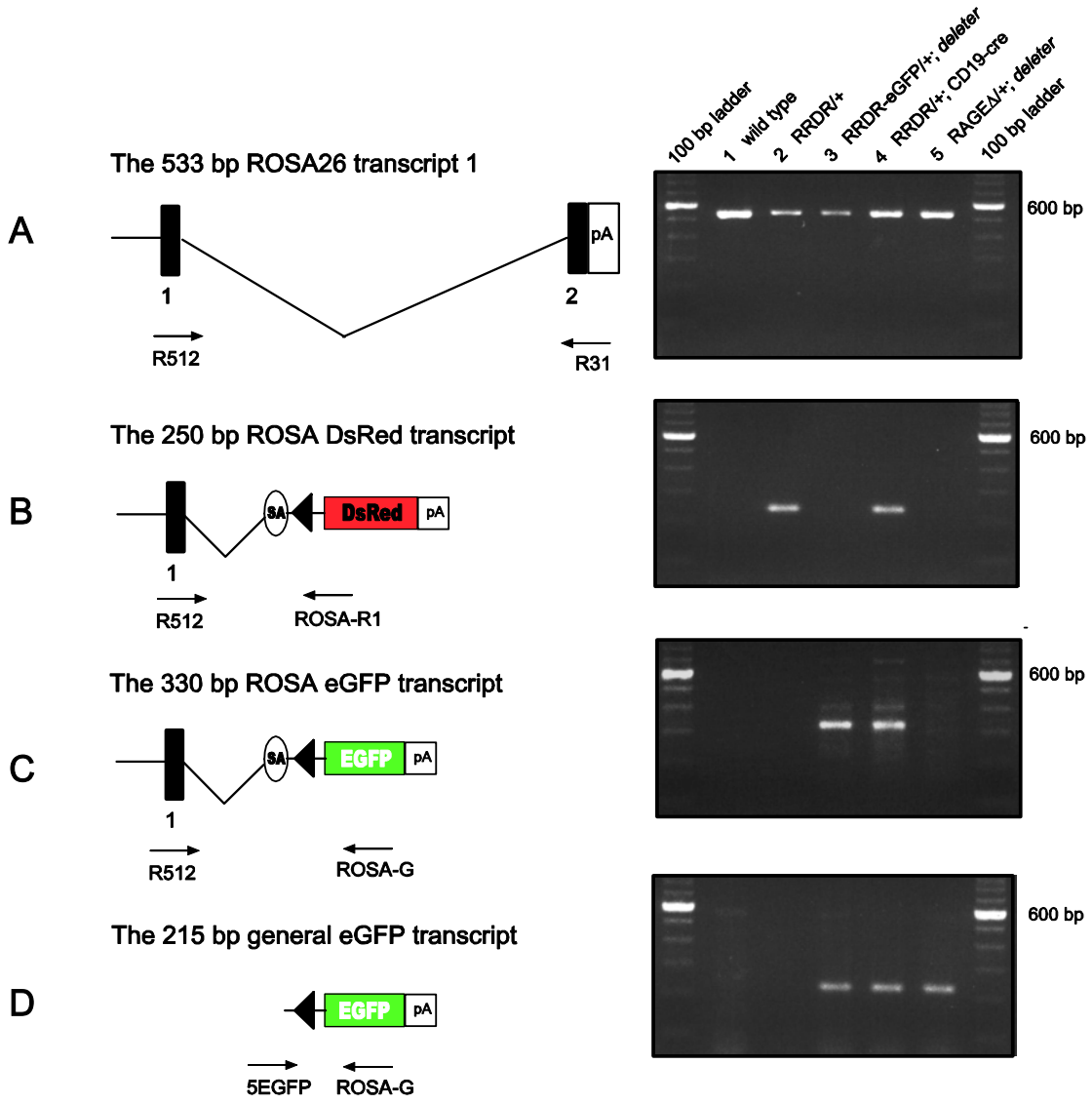


Fig. 20 mRNA expression of DsRed and eGFP in splenocytes.

Using RNeasy mini kit, mRNA from 10^6 splenocytes of different mice was prepared. RT reaction was carried out to produce cDNA, which was subsequently amplified in PCR with the primers indicated in (A) for ROSA26 transcript 1, in (B) for ROSA26 DsRed, in (C) for ROSA26 eGFP and in (D) for general eGFP.

3.3 The inducible Cre*PR system

Activity of various inducible Cre constructs

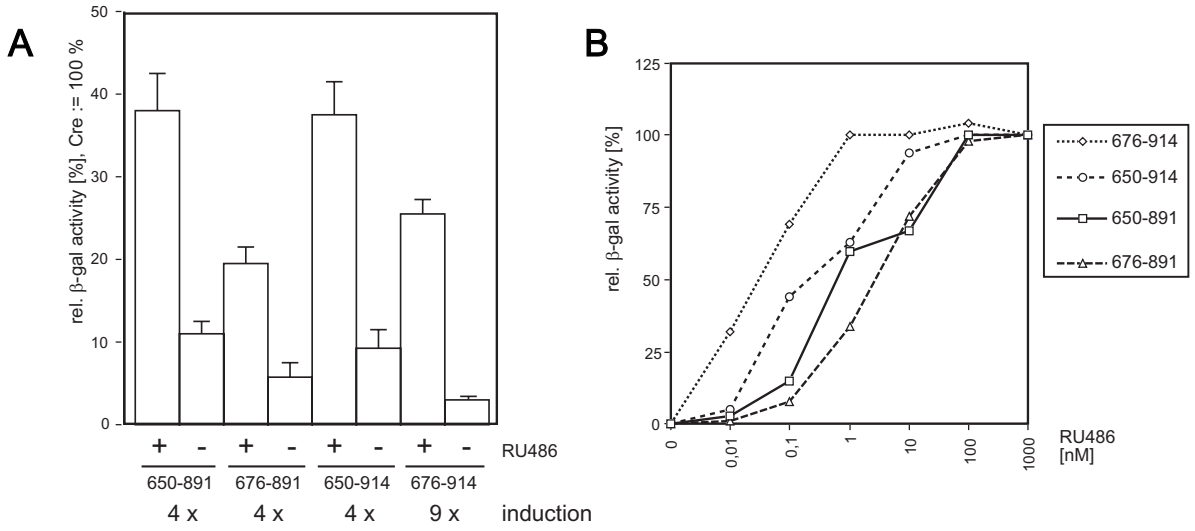
Cre activity of CrePR fusion proteins can be controlled by the synthetic steroid RU486 (Kellendonk *et al*., 1996). A fusion of NLS Cre (Cre containing the nuclear localisation signal NLS) to the ligand binding domain (LBD) of the progesterone receptor (PR) ranging from amino acid 641 to 891 has been used to achieve inducible recombination in brain (Kitayama *et al*., 2001) and heart (Minamino *et al*.,

2001). However, background activity in the absence of inducer occurs and administration of inducer leads only to incomplete recombination. The C-terminal deletion mutant PR891 (Fig. 5) is insensitive to the natural ligand progesterone, but it still has high affinity for the synthetic ligand RU486 (Kellendonk *et al.*, 1996).

In order to improve the inducibility of the system, new constructs were generated. Besides PR891 deletion mutants, I also cloned C-terminally extended versions ending with amino acid 914 of the PR, since previous reports suggested that PR914 mutants have a higher sensitivity to RU486 (Wang *et al*., 1997b; Xu *et al*., 1996). At the N-terminus of the PR-LBD, I introduced new truncations resulting in constructs starting with amino acids 650 and 676, respectively. These modifications are expected to decrease accessibility of the CrePR to proteases due to the shorter linker between Cre and PR (Fig. 6). Activity assays and dose response experiments were performed with constructs containing the new LBDs in combination with Cre lacking the first 18 amino acids, which were suggested to be devoid of structural and catalytic functions (Guo *et al*., 1997; Wierzbicki *et al*., 1987). Each construct was cloned into the same expression vector, pNN265E$^-$bPA, containing a CMV promoter, a synthetic intron, and a bovine polyadenylation signal (Fig. 6). In activity assays, $8x10^4$ CV1-5B cells were plated on a 6 well 24 h prior to transfection and then transfected with 1 µg of test plasmids together with 1 µg of pHD2-AP encoding alkaline phosphatase in order to determine transfection efficiencies. After 12 h, cells were split into two wells of a 12 well-plate and medium was supplemented either with 100 nM RU486 or with control medium. Three days later, cells were fixed and stained first for β-galactosidase activity.

β-galactosidase is expressed upon Cre-mediated excision of the loxP-flanked stop cassette of the reporter construct, which is stably integrated into the CV1-5B genome. Subsequently, cells were stained for alkaline phosphatase to monitor transfection efficiencies. In each well, blue (indicating β-galactosidase activity) and red (indicating alkaline phosphate activity) cells were counted in three different areas. The ratio of blue to red cells was determined and the ratio obtained with unmodified Cre was set to 100%. Each experiment was conducted at least in triplicate. Fig. 21 compares the activities of the CrePR constructs with different lengths of the LBDs. Constructs encoding LBDs PR676-914 or PR 676-891 fused to Cre show the lowest maximal activity in the presence of inducer, but also the lowest background activity. The Cre19PR650-891 and Cre19PR650-914 exhibit the same maximal activity of

about 40% of authentic Cre and background activities of about 10% in the absence of RU486. The resulting 'induction factors' are shown in Fig. 21A. The term 'inducibility' will be used to describe the fold increase of Cre activity after addition of inducer relative to the background activity in the absence of inducer.



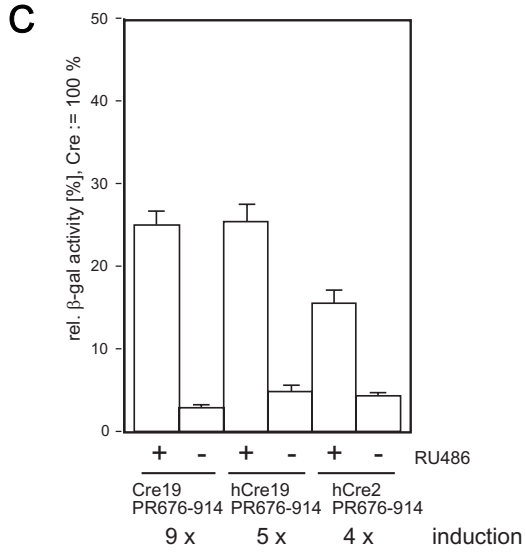


Fig. 21 Quantitative analysis of recombinase activities of various CrePR fusion proteins.

(A) Reporter cells were transfected with different Cre19PR expression vectors and cultured in the presence (+) or absence (-) of 100 nM RU486. Three days later, the relative β-galactosidase activity was determined as described in Materials and Methods. The columns show mean values of at least three different transfections with standard deviations indicated by vertical lines. (B) Dose response analysis of different Cre19PR fusions. Reporter cells were transfected with the indicated constructs and cultured in the presence of various concentrations of RU486. No β-galactosidase activity was detected in the presence of 1 µM progesterone. (C) Comparisons of activities of different Cre fused to PR676-914. The mean value from six different transfections are depicted with standard deviations indicated by vertical lines. Cre19, truncated Cre lacking the first 18 amino acids; hCre, humanized Cre; Cre2, full length Cre.

Dose response assays demonstrated that the two PR914 versions retain a higher affinity to the inducer. In these experiments, 5 µg of pNNCre19PR676-914, pNNCre19PR676-891, pNNCre19PR650-914 and pNNCre19PR650-891 were transfected in $8x10^5$ CV1-5B fibroblasts plated on a 9 cm dish. After 12 h, cells were trypsinized and split into seven wells in a 6 well-plate format, containing different concentrations of RU486. Seventy two hours after transfection, cells were stained for

β-galactosidase and counted as described above. For each CrePR construct the β-galactosidase activity obtained with the maximal concentration of inducer (1 µM) minus the background activity obtained in the absence of inducer was set to 100%. β-galactosidase activities obtained with lower RU486 concentrations were expressed as proportion (percent) of this maximal activity for each individual construct. Each experiment was performed in duplicate and mean values are given in Fig. 21B. The CreLBD construct with PR676-914 has a half maximal activity (C_{50} value) of 0.04 nM, whereas the other constructs used in this analysis display C_{50} values that are at least one order of magnitude lower.

Based on these results, further investigations concentrated on the construct displaying the lowest C_{50} value (concentration of half maximal activity) and the highest inducibility, namely Cre19PR676-914. This construct contains a truncated Cre recombinase lacking the first 18 amino acids at the N-terminus. In order to investigate the influence of both N-terminal truncation and optimization of codon usage for Cre, PR676-914 was fused to a Cre encoding cDNA which codon usage was improved for the expression in mammalian cells (Shimshek *et al*., 2002), designated hCre below, encoding either full length (hCre2) or a truncated Cre lacking the first 18 aa (hCre19). A comparison of the recombinase activities of these constructs with the corresponding truncated phage CrePR fusion (Cre19PR676-914) relative to unmodified Cre shows that both truncated Cre19PR variants containing either hCre or phage Cre display higher recombinase activity (26% of Cre) than the full length hCre (16%) in the presence of inducer (Fig. 21C). The background activity is slightly higher in case of the hCre constructs (5%) as compared to the construct originating from phage Cre (3%).

Analysis of aberrant splice events

Since the CrePR system is intended to be used as a genetic switch in mice, it has to be taken into account, that aberrant splicing of the CrePR-encoding mRNA may occur depending on the sequence context of the integrated construct. In order to check the presence of cryptic splice sites in the CrePR constructs, computer aided analysis of different CrePR mRNAs was performed using the programs GeneFinder and Splice Site Score Calculation. According to this analysis, wild type Cre contains four distinct sites displaying similarity to the consensus sequence of splice donors

(Fig. 22). Two of these cryptic splice donors are located at the 3' end of the Cre gene. The PR-LBD fusion partner contains at least three sequences within the region coding for PR650-914 that show high similarity to the splice acceptor (SA) consensus sequence. SA (8.3) and SA (11.2) were shown to be used in alternatively spliced isoforms of the PR (Balleine *et al*., 1999; Leygue *et al*., 1996). In RT-PCR experiments, a cryptic spliced CrePR product could be isolated, in which splicing occurred from SD (5.8) to SA (11.2) (Fig. 22) (Wunderlich, 1999). This cryptically spliced mRNA can result in a C-terminally truncated translation product because of a premature stop codon.

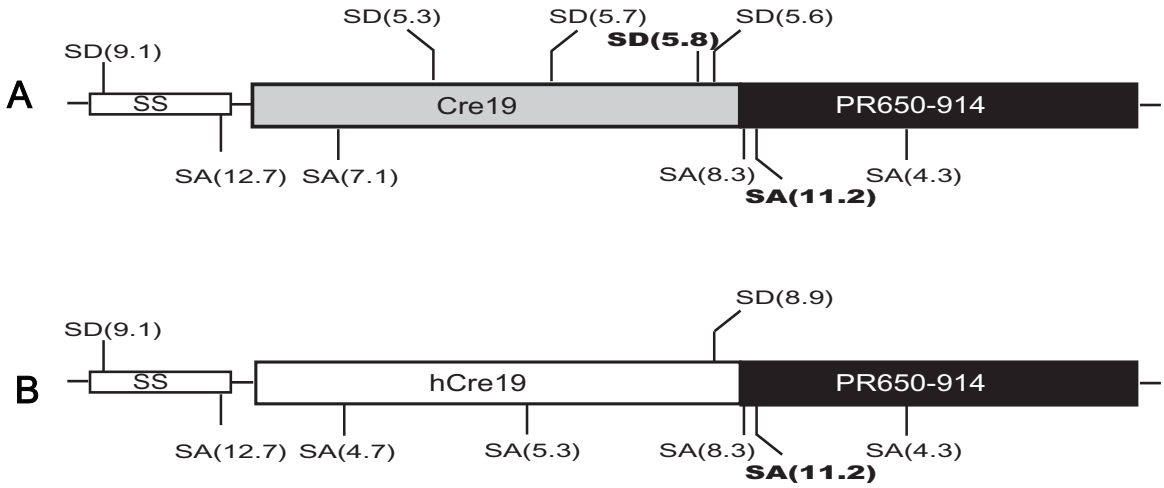


Fig. 22 Map of cryptic splice sites within mRNAs of (A) Cre19PR650-914 and (B) hCre19PR650-914.

Potential splice donors (SD) and acceptors (SA) are shown together with calculated scores in parentheses, which express how well the splice sites fit to the consensus sequence. Cryptic splice sites were identified by using the program GeneFinder (http://dot.imgen.bcm.tmc.edu:9331/gene-finder/gf.html). The corresponding scores were calculated by the program splice site score calculation (http://www2.imcb.osaka-u.ac.jp/splice/score.html). For example, a 100% match to the mammalian 3' splice site corresponds to a score of 14.2. A perfect 5' splice site would have a score of 12.6. The mean scores of the 5' and 3' splice sites in constitutive exons were 8.1 and 7.9, respectively. Sites that proved to function in a physiological context are shown in bold. SS, splice substrate.

I wanted to prevent that unwanted splicing which could result in truncated, constitutively active forms of CrePR due to a partial loss of the PR-LBD. Three of the potential splice donor sites in the hCre gene had been eliminated by introducing mammalian codon usage (Shimshek *et al*., 2002). However, one cryptic donor site was still present in the hCre gene (SD (8.9) in Fig. 22B). Since a silent mutation is

insufficient to unambiguously suppress the risk of splicing at this position, an amino acid substitution was introduced at the codon 336 of hCre by mutation of GTG (Val) to GCG (Ala) (Fig. 22). This mutation is expected to completely prevent splicing at this position, since the indispensable GT for splice donors is abolished (Fig. 23).

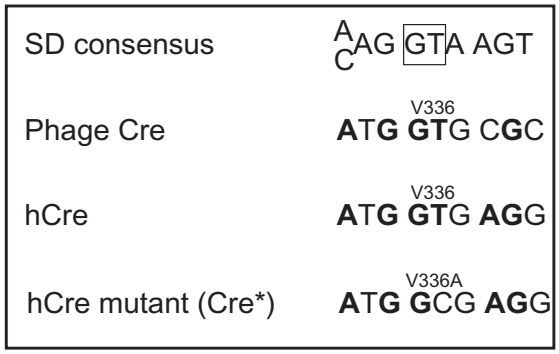


Fig. 23 Alignment of codons 335-337 of phage Cre, humanized Cre and its V336A mutant compared to the consensus sequence of splice donor sites.

The boxed GT indicate those nucleotides which are critical for splicing. Bases in bold indicate identity to the consensus sequence.

Activity of the Cre*PR constructs

The hCreV336A, designated Cre* below, was fused to either the LBD of PR676-914 or the LBD of PR650-914 and the inducibilities were compared in activity tests to the corresponding phage Cre constructs consisting of Cre19PR676-914 and Cre19PR650-914. Unmutated CrePR676-914 displays a recombinase activity of 2.5% in the absence of RU486, whereas the mutant Cre*PR676-914 exhibits at least a 10-fold decreased background activity of 0.2% (Fig. 24), close to the detection limit. No significant difference was observed with respect to the maximal activity of both PR676-914 constructs (26%), irrespective of the mutation. Due to the reduced background activity of Cre*PR676-914, it has an inducibility of 128-fold, compared to 10-fold for CrePR676-914.

As demonstrated in Fig. 21A, the maximal activity of the Cre19PR650-914 construct is significantly higher than that of the N-terminally truncated Cre19PR676-914 version, but this is accompanied with a higher background activity. However, by introducing the V336A mutation into Cre*PR650-914 construct, the background activity was reduced at least 25-fold to a negligible level. The maximal activity of the Cre*PR650-914 fusion protein in the presence of RU486 was 40% of that of authentic Cre, enhancing the inducibility to 200-fold (Fig. 24) (Wunderlich *et al*., 2001).

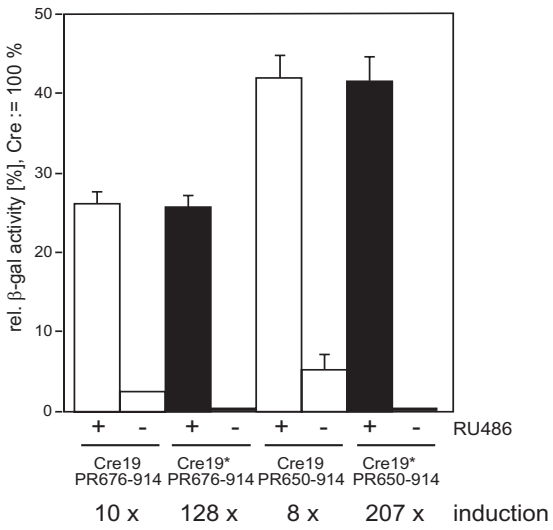


Fig. 24 Recombinase activities of various CrePR fusion proteins containing a V336A mutation within Cre in comparison to corresponding unmutated constructs.

White columns correspond to unmutated phage Cre and black columns represent mutated CreV336A. CV1-5B reporter cells were transfected with different Cre19PR expression vectors and cultured in the presence (+) or absence (-) of 100 nM RU486. Three days later the relative β-galactosidase activity was determined as described in Materials and Methods. The columns show mean values of three different transfections with standard deviations indicated by vertical lines.

In order to compare the previously described CrePR1 system (Kellendonk *et al*., 1996) with the newly generated Cre*PR system, the CrePR1 open reading frame was cloned into the pNN265E^{-}bpA vector. A side by side comparison of the two systems is shown in Fig. 25. Cre*PR exhibits a dramatically reduced background activity as compared to CrePR1, whereas both constructs show the same maximal activities of 50% in the presence of RU486 (Fig. 25A). Dose response analysis revealed the increased sensitivity of PR650-914 to RU486, due to the elongation of the C-terminus of PR-LBD. The C_{50} value of Cre*PR is at least 10 times lower than that of CrePR1 (Fig. 25B).

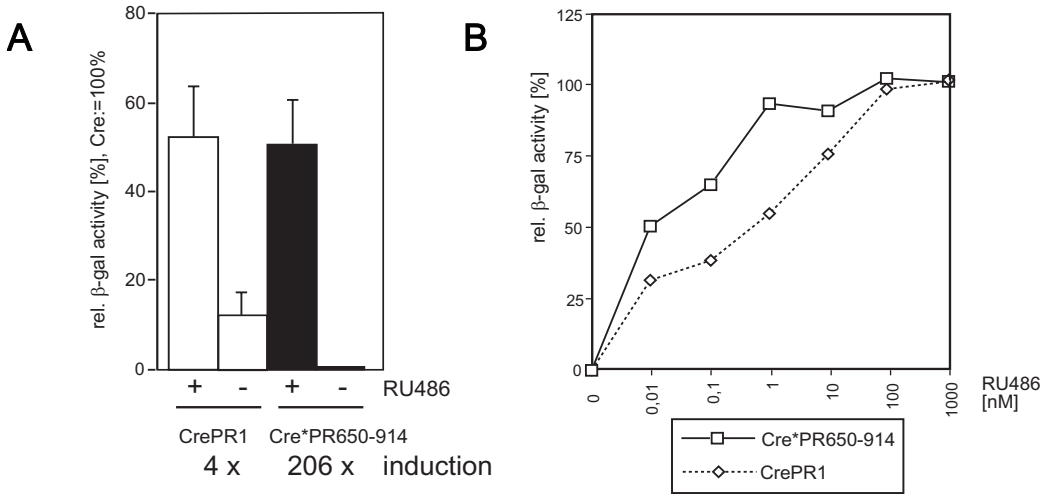


Fig. 25 Comparative analysis of inducible Cre recombinase activities of the improved Cre*PR system and the previously published CrePR1 system.

(A) CV1-5B reporter cells were transfected with the different CrePR1 or Cre*PR650-914 expression vectors and cultured in the presence (+) or absence (-) of 100 nM RU486. Three days later the relative β-galactosidase activity was determined as described in Materials and Methods. The columns show mean values of three different transfections with standard deviations indicated by vertical lines. (B) Dose response analysis of CrePR1 and Cre*PR650-914. Reporter cells were transfected with the indicated constructs and cultured in the presence of various concentrations of RU486.

Before using the Cre*PR system for the generation of inducible mouse strains, I attempted to completely prevent cryptic splicing of Cre* to PR-LBD. In order to achieve this, a version of the PR-LBD beginning with amino acid 678 was designed in which the amino acids S676 and P677 were mutated to glycines. Thereby, the SA (11.2) of the PR shown in Fig. 22 is mutated such, that it does not exhibit any similarity to the consensus of splice acceptors any more. Comparisons of Cre*PR676-914, Cre*PR650-914 and Cre*PR678-914 with the corresponding phage Cre constructs in activity assays are shown in Fig. 26. The data obtained with Cre*PR676-914 and Cre*PR650-914 shown in Fig. 24 were confirmed, whereas the newly generated Cre*PR678-914 shows the same properties as Cre*PR650-914: A dramatically reduced activity in the absence of inducer and a similar activity in the presence of RU486. Accordingly, the Cre*PR678-914, designated Cre*PR below, was used to establish the Cre*PR system in mice.

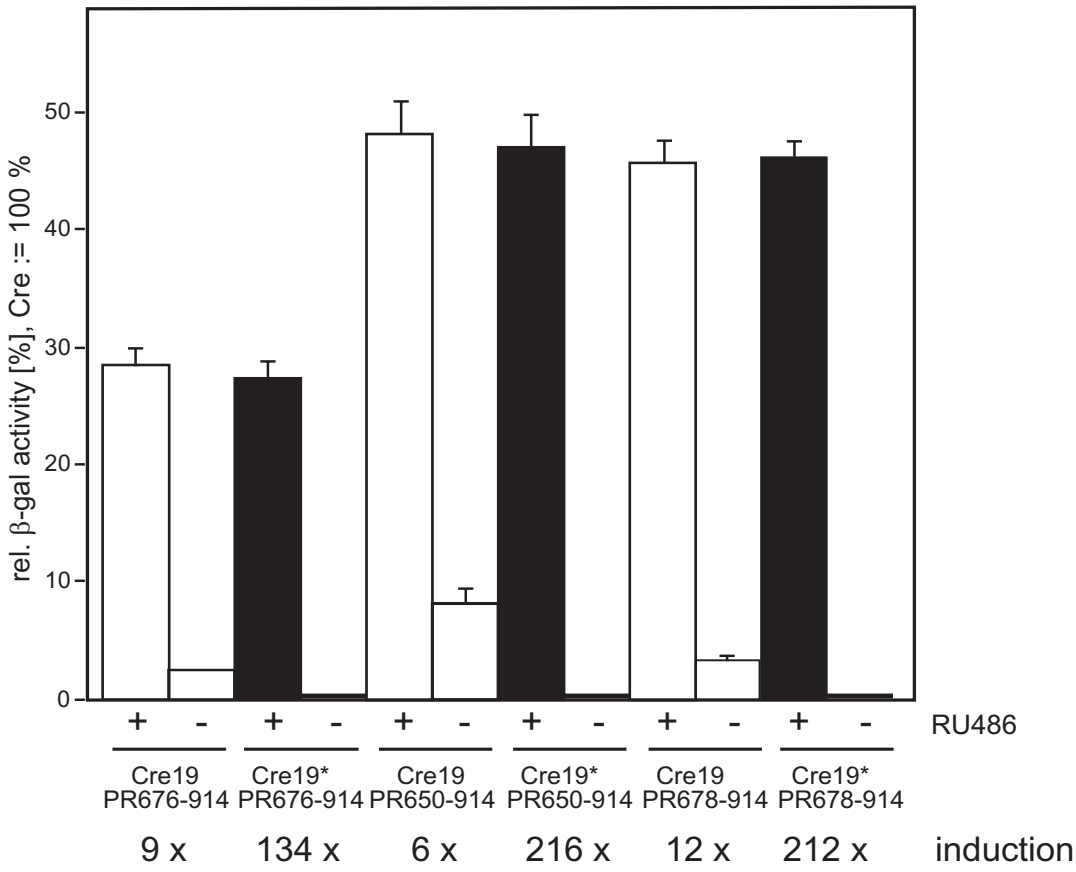


Fig. 26 Comparison of recombinase activities of different Cre*PR fusion proteins with unmutated constructs.

White columns correspond to unmutated phage Cre, black columns represent mutated Cre*PR constructs. CV1-5B reporter cells were transfected with different Cre19PR expression vectors and cultured in the presence (+) or absence (-) of 100 nM RU486. Three days later the relative β–galactosidase activity was determined as described in Materials and Methods. The columns show mean values of three different transfections with standard deviations indicated by vertical lines.

3.4 Targeted insertion of Cre*PR into ubiquitously expressed loci

In order to achieve ubiquitous, RU486 dependent deletion of loxP-flanked target genes in mice, three different approaches were followed: Cre*PR was inserted into the HPRT locus under the control of either the CAGGS or the EF1α promoter, or into the ROSA26 locus under the control of the endogenous ROSA26 promoter.

3.4.1 Knock-in of CAGGS- and EF1α-driven Cre*PR into the HPRT locus of RRDR ES cells

Since Cre*PR678-914 (designated Cre*PR) showed the best properties in *in vitro* experiments, it was used for generating constructs allowing the introduction of the transgene into defined loci of the mouse genome. According to the original strategy depicted in Fig. 10, Cre*PR constructs expressed under the control of the CAGGS or the EF1α promoter were targeted into the HPRT locus of RRDR ES cells. Successfully targeted ES cells were identified on day 9 of HAT selection and picked as single clones. Subsequently, HT medium was applied for the next 5 days to dilute out aminophterin, since it is thought to interfere with proper blastocyst development. Subsequently, the cells were cultured in normal ES cell medium. Southern blot analysis of StuI digested genomic DNA using an internal probe against the Cre* part of Cre*PR revealed correctly targeted clones and excluded random integration sites of the transgene (Fig. 27). Targeting frequencies were always 100%, i.e. each surviving ES cell colony arose from a homologous recombinant. When 10^7 ES cells were transfected with 30 µg of the targeting vector, usually 20 to 30 colonies were observed after selection. The same proportion was obtained, when 10 times less cells were transfected with 10 times less DNA, resulting in 2 to 3 colonies per transfection.

Analysis of CAGGS- and EF1α-driven Cre*PR in RRDR ES cells

In order to test RU486 inducible recombinase activity in RRDR ES cells, ES cell clones containing homologous integrations of the RRDR-DsRed allele and CAGGS Cre*PR or EF1α Cre*PR were treated with 100 nM RU486 for 16 days as detailed in Fig. 28. Southern blotting using a probe to detect the RRDR indicator showed RU486 inducible Cre activity in the CAGGS Cre*PR clone, but not in that clone in which Cre*PR was expressed from the EF1α promoter (Fig. 28B, C). No recombination was observed in both cases in the absence of inducer. To detect the Cre*PR protein in

ES cells, Western blot analysis was performed with whole ES cell lysates using a polyclonal anti-Cre rabbit serum. In lane 1 of Fig.28 D, the lysates from clone 9 of the CAGGS Cre*PR were loaded and a specific band of 67 kDa was observed, whereas no significant signal was detected in EF1α Cre*PR lysates (lane 2).

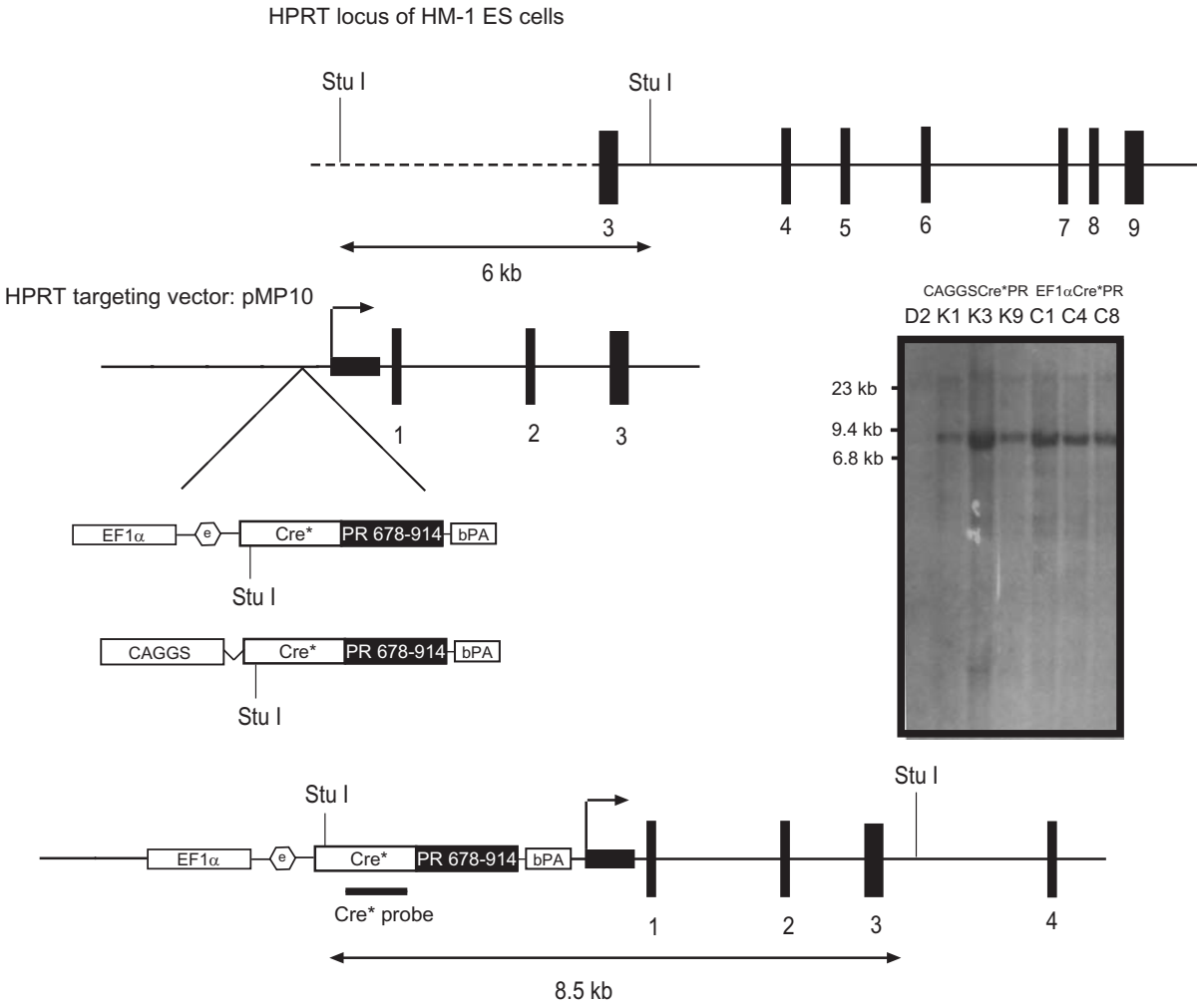


Fig. 27 Knock-in of CAGGS Cre*PR and EF1αCre*PR into HPRT locus of RRDR ES cells.

The HPRT locus of HM-1 ES cells contains a spontaneous deletion of 50 kb including the promoter and the first two exons of the HPRT gene. With the targeting vector (pMP10) the HPRT gene is complemented. This targeting event can be selected with HAT medium. Upstream of the HPRT promoter, the Cre*PR either driven by the CAGGS or the EF1α promoter was inserted. Homologous recombination can be confirmed in Southern blots of StuI digests. A probe against Cre* was used and in correctly targeted clones appeared a 8.5 kb band. Since StuI cuts only once in the construct, also random integrants can be excluded from analysis. Size marker was the λ-Hind from Gibco.

In order to examine whether high degrees of recombination of the DsRed-stop cassette can be achieved with the CAGGS Cre*PR, RRDR-DsRed/CAGGS Cre*PR ES cells were treated with 100 nM RU486 for 22 days. Cells were trypsinized every second day to keep the cells undifferentiated and to synchronize the cell cycle. Southern blot analysis revealed absence of Cre-mediated recombination in samples without RU486, but up to 81% deletion of the RRDR indicator after 22 days (Fig. 29C).

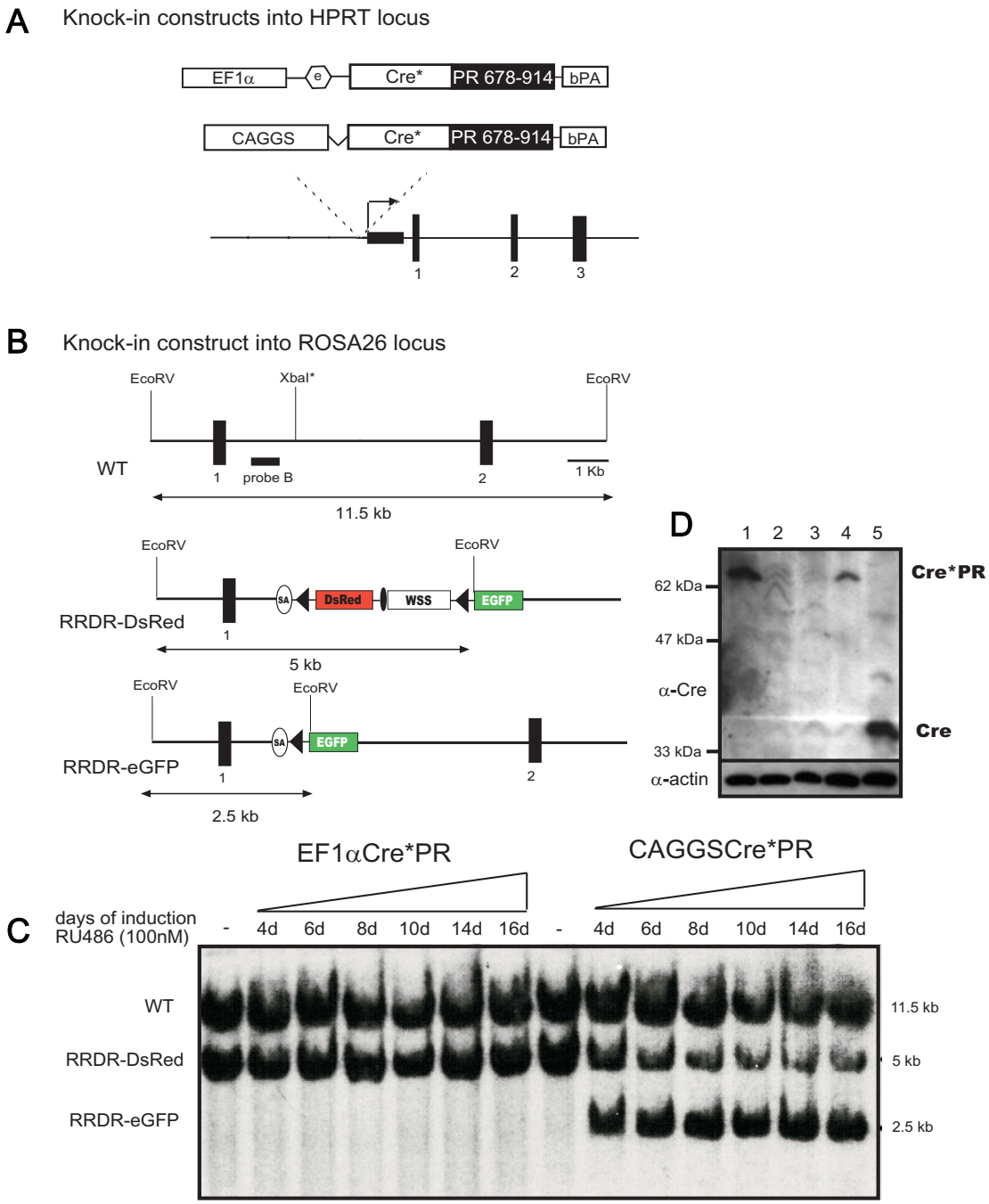


Fig. 28 Cre*PR in RRDR ES cells.

(A) Knock-in constructs in the HPRT locus of RRDR ES cells. Cre*PR is either expressed from EF1α or CAGGS promoter.
(B) Knock-in construct of the RRDR Cre indicator in the ROSA26 locus. The locus is shown before (RRDR-DsRed) and after Cre-mediated excision of the DsRed-stop cassette (RRDR-eGFP) with indicated sizes of DNA fragments.
(C) $2x10^5$ twice targeted ES cells of EF1α Cre*PR and CAGGS Cre*PR were treated with 100 nM RU486 for the indicated days. Genomic DNA of samples were digested with EcoRV and used in Southern blot against the indicator as shown in B.
(D) Western blot of cell lysates from ES cells of CAGGS Cre*PR (1), EF1αCre*PR (2), wild type (3), ROSACre*PR (4) and spleen of CD19-cre-/+ (5). Sizes of proteins were estimated by comparison to a standard protein marker from NEB.

Fig. 29D shows an experiment, which should address the question, whether there is a difference in deletion efficiency when adding the inducer only once or four times. RU486 at a concentration of 1000 and 100 nM, respectively, was added only once or

four times to the cells by daily exchanging the medium and cells were kept in culture for the period of four days. Southern blotting using a probe to detect the Cre indicator revealed that Cre-mediated excision was influenced in a dose dependent manner of RU486. Also, Cre*PR activity was increased when the inducer was added four times with 1000 nM and 100 nM, respectively (Fig. 29D).

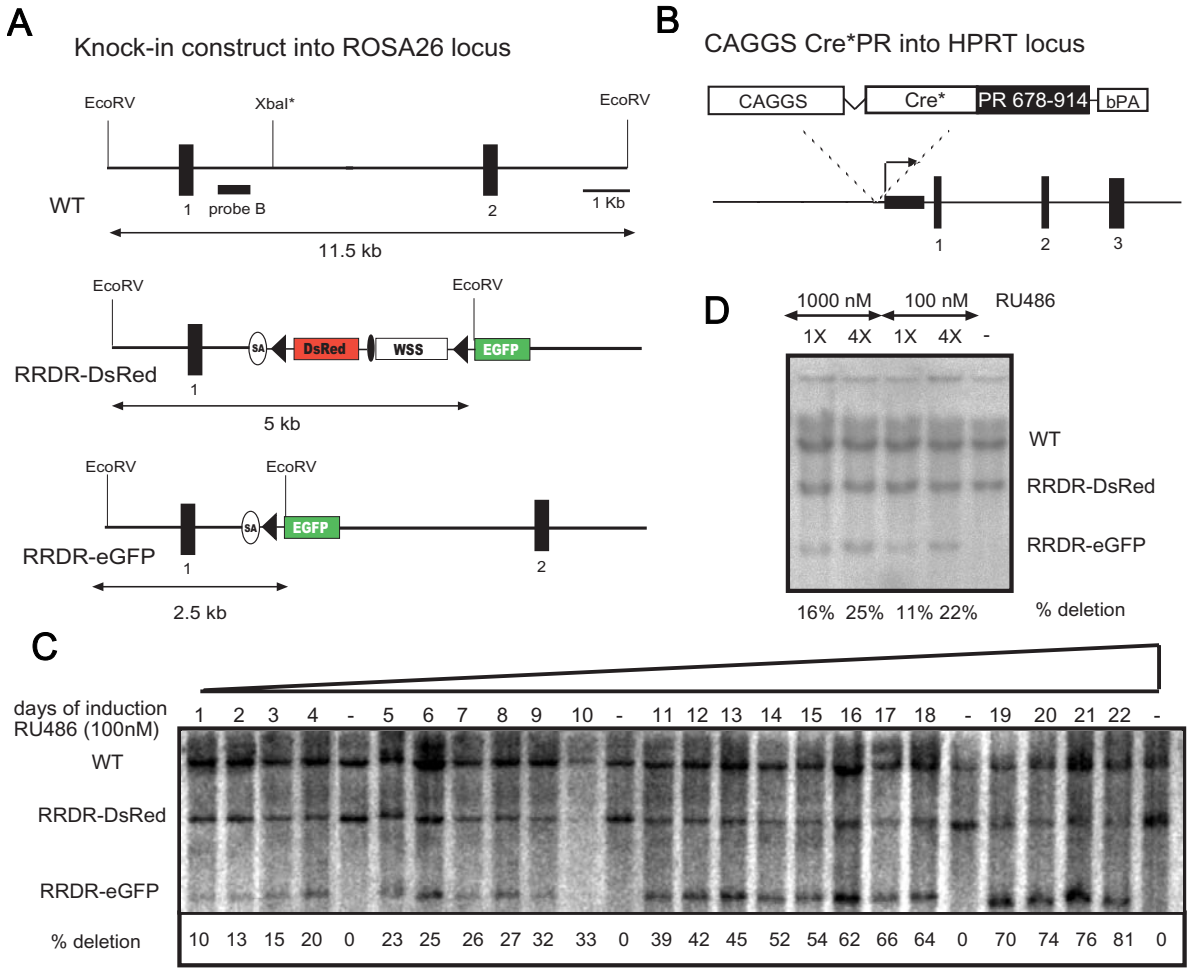


Fig. 29 Kinetics of CAGGS Cre*PR in RRDR ES cells.

ES cells were targeted twice into the ROSA26 locus (A) and the HPRT locus (B). (A) Screening strategy for detection of the WT, RRDR-DsRed and RRDR-eGFP alleles in Southern blots. (B) Knock-in construct into HPRT locus. (C) $2x10^5$ twice targeted ES cells were treated with 100 nM RU486 for the indicated days. Deletion efficiencies are depicted below. (D) Dose dependence of Cre*PR on RU486. $2x10^5$ cells were treated either with 1000 nM or 100 nM RU486 one or four times, respectively. Deletion efficiencies are depicted below and were quantified with Fuji Bias reader.

Dose responses of CAGGS Cre*PR in RRDR ES cells

In order to investigate the hormone inducibility of Cre*PR, assays were performed with increasing doses of RU486 and a new progesterone antagonist Org31710 (Kloosterboer *et al*., 1994) kindly provided by G. Esposito (Organon, Oss, the Netherlands). The latter hormone acts similar as RU486, but differs with respect to

structure and pharmacokinetics (Kloosterboer *et al*., 1994). Also, Org31710 is much better soluble in the vehicles trioctanoin and sesame oil, which have to be used for the applications in mice. $2x10^5$ twice targeted ES cells of clone K9 (CAGGS Cre*PR in HPRT, RRDR in ROSA26) were plated on 12 wells and inducer (RU486 or Org31710) was applied once at the indicated concentrations (Fig. 30B, C). Cells were grown for three days without changing the medium, then harvested and analyzed by Southern blot for the RRDR Cre indicator using a probe to detect eGFP on EcoRI digested DNA. In this case, the smaller 2.2 kb band indicates the loxP-flanked Cre indicator RRDR-DsRed, whereas the appearance of the bigger 5.4 kb band shows deletion of the DsRed-stop cassette indicating the RRDR-eGFP (Fig. 30A). In this experiment, RU486 could induce higher Cre*PR activity than Org31710, as assessed by excision of the loxP-flanked DsRed-stop cassette from the RRDR-DsRed indicator in inducer-treated ES cells. In order to test the activity of RU486 *in vivo*, 8 week old mice were *i.p.* injected once with 2.5 mg RU486 either resuspended in trioctanoin or sesame oil. One and four hours after injection, blood was taken from the tail vein and serum was prepared. A 1/50 dilution of the sera was applied to RRDR/CAGGS Cre*PR ES cells and maintained on the cells for three days. Subsequently, cells were harvested and genomic DNA was prepared and analyzed by Southern blot as described above. Excision efficiencies of the RRDR indicator are shown in Fig. 30C. The sera taken from mice after 1 h induce Cre*PR activity corresponding to 1 µM of RU486. Sera taken 4 h after injection contained less, but still considerable RU486 activity (Fig. 30B, C).

Since the CAGGS Cre*PR construct worked in ES cells, two clones (K1 and K9) were chosen for injection into C57BL/6 blastocysts. Clone K1 gave 11 chimeras with 20 to 80% chimerism, clone 9 gave two female chimeras with 30% and 50% chimerism, respectively, as indicated by coat colour. Chimeras generated from clone K1 did not transmit the transgene through the germ line at all, whereas both chimeras generated from clone K9 did. All male progenies from this breeding with the right coat colour (in total four animals) carried the transgene as determined by Southern blot and/or PCR. None of the female offspring carried the transgene. Since the CAGGS Cre*PR construct was inserted into the X-linked HPRT locus, the male transgenic offspring had to be backcrossed again two times to obtain experimental male mice with the desired genotype.

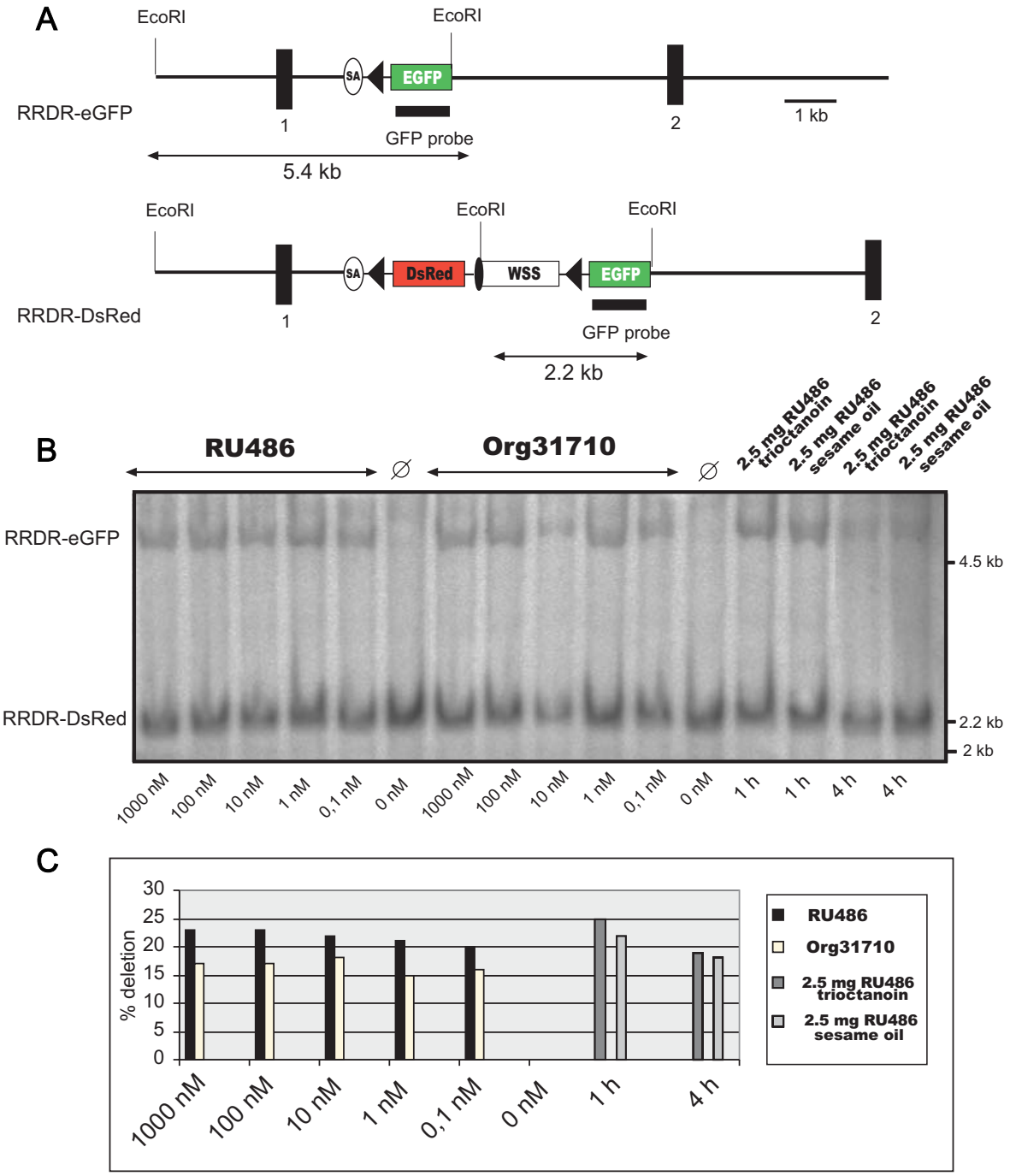


Fig. 30 Dose response assays of CAGGS Cre*PR in RRDR ES cells.

(A) Southern blot screening strategy to distinguish between RRDR-DsRed and the excised RRDR-eGFP allele. Using a probe against eGFP, the 2.2 kb band indicates the RRDR-DsRed and the 5.4 kb band the RRDR-eGFP allele.

(B) Southern blot of twice targeted ES cells (CAGGSCre*PR in HPRT locus; RRDR in ROSA26 locus) treated with increasing doses of RU486 and Org31710. The four lanes on the right are from sera of mice injected *i.p.* with 2.5 mg RU486 dissolved in trioctanoin or sesame oil. Sera were taken at 1 h and 4 h after injection and applied to twice targeted ES cells for three days. Size marker belongs to the λ-Hind from Gibco.

(C) Quantification of excision efficiencies was performed with Fuji Bias reader. Black bars correspond to samples treated with RU486; white bars show Cre*PR activities in the presence of Org31710. Dark grey bars indicate recombination efficiencies achieved with serum of mice injected with 2.5 mg RU486 in trioctanoin and light grey bars correspond to serum of mice injected with 2.5 mg RU486 in sesame oil.

3.4.1.1 Analysis of chimeras generated from RRDR/CAGGS Cre*PR ES cells

Chimeras were generated from ES cell clones K1 and K9, which were targeted first in the ROSA26 locus with the DsRed/eGFP Cre indicator and then in the HPRT locus with the inducible Cre*PR (Fig. 31A and B). This allows to test the ubiquitously expressed Cre*PR driven by the CAGGS promoter *in vivo*. Chimeras showing the highest degree of coat colour chimerism were fed for 5 consecutive days with 5 mg RU486 dissolved in trioctanoin, or injected *i.p.* with 5 mg RU486, and subsequently kept under standard conditions for 5 additional days. Mice were sacrificed and DNA was prepared from all major organs indicated in Fig. 31C.

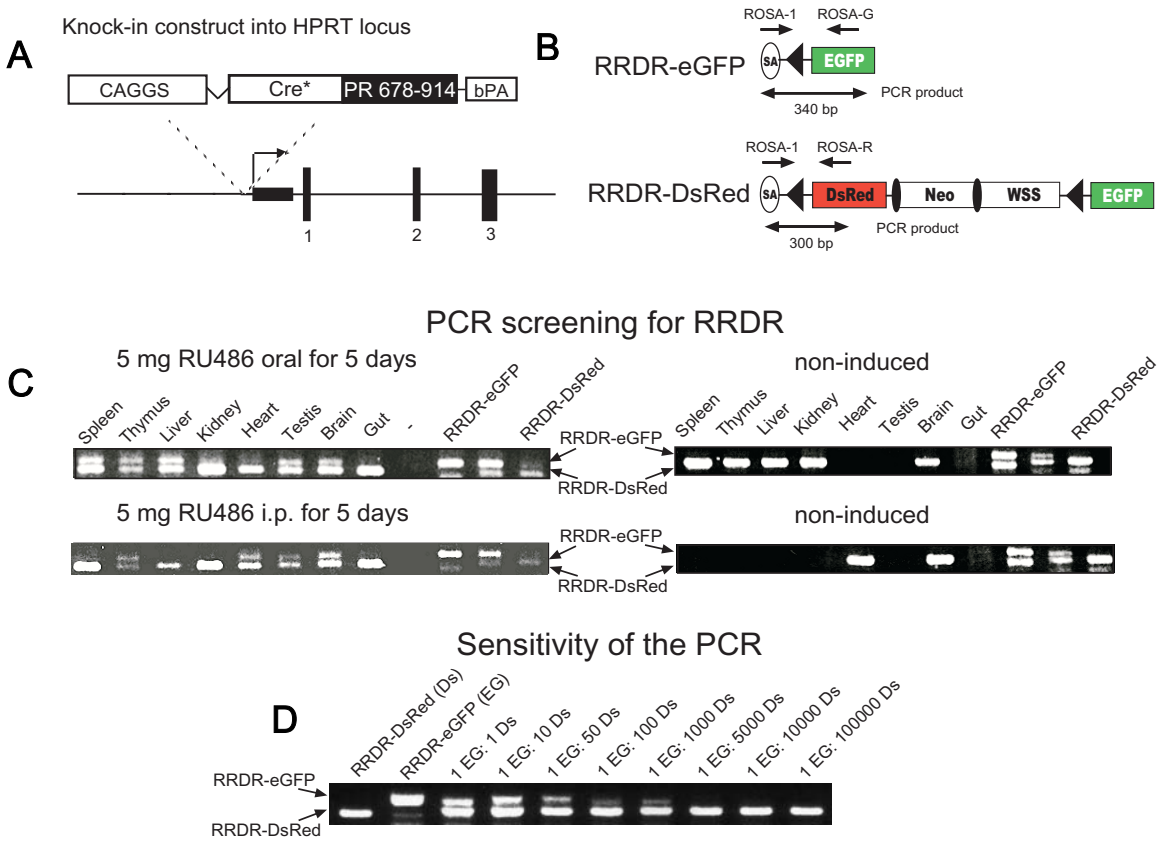


Fig. 31 Analysis of chimeras generated from RRDR/CAGGS Cre*PR ES cells.

ES cells were targeted twice with the constructs shown in (A) and (B). Subsequently, they were injected into C57BL/6 blastocyst and chimeric mice were generated. (B) PCR screening strategy to distinguish between RRDR-DsRed (300 bp) and excised RRDR-eGFP (340 bp) alleles. (C) Chimeras were either fed or injected *i.p.* with 5 mg RU486 in trioctanoin for 5 consecutive days. Five days later, mice were sacrificed and genomic DNA was prepared from indicated organs and used in PCR experiments. (D) The sensitivity of the PCR screening was determined by dilutions of RRDR-eGFP (EG) DNA with RRDR-DsRed (Ds) DNA at indicated concentrations.

Southern blotting to detect recombination events at the Cre indicator was not successful, even when a probe against the internal eGFP was used (data not shown). This indicates that the proportion of ES cell-derived cells in the chimeras was

not as high as expected from the coat colour observation. Therefore, deletion efficiencies were analyzed by a PCR approach, which was designed to detect the loxP-flanked RRDR-DsRed allele and the RRDR-eGFP allele of the indicator (Fig. 31B). When using a three primer approach with the primer ROSA-1 in combination with the primers ROSA-R and ROSA-G (Table 4 under 2.2.8), it is possible to distinguish between the RRDR-DsRed (300 bp) and the excised RRDR-eGFP (340 bp) alleles of the indicator on agarose gels. A band representing the RRDR-eGFP allele, resulting from Cre-mediated excision of the loxP-flanked DsRed-stop cassette from RRDR-DsRed, could only be amplified from mice which had been treated with the inducer, while untreated mice showed only the band amplified from the loxP-flanked RRDR-DsRed allele (Fig. 31C). In order to test the sensitivity of this PCR approach, DNA of tails from RRDR-eGFP/+ and RRDR-DsRed/+ mice was prepared, and DNA from both preparations was mixed in various ratios. Decreasing dilutions of RRDR-eGFP/+ with RRDR-DsRed/+ DNA were used in PCR experiments to determine the detection limit of the RRDR-eGFP. One RRDR-eGFP allele can be detected in thousand RRDR-DsRed alleles in this three primer PCR. Therefore, the absence of the PCR product of the RRDR-eGFP allele from PCR reactions of RRDR/CAGGS Cre*PR chimeras which were not treated with inducers indicates the absence of background recombination, i.e. the ‘tightness’ of the Cre*PR system in this experiment (Fig. 31D).

3.4.1.2 Analysis of the CAGGS Cre*PR system *in vivo*

In order to further determine the *in vivo* inducibility of the CAGGS Cre*PR strain with a different indicator mouse strain, female CAGGS Cre*PR/+ mice were intercrossed with male mice of the genotype RAGE-EG-/- containing an eGFP indicator construct for Cre in the RAGE locus (Constien *et al*., 2001) (Fig. 32A). Since the CAGGS Cre*PR transgene is located on the X-chromosome, only male offspring were analyzed by PCR for the presence of the CAGGS Cre*PR transgene and used for further experiments. Four week old double transgenic males were *i.p.* injected with 2.5 mg RU486 or 2.5 mg Org31710 daily for five days. Five days after the last injection, mice were sacrificed and organs were analyzed by fluorescence microscopy and flow cytometry. Only in positive controls (RAGEΔ/+; *deleter*) widespread GFP expression could be observed (data not shown). To exclude the possibility that, in injected mice, Cre*PR induced recombination took place without

inducing GFP expression from the RAGE locus, DNA isolated from organs of these mice was analyzed by Southern blot using a probe to detect GFP. In KspAI digested DNA, the RAGE allele that underwent Cre-mediated recombination can be distinguished from the loxP-flanked allele as shown in Fig. 32A. A low degree of Cre-mediated excision of exons II-VII could be observed in liver, kidney and brain of RU486 injected mice (~13%), whereas in Org31710 treated mice, excision took place only in liver (Fig. 32B). No Cre activity was observed in mice that were not injected with synthetic hormones. Since no recombination of the loxP-flanked target was

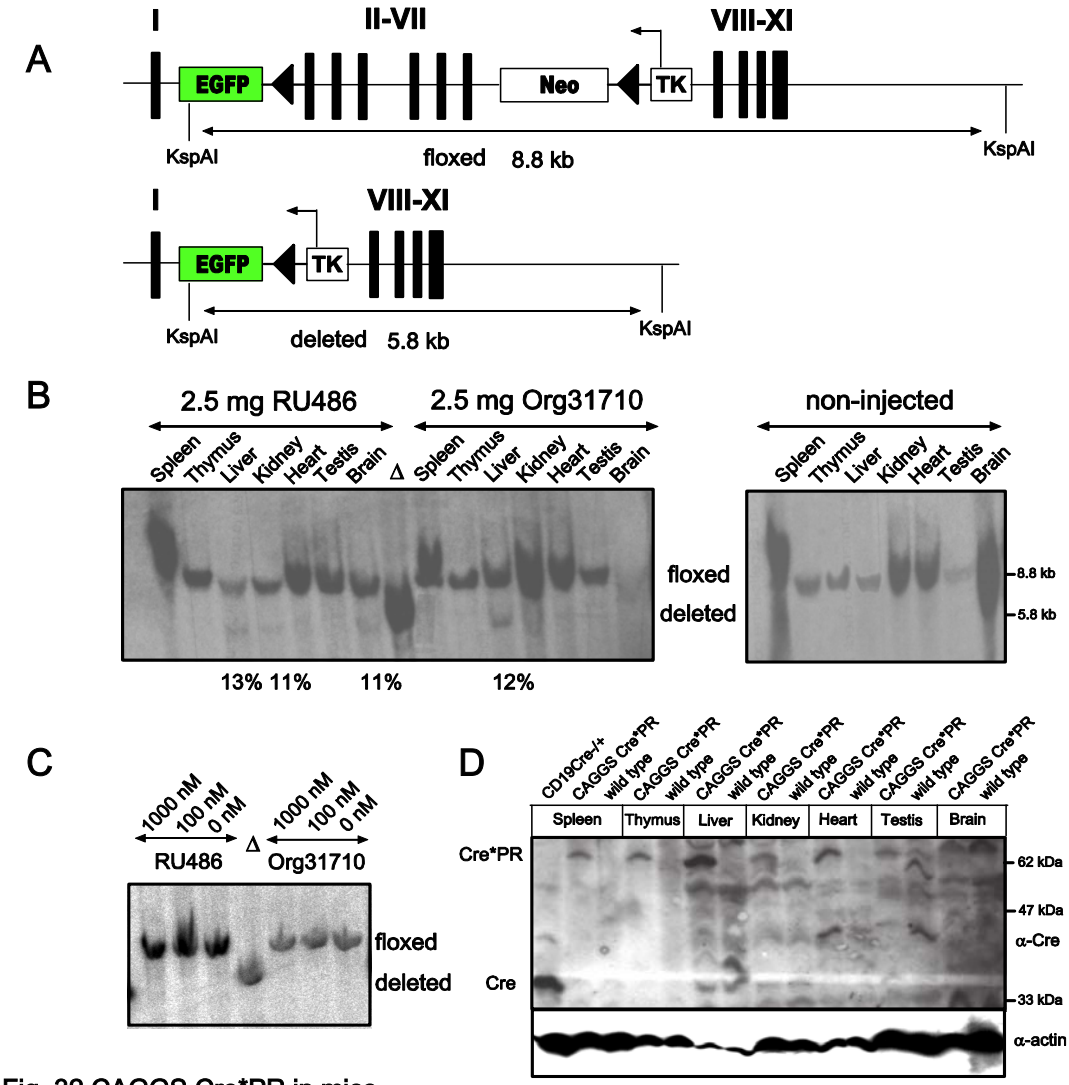


Fig. 32 CAGGS Cre*PR in mice

Female mice of the CAGGS Cre*PR strain were intercrossed with RAGE-EG-/- males to obtain male experimental mice.

(A) Screening strategy for the deletion of the conditional RAGE-EG allele according to Constien *et al.* (2001).

(B) Southern blot against GFP of KspAI digested DNA from organs of mice which were *i.p.* injected with 2.5 mg of RU486 and Org31710 daily for five days, respectively.

(C) Southern blot of *ex vivo* splenocytes, which were kept in medium supplemented with LPS and increasing amounts of RU486 and Org31710.

(D) Western blot against Cre of lysates from organs of the indicated mice.

observed in spleen, splenocytes were cultured in the presence of LPS and increasing doses of either RU486 or Org31710 in order to determine whether Cre activity could be induced *in vitro*. Three days later, these splenocytes were analyzed by flow cytometry. No GFP expression could be detected by FACS (data not shown). In accordance, Southern blot analysis of the DNA purified from these splenocytes did not reveal any recombination of the RAGE-EG reporter (Fig. 32C). Western blot analysis of CAGGS Cre*PR mice revealed, however, that Cre*PR was expressed in various organs (Fig. 32D).

These data could indicate that the inducible Cre*PR system can only mediate recombination of loxP-flanked gene segments *in vivo*, when the Cre*PR fusion protein is highly expressed. This view is supported by the fact that the highest expression level of Cre*PR protein is detected in liver, as indicated by Western blot, and that Cre*PR mediates recombination in this organ *in vivo* upon injection of either RU486 or Org31710. The excision of the RAGE-EG reporter in kidney and brain of RU486 injected mice could be explained by high expression of the Cre*PR in defined subpopulations of cells in these organs, since Western blot analysis of lysates prepared from these organs revealed only intermediate (kidney) to low (brain) expression levels of Cre*PR.

3.4.2 Knock-in of Cre*PR into the ROSA26 locus

Knock-in strategies into the ROSA26 have been described to confer ubiquitous expression of transgenes in mice (Mao *et al*., 2001; Srinivas *et al*., 2001). In order to achieve ubiquitous expression of Cre*PR, the original strategy of Soriano (1999) was applied to insert the Cre*PR into this locus. After the initiation of this project, two publications appeared, in which the authors reported the knock-in of Cre fusion proteins in combination with the estrogen ligand binding domains into the ROSA26 locus (Voojis *et al*., 2001; Seibler *et al*., 2003). Especially, the improved CreERT2 version showed a high degree of inducible recombination after administration of the synthetic ligand tamoxifen to transgenic mice. The unwanted background activity in the absence of inducer appeared only when short loxP-flanked gene segments as that from the conditional allele of the retinoblastoma gene were crossed to the respective transgenes (Voojis *et al*., 2001; F.Schwenk, personal communication).

The knock-in strategy of Cre*PR into the ROSA26 locus is shown in Fig. 33A. Southern blot revealed the 16 kb wild type band of EcoRI digested DNA probed with

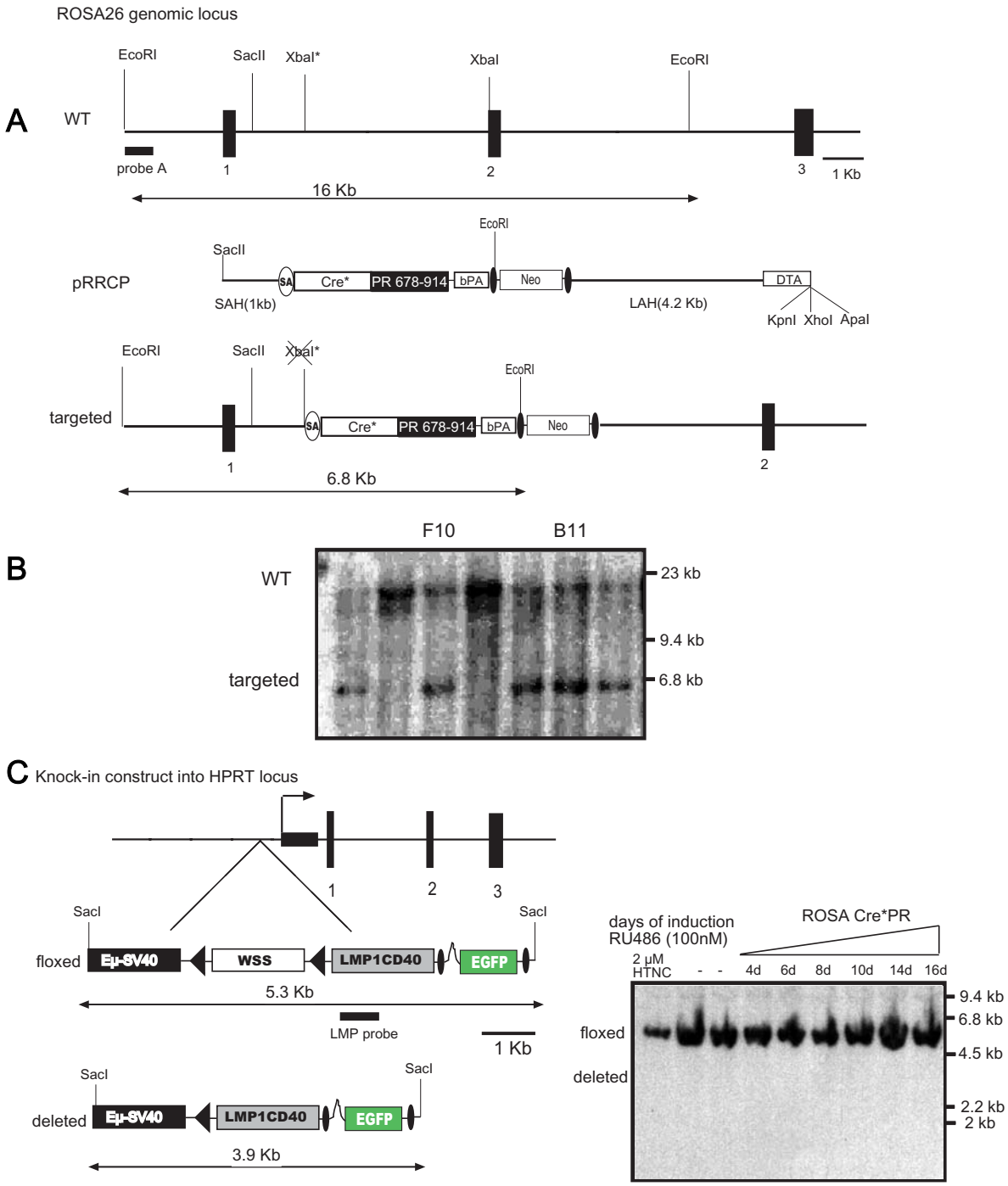


Fig. 33 Knock-in of Cre*PR into the ROSA26 locus.

(A) Knock-in strategy of Cre*PR in ROSA26. In Southern blots of EcoRI digested DNA using probe A, the 16 kb wild type (WT) band appeared and a 6.8 kb band in correctly targeted clones.

(B) Southern blot of homologous recombinant clones. Size marker is the λ-Hind from Gibco.

(C) ES cells used for the knock-in of ROSACre*PR were targeted previously with a conditional LMP1-CD40 allele into the HPRT locus (Strobel & Rajewsky, unpublished). In Southern blots against LMP1 on SacI digested DNA, the floxed band appears at 5.3 kb, whereas a 3.9 kb band arises after Cre-mediated deletion of the WSS. Southern blot of twice targeted ES cells (F10) induced for the indicated days with 100nM RU486. Size marker is the λ-Hind from Gibco.

the external Eco/Pac probe A and an additional 6.8 kb band in correctly targeted ES cell clones (Fig. 33B). ES cells from U.Strobel, which were previously targeted with a conditional allele for an artificial LMP1-CD40 construct into HPRT locus, were used

for the targeting of Cre*PR into the ROSA26 locus. This allows to test the efficiency of ROSACre*PR in ES cells (Fig. 33C). The targeting frequencies were 1 in 8 in this experiment and the two ES clones F10 and B11 were chosen for further experiments. Induction of Cre activity was achieved by supplementing the medium with 100 nM RU486 for 16 days. In order to test the extent of excision, genomic DNA was digested with SacI and probed with an internal LMP1 probe, which should detect the loxP-flanked LMP1-CD40 allele as a 5.3 kb band, whereas the allele from which the loxP-flanked stop cassette had been excised should display a band of 3.9 kb (Fig. 33C). However, no recombination was observed in this experiment, even when ES cells were treated with HTNC, which has been shown to delete loxP-flanked DNA segments with high efficiency in ES cells. Expression of Cre*PR was shown in Western blot analysis, in which a band of 67 kDa appeared after incubation of membranes onto which protein extracts from ROSACre*PR ES cells had been blotted with the polyclonal Cre antiserum (Fig. 28D, lane 4). Correct integration and integrity of the inserted transgenic sequences into the genome of ES clones F10 and B11 was confirmed by sequencing. To this end, a 3.3 kb PCR product amplified from F10 and B11 DNA with the primers 5BstEIIROSA and 3PR914 containing the first exon of ROSA26, intronic sequences, and the Cre*PR ORF was sequenced. F10 and B11 were then injected into C57/BL6 blastocysts. Chimeras from clone B11 failed in germ line transmission, whereas a chimera of clone F10 (90% chimerism by coat colour) transmitted the transgene to its offspring at a high extent. Mice carrying the ROSA26Cre*PR insertion were further intercrossed to RAGE-EG-/- mice, pol-β flox/flox mice and RRDR/RRDR mice in order to test its applicability *in vivo*.

3.4.2.1 Analysis of the ROSACre*PR system *in vivo*

In order to test deletion efficiencies of ROSACre*PR *in vivo*, ROSACre*PR mice were intercrossed with conditional alleles for the DNA polymerase-β gene (Gu *et al*., 1994), the RAGE-EG gene (Constien *et al*., 2001) and the RRDR indicator.

Double transgenic offspring carrying ROSACre*PR and pol-β flox/+ were either *i.p.* injected or orally fed with 5 mg RU486 for five days. Afterwards, mice were further kept for five days, before they were sacrificed. Several organs were isolated and genomic DNA was prepared for analysis by Southern blot. DNA isolated from organs of ROSACre*PR-/+; pol-β flox/+ was digested with BamHI and probed with the 900

bp pol-β probe to detect the wild type (10 kb), the loxP-flanked (4.5 kb) and the deleted (3 kb) alleles of pol-β (Fig. 34A). Fig. 34B shows that no inducible recombination of the loxP-flanked pol-β allele could be detected in any organ in the presence and absence of RU486. Also, in *ex vivo* splenocytes of these mice, which were isolated and kept in culture in the presence of 1 µM RU486, Cre*PR failed to excise the loxP-flanked allele (data not shown). In order to confirm that Cre*PR is expressed *in vivo*, cell lysates of all major organs were analyzed by Western blot (data not shown). This experiment revealed that Cre*PR is expressed in all the organs that were analyzed, but at various levels. Similar results were obtained with experimental mice of the genotype ROSACre*PR-/+; RAGE-EG-/+ (data not shown).

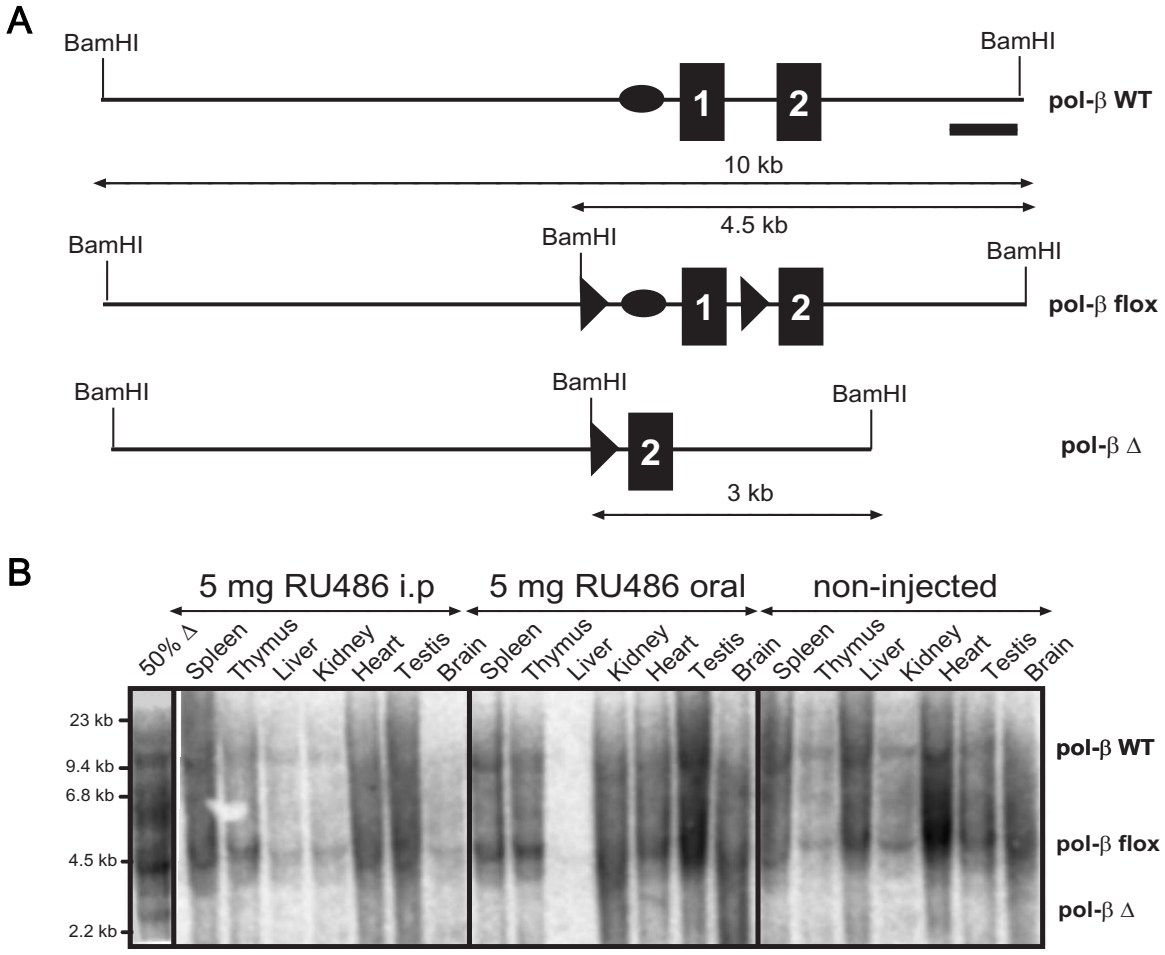


Fig. 34 ROSACre*PR in mice

ROSACre*PR mice were intercrossed with pol-β flox/flox mice (Gu *et al.*, 1994) to obtain experimental mice. (A) Screening strategy for the deletion of the conditional pol-β allele adapted from Gu *et al.* (1994). (B) Southern blot against the pol-β gene of organs from mice which were either *i.p.* injected or orally fed with 5 mg RU486 daily for five days. Size marker was the λ-Hind from Gibco.

3.4.2.2 Analysis of ROSACre*PR in mouse embryonic fibroblasts

Mouse embryonic fibroblasts (MEFs) of embryos of ROSACre*PR/RRDR-DsRed and RRDR-DsRed/+ were generated in order to test the functionality of ROSACre*PR *in vitro*. In addition, experiments with MEFs should address the question, whether the Cre*PR protein translocates to the nucleus upon binding of RU486 (Fig. 35A, C). In order to induce Cre*PR activity in these cells, 100 nM RU486 was added to 10^6 MEFs. Two days later, cells were harvested and separated in two samples for the analysis by Southern and Western blots. No recombination of the DsRed-stop cassette was observed in the presence or absence of inducer (Fig. 35B). To test

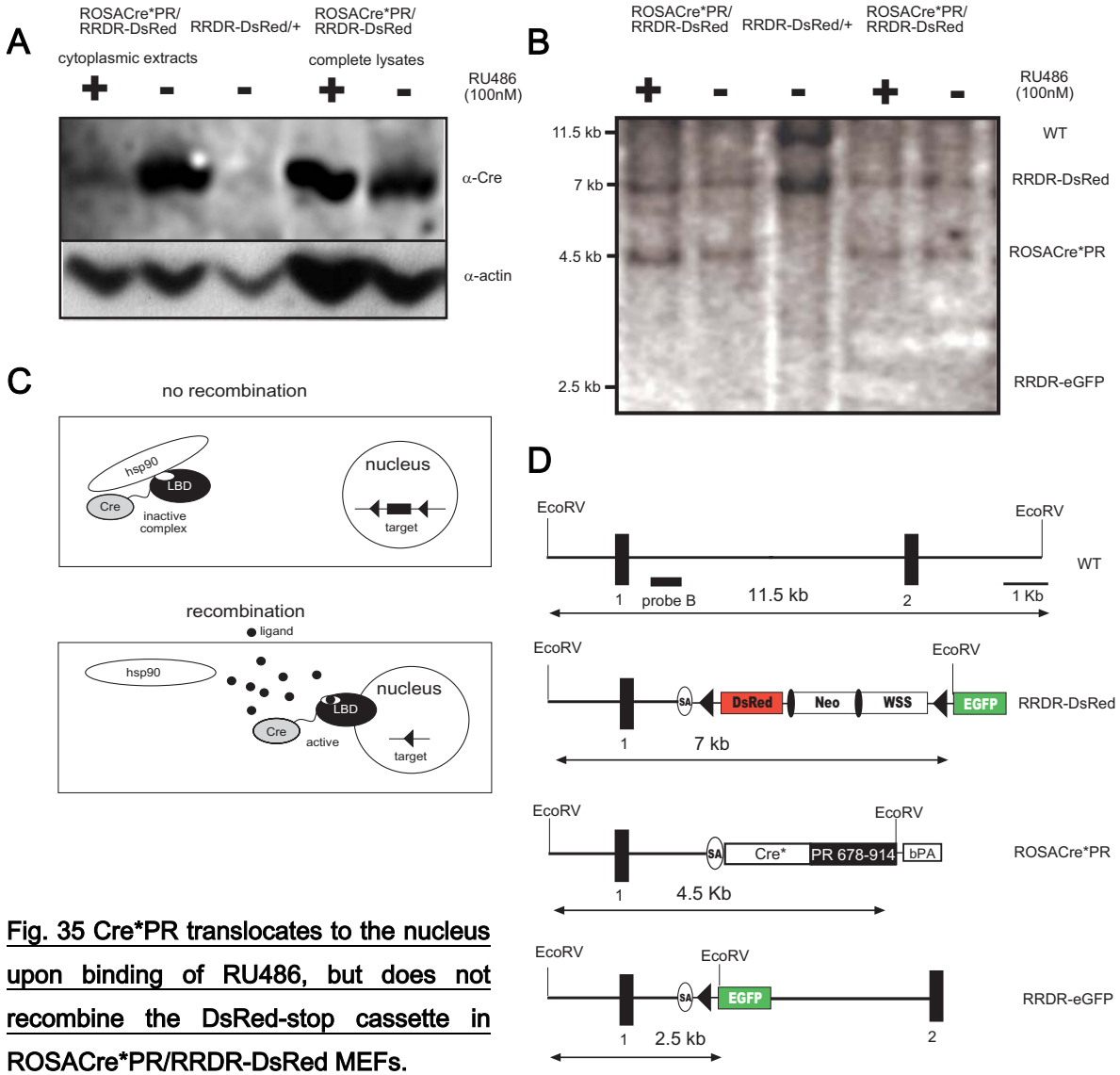


Fig. 35 Cre*PR translocates to the nucleus upon binding of RU486, but does not recombine the DsRed-stop cassette in ROSACre*PR/RRDR-DsRed MEFs.

10^6 MEFs of embryos with the genotype ROSACre*PR/RRDR-DsRed were seeded on 6 cm plates and induced with 100 nM RU486. Two days later, cells were harvested analyzed by Western blot (A) and by Southern blot (B). (C) Schematical outline of the function of CrePR. (D) Sizes of DNA fragments obtained from these MEFs after EcoRV digest and probing with probe B in Southern blot analysis.

whether the Cre*PR protein translocates to the nucleus upon RU486 treatment (Fig. 35C), cytoplasmic and nuclear protein extracts were prepared from the MEFs by NP-40 lysis and the cytoplasmic fractions were subsequently analyzed by Western blot. Complete MEF cell lysates served as controls (Fig. 35A). In the cytoplasmic fraction isolated from RU486 treated ROSACre*PR/RRDR-DsRed MEFs, nearly no Cre*PR protein could be detected, whereas a specific band for Cre*PR is present in non-induced samples. To exclude the proteolytical degradation of the Cre*PR upon ligand binding, complete protein lysates of induced and non-induced MEFs were loaded as controls. In both samples, Cre*PR protein is present at equal amounts. These data suggest that indeed the Cre*PR protein translocates to the nucleus upon RU486 binding as demonstrated by the disappearance of the Cre*PR protein from cytoplasmic fractions of induced MEFs, whereas the Cre*PR protein is present in the cytoplasm of non-induced cells. However, once in the nucleus, the Cre*PR does not recombine the DsRed-stop cassette of the RRDR indicator as shown by Southern blot (Fig. 35B). A possible explanation could be that the Cre*PR protein sticks at the nuclear pores and is not imported in the nucleus. However, this appears rather unlikely, since lysis of MEFs with 1% NP-40 to obtain the cytoplasmic fraction also solubilizes the nuclear envelope including the nuclear pore complexes (Wunderlich *et al*., 1976). By this method, the solubilized compartment of the nuclear envelope is therefore in the cytosolic fraction.

3.5 B lymphocyte restricted expression of Cre*PR

One step forward in inducible gene targeting is to restrict the inducible Cre system to certain cell types by the usage of cell type specific promoters. In order to achieve a B cell restricted expression of transgenes, the B cell specific CD19 locus was shown to be an excellent candidate for integration of transgenes (Zhou *et al*., 1992). CD19 is a B lymphocyte cell surface marker that is expressed early during pre-B cell differentiation and its expression persists until terminal differentiation into plasma B cells (for review, see e. g. Fearon, 1993). The murine CD19 gene (mCD19) is composed of 15 exons and is a member of the Ig gene superfamily. CD19 contains two extracellular Ig-like domains separated by a non Ig-like domain with a hydrophobic membrane-spanning domain and an extensive cytoplasmic domain. When Cre recombinase was knocked-in into the mCD19 locus, its expression yielded very efficient Cre-mediated excision of loxP-flanked target sequences in B

lymphocytes (Rickert *et al*., 1995). In this knock-in strategy, the ORF of the Cre was inserted into the second exon of the CD19 gene and it was attempted to mutate the endogenous start AUG of CD19. The targeting was successful, but replacement of the endogenous AUG codon could not be achieved (J. Roes, personal communication). A possible reason is that, in the CD19 targeting vector used, the short arm of homology was not long enough. Nevertheless, the CD19-cre mouse is an extremely useful tool for conditional gene targeting in B cells (Rickert *et al*., 1997).

However, the original CD19-cre targeting vector has the disadvantage that it cannot be used for insertion of other transgenes. Therefore, I constructed a general CD19 targeting vector termed pCD19, into which the gene of interest can be inserted with a single cloning step to achieve B cell specific expression (Fig. 9). In addition, the short arm of homology was elongated to increase the chances for cointegration of the mutated endogenous translation start of CD19 in comparison to the former strategy. Because of its importance for immunological experiments, I generated not only CD19Cre*PR (Fig. 36), but also CD19CreERT2 constructs (Fig. 9). At the time when the project was started, I had only access to sequence information of the 129 ola mouse strain and therefore amplified the homology arms of pCD19 from 129 ola genomic DNA. Nevertheless, targetings were not only performed in isogenic ES cells, but also in C57BL/6 derived ES cells (Kontgen *et al*., 1993), since the B6 genetic background is particularly well analyzed in the context of immunological experiments. These two ES cell lines do not differ with respect to restriction site polymorphisms in Southern blot analysis using at least seven different restriction enzymes in the mCD19 gene (data not shown).

Targeting experiments were performed in HM-1 ES cells (Magin *et al*., 1992) and Bruce-4 ES cells (Kontgen *et al*., 1993), as already described. From each transfection, 130 G418 and GANC resistant ES cell colonies were isolated as single clones and their genomic DNAs were analyzed by Southern blot. The identification of a 9.4 kb band besides the 28 kb wild type band by probing SpeI digested DNA with the CD19 probe B indicates correctly targeted clones (Fig. 36A, B). From 130 HM-1 clones only a single clone, C9, showed the additional band (Fig. 36). The targeting of Bruce-4 cells was not successful. A second digest of DNA isolated from clone C9 with BamHI revealed the cointegration of the mutated start AUG in a Southern blot

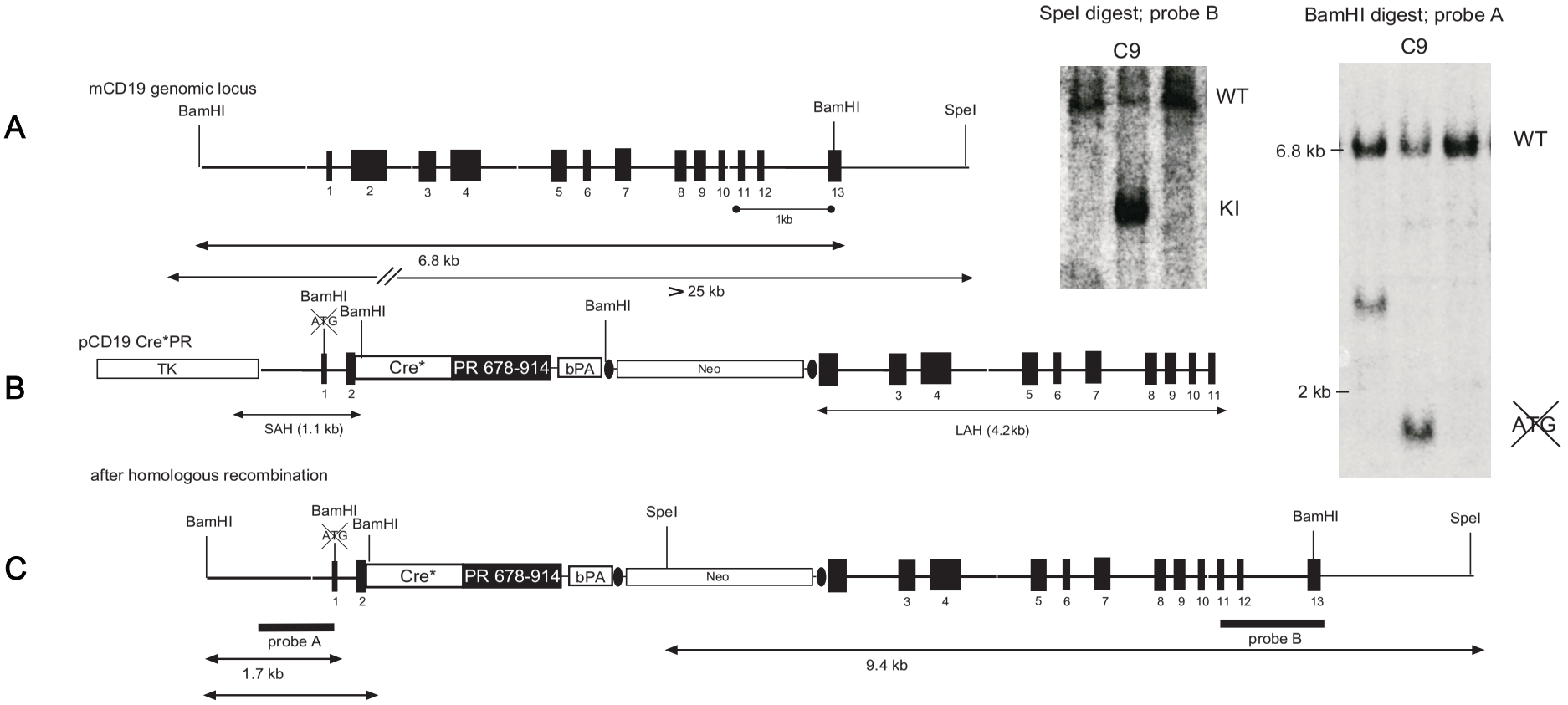


Fig. 36 Generation of B cell specific inducible Cre*PR: Knock-in into the mCD19 locus.

(A) The upper part displays the wild type mCD19 locus. In Southern blots of BamHI and SpeI digested DNA, there appear bands of 6.8 kb and more than 25 kb, using probe A and B, respectively. (B) The pCD19 Cre*PR targeting vector consists of theTK gene from HSV followed by the short arm of homology (SAH), which has a size of 1.1 kb. The Cre*PR ORF was placed in the second exon, together with a neomycin resistance gene flanked by FRT sites. The long arm of homology (LAH) is 4.2 kb long. Instead of the endogenous mCD19 start codon, the SAH contains a BamHI site, which can be used for screening. (C) If homologous recombination will take place in front of the destroyed ATG, a band of 1.7 kb will appear, using probe A in BamHI digests in Southern blots, whereas a band of 2.3 kb will arise when the BamHI site is not cointegrated. But the main screening for homologous recombination is performed by a SpeI digest, using external probe B in Southerns. Correctly targeted clones will reveal a 9.4 kb band. The blots show that clone C9 was correctly targeted and that the destroyed start ATG was cointegrated.

using CD19 probe A (Fig. 36C). Sequencing of a PCR fragment amplified with the primers 5NotEx1e and 3PR914 from the CD19 locus of clone C9 confirmed the expected sequences and clone C9 was therefore used for injection into blastocysts of CB20 or C57BL/6 donors. Backcrosses of the resulting chimeric mice led to germ line transmission and CD19Cre*PR mice were intercrossed with mice carrying the RRDR indicator and with a mouse strain which carries a conditional allele for the insulin receptor (Brüning *et al.*, 1998).

In order to examine the B cell specific expression of Cre*PR in CD19Cre*PR mice, I performed RT-PCR, Western blot analysis and flow cytometry (Fig. 37). B cells and T cells of the spleen were labelled with anti-B220 FITC and anti-TCR-β PE, respectively, and separated by FACS sorting. The purity of B cells and T cells was more than 95% after FACS sorting. Subsequently, mRNA of the purified lymphocytes from wild type mice and CD19Cre*PR-/+ mice were isolated and subjected to cDNA synthesis. The specific cDNA of Cre*PR expressed from the CD19 promoter was amplified with the primers 5BamEx1 and hCreintrev yielding a 440bp PCR product, when splicing from CD19 exon 1 is directed to the splice acceptor of Cre*PR (Fig. 37B). The 440bp product appears only in splenic B cells of CD19Cre*PR mice, but not in splenic T cells (Fig. 37B). Sequencing of the PCR product verified the expected sequences and confirmed the correct splice junctions. In order to detect the Cre*PR protein, Western blot of $3x10^6$ splenocytes from wild type mice, CD19-cre-/+ mice and CD19Cre*PR-/+ mice were performed with the polyclonal Cre anti-serum (Fig. 37C). The Cre*PR protein was detectable in lysates of spleen cells of CD19Cre*PR mice.

Correct integration of Cre*PR into the CD19 locus was confirmed by flow cytometry of blood lymphocytes. Peripheral blood mononuclear cells (PBMCs) were isolated from the blood of CD19Cre*PR mice by Ficoll gradient centrifugation and stained with CD19 FITC to identify B lymphocytes (Fig. 37C). Both B cells from heterozygotes CD19-cre-/+ mice and those from CD19Cre*PR-/+ mice expressed only half of the CD19 molecules on their surface in comparison to wild type mice, whereas no CD19 expression was observed on the surface of B cells from homozygotes CD19-cre-/- mice (Fig. 37C). Obviously, the Cre*PR is correctly integrated into the second exon of the CD19 gene.

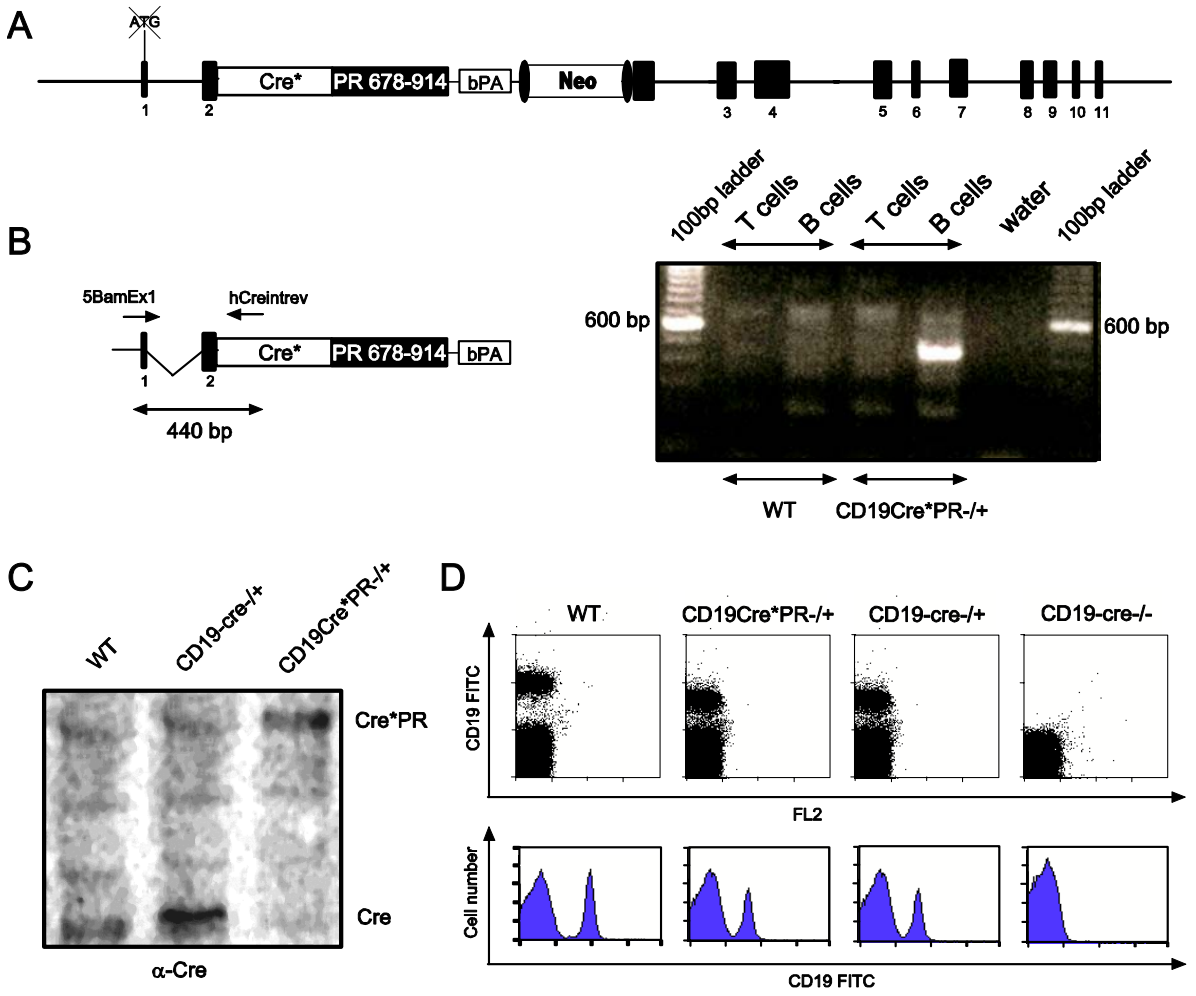


Fig. 37 B cell specific expression of CD19Cre*PR.

(A) Insertion of Cre*PR in the CD19 locus.

(B) RT-PCR of cDNAs from 10^6 sorted splenic T cells and B cells of wild type (WT) and CD19Cre*PR-/+ mice. Primers 5BamEx1 and hCreintrev were used to amplify the correctly spliced 440bp product of Cre*PR expressed from CD19.

(C) Western blot of $3x10^6$ splenocytes from the indicated mice using a polyclonal Cre anti serum.

(D) Blood lymphocytes were isolated by Ficoll gradient centrifugation and stained with CD19 FITC. Dot blots and corresponding histograms of the indicated mice are shown.

In order to determine the inducibility of Cre*PR in B cells *in vivo*, CD19Cre*PR mice were intercrossed with the RRDR indicator mouse strain. Subsequently, mice carrying the CD19Cre*PR and RRDR-DsRed alleles were *i.p.* injected with 2.5 mg RU486 daily for five days, before sacrificing the mice and preparing single cell suspensions from bone marrow and spleen. Pro/pre B cells were purified as $B220^+$, IgM^- fraction by FACS sorting from bone marrow cells labelled with anti-B220 FITC and anti-IgM PE. Splenic $B220^+$ cells were separated from splenic TCR-β^+ cells by FACS sorting. DNA was prepared from 10^6 cells in order to detect the loxP-flanked

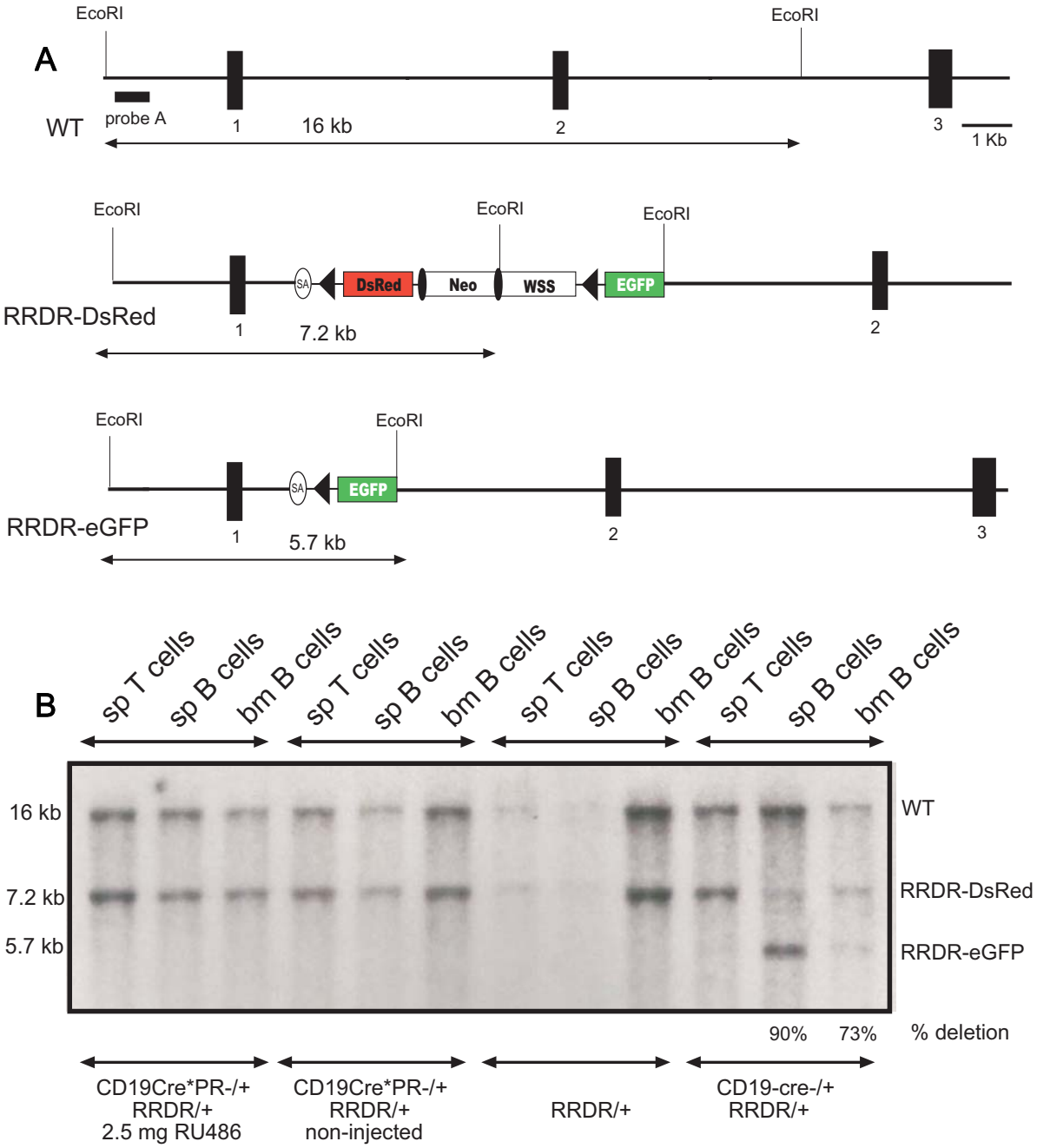


Fig. 38 Inducibility of CD19Cre*PR *in vivo*.

(A) CD19Cre*PR mice and CD19-cre mice were intercrossed with RRDR mice. Southern blot analysis of EcoRI digested DNA reveals the 16 kb wild type ROSA26 allele (WT), the 7.2 kb loxP-flanked RRDR-DsRed, and the 5.7 kb excised RRDR-eGFP alleles using probe A.

(B) CD19Cre*PR; RRDR mice were *i.p.* injected with 2.5 mg RU486 daily for 5 days. Splenic (sp) T cells and B cells were separated by FACS sorting. Pro/pre B cells from bone marrow (bm) were isolated by FACS sorting as $B220^{+}$, IgM^{-} cells. The same cell fractions from non-injected CD19Cre*PR; RRDR mice and CD19-cre; RRDR mice served as controls. Deletion efficiencies were quantified using the Fuji Bias reader and are indicated as percent.

RRDR-DsRed (7.2 kb) and the excised RRDR-eGFP (5.7 kb) alleles by Southern blot analysis (Fig. 38A, B). No RU486 inducible recombination mediated by Cre*PR was

observed in this experiment. The RRDR-eGFP allele was only detectable in approximately 90% of splenic B cells and 73% of pro/pre B cells from control CD19-cre-/+; RRDR-DsRed/+ mice.

3.6 An attempt to generate a tet-inducible system controlling Cre activity

Tet-inducible systems are elegant tools to regulate gene expression inducibly in a quantitative fashion.

I attempted to generate a general tet-inducible system by construction of the pEXchange vector, in which all elements necessary for the tet-on system are included. This system has the advantage that the promoter, which drives the expression of the reverse tet-transactivator rtTA-M2, can be exchanged in order to obtain cell type restricted expression of the rtTA-M2. The pEXchange construct was designed in such a manner that one additional cloning step results in the generation of a HPRT targeting vector. The HPRT locus was chosen for these experiments, since the HPRT gene is a housekeeping gene. Therefore, expression of transgenes driven by any inserted promoter is presumably not restricted by unfavourable chromatin configurations. In addition, the HPRT locus is known to be far away from any enhancer activity of adjacent genes (Cvetkovic *et al*., 2000; Su *et al*., 2002).

The basic construct and the principle of the system are depicted in Fig. 39. A cell type specific or ubiquitous promoter drives the expression of the improved rtTA-M2 (Urlinger *et al*., 2000), that is separated from the tet-responsive Cre cassette by two SV40 pA and a WSS to prevent transcriptional read-through. The WSS was inserted in front of the tet-responsive cassette such that any transcription occurring from 5' will be spliced from the strong splice donor of the WSS to the splice acceptor in front of the Cre ORF and a wrong start codon will result in out of frame translation of Cre. To drive the doxycycline inducible expression of Cre, the tetO7 promoter was used which consists of seven tet-responsive elements and the CMV minimal promoter combined with a synthetic intron (kindly provided by F.Schwenk and J.Seibler, Artemis Pharmaceuticals, Cologne, Germany). In order to achieve ubiquitous expression of rtTA-M2, the EF1α and the CAGGS promoters were cloned into the pEXchange construct, respectively (Fig. 40A).This allows testing the tet-on system in ES cells. The HPRT targeting vectors were generated as described in Fig. 8 and transfected into RRDR ES cells which contain the DsRed/eGFP indicator into the ROSA26 locus (Fig. 40B). Transfection of 10^6 ES cells with 3 µg of each targeting

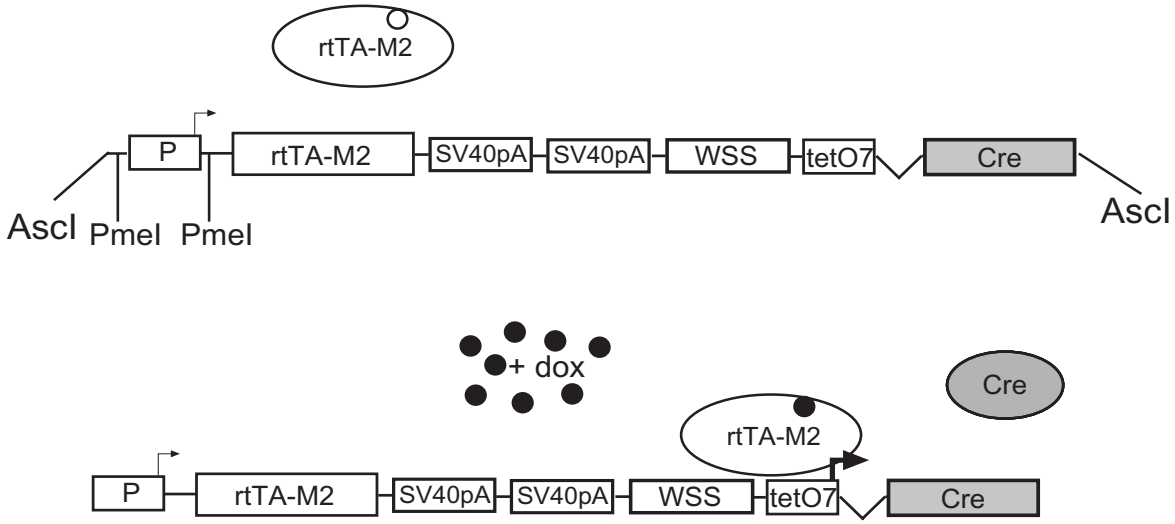


Fig. 39 Priniple of the tet-on system for Cre.

All elements of the tet-on system are combined in the construct. The promoter (P) drives expression of rtTA-M2 and can be exchanged by a PmeI digest. The rtTA-M2 is separated from the tet-responsive cassette by two SV40 pA and the WSS to prevent transcriptional read-through. The tetO7 promoter consists of seven tet-responsive elements and the CMV minimal promoter. After doxycycline administration, the rtTA binds to tetO7 and activates expression of Cre. The whole construct can be cut out from the plasmid by AscI digestion and can be cloned into the HPRT targeting vector pMP10.

vector resulted in HAT resistant colonies, i.e. two for the EF1α construct and three for the CAGGS construct. Correct homologous recombination was confirmed by Southern blot analysis of StuI digested DNA using a Cre probe (data not shown).

Initial experiments, in which expression of Cre was induced by doxycycline administration revealed a high extent of deletion of the DsRed/eGFP indicator in both the EF1α and the CAGGS systems also in the absence of the inducer (data not shown). In order to select for clones that still retained the loxP-flanked DsRed-stop cassette including the neomycin resistance gene, ES cell clones were treated with 300 µg/ml G418 for 14 days. Subsequent Southern blot analysis revealed absence of cells of the RRDR-eGFP genotype lacking the DsRed-stop cassette (data not shown). After two days without G418, $2x10^5$ ES cells containing knock-ins of the EF1α and CAGGS tet-systems for Cre were plated on a well of a 6 well-plate and were induced with 1 µg/ml doxycycline for 9 days. Control cells that were not treated with doxycyline were harvested at the end of the experiment on day 9. Southern blot analysis to distinguish between the RRDR-DsRed and the RRDR-eGFP alleles of the Cre-indicator showed no recombination without doxycycline in case of the EF1α tet-system, whereas the CAGGS tet-system exhibited background activity in the absence of doxycycline.

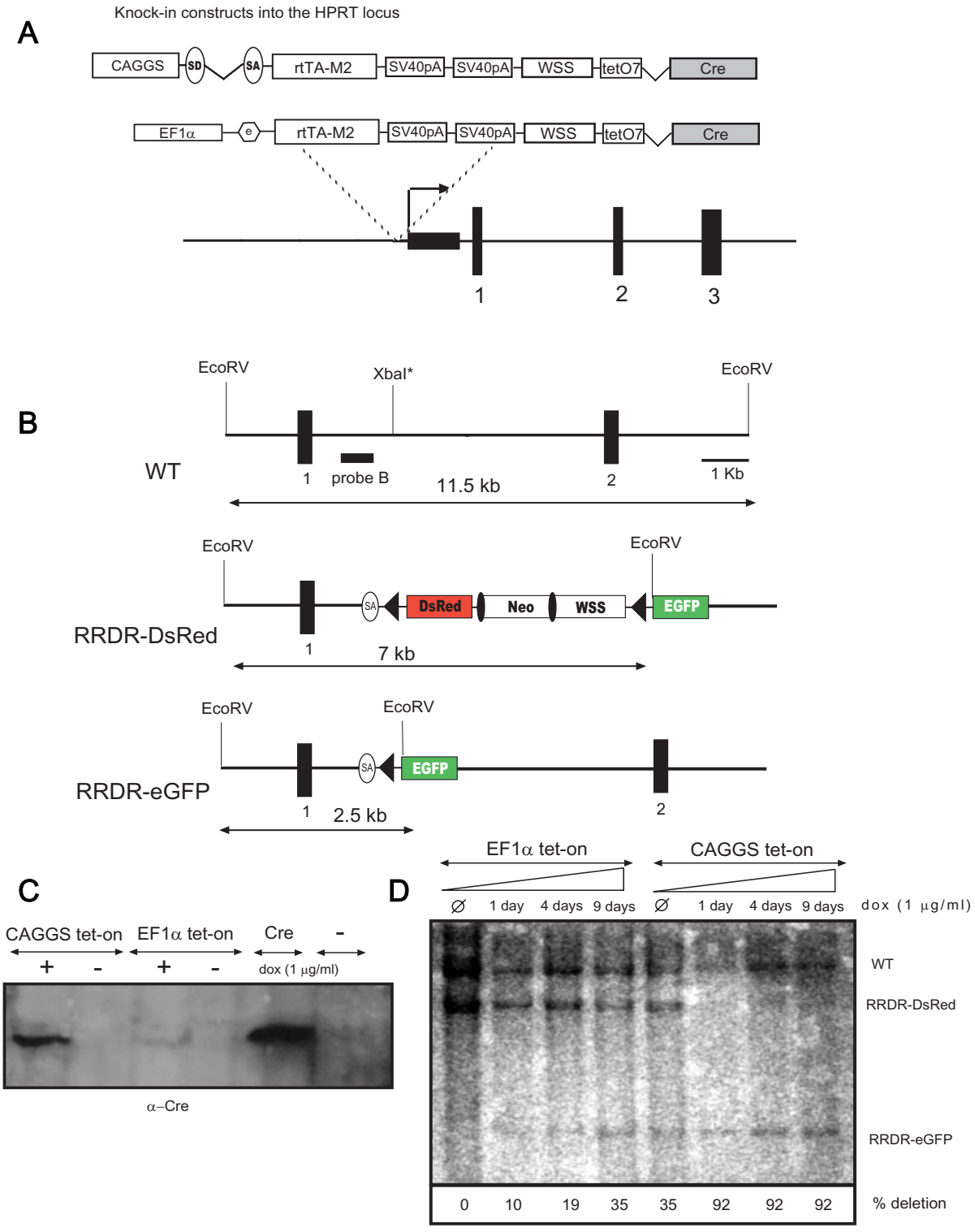


Fig. 40 Tet-on systems for Cre in RRDR ES cells.

(A) The two different tet-systems for Cre are shown which were inserted into the HPRT locus of RRDR ES cells.

(B) RRDR ES cells contain the DsRed/eGFP indicator for Cre activity into ROSA26 locus. The screening strategy to detect the loxP-flanked RRDR-DsRed and the excised RRDR-eGFP alleles by Southern blot is shown.

(C) Western blotting to detect inducible Cre expression in ES cell lysates of twice targeted ES cells (RRDR and CAGGS tet-on or EF1α tet-on). Cells were kept in culture for 4 days.

(D) Southern blot of twice targeted ES cells with RRDR and EF1α tet-on or CAGGS tet-on which were incubated in the presence of 1 µg/ml doxycycline (dox) for the indicated days.

Deletion of the loxP-flanked DsRed-stop cassette of the indicator was achieved in the EF1α tet-system ranging from 10% on day 1 to 35% on day 9 (Fig. 40D). With the CAGGS tet-system, however, nearly complete excision of the DsRed-stop cassette was obtained after 1 day of doxycycline treatment (92%), which was not further increased by prolonged incubation in the presence of doxycycline (Fig. 40D). Western blot of cell lysates of the EF1α and CAGGS tet-systems revealed that Cre expression was dependent on the addition of doxycycline (Fig. 40C). Cell lysates from ES cells transgenic for the CAGGS tet-system knock-in revealed a strong band at 38 kDa when ES cells were induced with doxycycline (Fig. 40C). A weak, but distinct band in non-induced samples of the CAGGS tet-system was observed only after longer exposure of the X-ray films (data not shown). In cell lysates from ES cells containing the EF1α tet-system, a weak band specific for Cre appeared only when the ES cells had been induced with doxycycline, whereas no band appeared without doxycycline treatment (Fig. 40C).

4. DISCUSSION

Conditional gene targeting using the Cre/loxP system is a widely used technique to analyze gene function *in vivo*. At present, efforts are undertaken to improve the control of the Cre recombinase by inducible systems. The aim of this study was to control Cre activity by a posttranslational and by a transcriptional inducible system in mice. Hence, I developed and tested inducible systems for Cre *in vitro* controllable by RU486 and doxycycline. Subsequently, the most promising constructs were further analyzed in transgenic mice. Specifically, a strategy was developed, which allowed to test the systems quantitatively in transiently transfected fibroblasts, then in ES cells, and finally in chimeric mice. In order to quantify the inducibility of the Cre constructs, a novel reporter for Cre activity had been established.

4.1 RRDR as a new indicator for Cre activity

Many different mouse indicator strains are available at present which are used to characterize the expression pattern of Cre transgenic mouse strains (Tsien *et al*., 1996; Lobe *et al*., 1999). A common feature is the activation of an indicator gene after Cre-mediated excision of a transcriptional stop sequence. Subsequently, the promoter which drives the indicator gene will come into close proximity to the ORF of the reporter gene, thus initiating transcription followed by protein synthesis, which can then be monitored. This approach implicates a constitutive transcriptional activity of the promoter after excision of the loxP-flanked stop sequence to express the reporter gene in any cell at any time. A much better indicator system would be a binary indicator construct which couples the Cre-mediated shut down of one indicator gene with the activation of a second indicator gene. This would allow discriminating between those cells in which Cre-mediated recombination took place, and those cells which had not undergone Cre-mediated recombination.

In the present study, I have tried to combine the DsRed and eGFP genes in a binary shut down/activation coupled construct for Cre activity. The DsRed gene encodes an autofluorescent protein originally identified in *Discosoma sp.* (Baird *et al*., 2000) and the eGFP gene the autofluorescent protein of *Aequorea victoria* (for review, see e. g. Hadjantonakis & Nagy, 2001). The emission spectrum of DsRed does not overlap with that of the eGFP making it an excellent candidate for double fluorescent labelling *in vivo* (Gross *et al*., 2000; Heikal *et al*., 2000).

I constructed the pTE RFP/GFP plasmid such that the CAGGS promoter drives the expression of a slightly modified DsRed gene. The latter is part of a loxP-flanked cassette that additionally contains a FRT flanked neo and the WSS (Lakso *et al*., 1992). Downstream of the DsRed-stop cassette, the promoterless eGFP gene is located. Excision of the DsRed-stop cassette should result in shut down of DsRed expression and activation of eGFP, which can be monitored as a switch from red to green fluorescence. In addition, the DsRed gene was modified with a NLS sequence to restrict it to the nucleus, whereas the eGFP gene is expressed in the cytoplasm. Indeed, I could show that the pTE RFP/GFP system worked as anticipated in transiently transfected CV1-5B fibroblasts. Transfected cells revealed DsRed-derived red fluorescence in nuclei and cytoplasmic eGFP-derived green fluorescence only after excision of the DsRed-stop cassette by a cotransfected Cre coding plasmid. Since the DsRed/eGFP indicator worked properly in transiently transfected fibroblasts, I attempted to extend its usage to ES cells. To this end, the indicator construct was targeted into the ROSA26 locus of HM-1 ES cells.

At that time, the ROSA26 locus appeared to be the only convenient option for this approach, since it was shown to be expressed in all cells of the adult mouse (Wunderlich & Rajewsky, unpublished results). Indeed, the ROSA26 locus was widely used for the ubiquitous expression of transgenes, e.g. for CreERT (Vooijs *et al*., 2001), CreERT2 (Seibler *et al*., 2003), indicators for Cre in combination with lacZ (Soriano, 1999), eGFP (Mao *et al*., 2001), eYFP, eCFP (Srinivas *et al*., 2001), and a conditional allele for LMP2a (Casola & Rajewsky, unpublished results). However, a few abnormalities of the ROSA26 locus have been noted during that time. First, the ROSA26 mutant allele is not inherited according to Mendelian segregation in intercrosses between heterozygotes mutants. In contrast, homozygous mutants do not show any obvious phenotype (Zambrowicz *et al*., 1997; personal observation). The only phenotypic effect described so far is, that $Apc^{Min/+}$ mice carrying in addition the ROSA26 insertion are resistant to ethylnitrosourea (ENU)-induced mammary tumor development, whereas $Apc^{Min/+}$ mice without the ROSA mutant allele are susceptible to ENU-induced mammary tumors (Kohlhepp *et al*., 2000; Kohlhepp *et al*., 2001). Second, the expression pattern of some ROSA26 driven transgenes was abnormal. For instance, Jonkers and coworkers could not detect any β–galactosidase activity from a ROSA26 expressed lacZ transgene when analyzing old mice, whereas younger mice displayed the overall expression of ROSA26 (J. Jonkers, personal

communication). Third, transgenes driven from the ROSA26 locus seem to be expressed at low levels. For instance, Mao *et al*. (2001) inserted an indicator transgene for Cre activity into the ROSA26 locus that activates eGFP expression after Cre-mediated recombination, but achieved only minimal eGFP expression.

To obtain a higher expression from the ROSA26 locus, I modified the targeting vector pROSA26-1 such that a putative start codon in the first exon of ROSA transcripts 1 and 2 was mutated. Therefore, I elongated the short arm of homology of the targeting vector and inserted two cytosines into the start codon. With this modified targeting vector, I expected the first AUG of the downstream ORFs of DsRed and eGFP to be used as start codons, thus causing higher expression levels. However, I could not detect any red or green fluorescence with this construct *in vivo*. Although the reason for this failure is not clear, I can exclude some possible explanations. First, the design of the pTE RFP/GFP plasmid appears to be successful, since S. Casola and T. Buch used this plasmid to generate mice with conditional alleles in the ROSA26 locus for LMP2a and the diphtheria toxin receptor, respectively (personal communication). Second, the expressed transgenic mRNAs from the ROSA26 locus are not instable. The properly spliced transcripts of DsRed and eGFP were detected in RT-PCR experiments and sequencing of the corresponding PCR products revealed that the first AUG codons in the mRNAs were those which were introduced with the Kozak consensus sequence for DsRed and eGFP, respectively. In addition, splenocytes, in which the DsRed-stop cassette - and therefore the DsRed gene - had been excised by HTNC treatment, revealed significant amounts of the DsRed mRNA after three days, thus indicating the stability of the corresponding mRNA. Thus, translation of the mRNA into protein appears to be impaired, since mRNAs of DsRed and eGFP are still expressed without any detectable protein fluorescence. In accordance, Western blot analysis did not reveal any significant amount of expressed eGFP protein in lysates from all major organs of RRDR-eGFP/+ mice, which had the loxP-flanked DsRed-stop cassette excised in the germ line, though this method is sensitive enough to detect eGFP in the positive control. At the present state of experiments, I can only speculate about the fact that, in the newly generated RRDR indicator mouse strain, transcription and processing of mRNAs of the introduced transgenes take place, but translation is inhibited for unknown reasons. I used a very similar targeting strategy as Srinivas *et al*. (2001) and Mao *et al*. (2001), which showed protein synthesis of fluorescent proteins from ROSA26. A major difference in

my strategy was the disruption of the endogenous start codon of ROSA26. It is therefore conceivable that cis acting elements in the 5'UTR of ROSA26 have been destroyed by introducing the two cytosine nucleotides into the AUG. This view is supported by the fact that posttranscriptional regulation of protein expression has been shown for nNOS and those genes, which are involved in iron metabolism (for review, see e. g. Kaempfer, 2003; Henderson *et al*., 1996). In case of nNOS, an alternatively spliced non-coding exon in the 5'UTR caused a drastic repression of protein synthesis, although mRNA levels were not altered (Newton *et al*., 2003). This non-coding exon has been proposed to form a stem loop motif at the mRNA, which serves as binding site for inhibitory molecules that interfere with translation.

Such a hypothesis could also reasonably explain my results: the mRNAs of DsRed and eGFP are expressed from the ROSA26 locus, but are not translated into protein due to inhibitory accessory proteins. In order to test a possible stem loop formation of the ROSA26 5'UTR, a computer aided analysis was performed. The 5'UTR consists of highly repetitive G-rich sequences which are capable of forming a stem loop structure at the RNA level, as shown in Fig. 41. The two cytosine nucleotides were inserted at the tip of the putative loop, which possibly disrupted the secondary structure or essential protein binding sites. In this context, it is noteworthy that several stem loop like motifs in the 5'UTR of cellular mRNAs were shown to act as internal ribosome entry site (IRES) (Kim *et al*., 2001; Rubtsova *et al.*, 2003; Jopling *et al*., 2004) as that from the EMCV virus (for review, see e. g. Martinez Salas, 1999). Incidentally, IRES do not require any 5'CAP of the mRNA to initiate translation (Bonnal *et al*., 2003). The EMCV IRES recruits the ribosome by its secondary structure through RNA-RNA interactions and RNA-protein interactions specifically to AUG number eleven in the viral mRNA to promote translation of the respective protein (Jang & Wimmer, 1990, Davies & Kaufman, 1992). The conserved loop motifs of the IRES as the GNRA loop and the RAAA loop (where N is any nucleotide and R is a purine) serve as binding sites for proteins and do not tolerate many changes in their sequence context, as these changes often lead to disruption of the IRES function (for review, see e. g. Sachs *et al.*, 1997). Provided the 5'UTR of the ROSA26 transcripts functions in a similar way, the insertion of the two cytosine nucleotides into the AUG could destroy the putative cellular IRES function. I am currently in the process of testing this hypothesis. In one approach, I have already constructed a modified targeting vector in which the U of the AUG was mutated to a C. Since this is

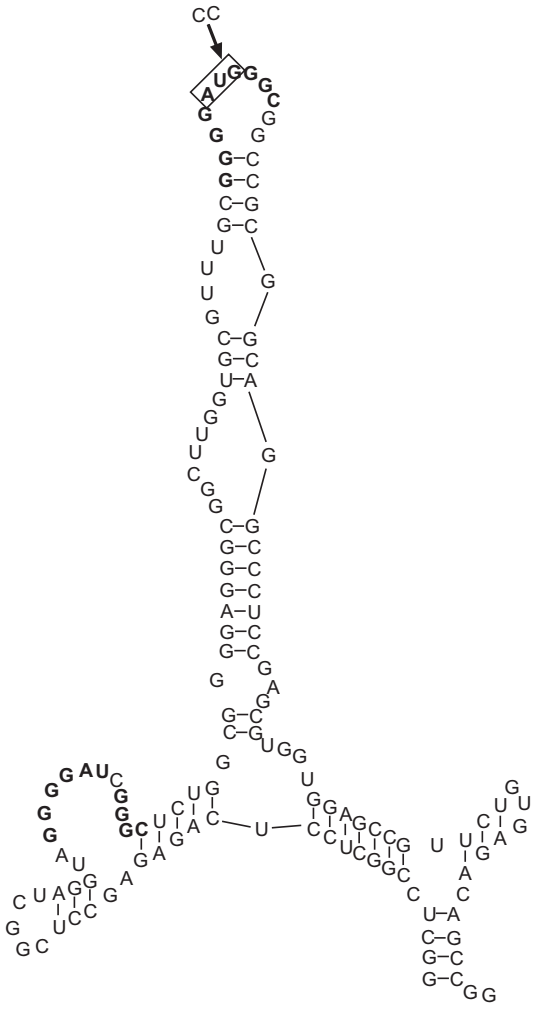


Fig.41 Predicted RNA secondary structure of the 5'UTR of ROSA26.

The computer program RNA2 prediction at http://www.genebee.msu.su/cgi-bin/nph-rna2.pl predicted this secondary structure for the 5'UTR of ROSA26. The endogenous putative start codon is boxed and the insertion site of the two cytosines is indicated by an arrow. Note that nucleotides marked prominent in the predicted loops are equal. The last two G nucleotides of this sequence do still belong to the first ROSA26 exon, whereas the intronic sequences are not shown.

a pyrimidine to pyrimidine exchange and no additional nucleotide is inserted, this could keep the predicted stem loop structure intact and, in addition, destroys the first AUG in the mRNA. In another approach, a complete expression cassette including the RRDR indicator is introduced into the ROSA26 locus. The RRDR indicator can be cut out from the pTE RFP/GFP plasmid together with the CAGGS promoter and inserted in the targeting vector of ROSA26. This would result in independent transcription and translation of RRDR from ROSA26 and would reflect the expression pattern of the CAGGS promoter. Transgenes driven by the CAGGS promoter were shown to be ubiquitously expressed (Ikawa *et al*., 1995; Okabe *et al*., 1997). A third approach to generate a strongly ubiquitously expressed transgene from the ROSA26 locus would be to exchange the complete 5'UTR to a well characterized example as the 5'UTR from the β-globin gene (Strong *et al.*, 1997). This might combine the widespread expression from the ROSA26 promoter together with the translational properties of the β-globin gene.

4.2 The inducibility of Cre*PR in fibroblasts

Cre activity of CrePR fusion proteins has been shown to be dependent on the presence of the synthetic steroid RU486 (Kellendonk *et al*., 1996; Kellendonk *et al*., 1999). However, there exist two major obstacles in the CrePR1 system used to date. First, the CrePR fusion proteins still exhibited a residual activity in the absence of inducer (Psarras *et al*., 2004). Second, the mutation introduced in the LBD of the PR to induce unresponsiveness to progesterone also leads to a decreased affinity to RU486. In this thesis, I have established a CrePR system without any detectable background activity and with increased affinity to RU486 (Wunderlich *et al*., 2001).

In order to improve the affinity of CrePR fusion proteins to RU486, I have examined different lengths of the PR-LBD in CrePR fusion proteins. The two previously used C-terminal truncations PR891 and PR914 were not responsive to progesterone, but still were responsive to RU486 (Vegeto *et al*., 1992; Xu *et al*., 1996). The variants ending with amino acid 914 displayed a significant higher affinity to RU486 than the variants ending with amino acid 891. Extension of the C-terminus of the PR-LBD was likely to result in an enhanced response to the synthetic ligand, since elongation from amino acid 891 to 914 increased the activation potential (Wang *et al*., 1997b). In accordance, I showed that the elongated Cre19PR676-914 reached half maximum activation at a 100-fold lower concentration of RU486 than the shorter Cre19PR676-891. The improvements I have obtained *in vitro* are also expected to hold true in *in vivo* applications, as similar results have been obtained with an improved version of a fusion protein between Cre and the LBD of the estrogen receptor (Indra *et al*., 1999).

In order to increase expression of CrePR fusion proteins, I deleted the first 18 amino acids of Cre, which were considered non-essential for the recombinase activity (Guo *et al*., 1997). Indeed, it turned out that the truncated Cre fusion protein exhibited definitively higher activity as compared to full length constructs. This result was more recently confirmed in a rapamycin-inducible Cre system (Jullien *et al*., 2003).

However, these novel CrePR fusion proteins still exhibited a substantial residual activity in the absence of inducer, varying between three and five percent, which may result from proteolytic cleavage and/or aberrant splicing. Both events could lead to C-terminally truncated forms of CrePR, thus exhibiting uncontrolled recombinase activity. Indeed, aberrant splicing using two cryptic splice sites within CrePR mRNA

are functional in eukaryotic cells (Wunderlich, 1999). Even if this supposed event may result in less then 1% aberrantly spliced mRNAs, this could serve as an explanation for a background activity of a few percent.

In order to reduce the risk of aberrant splicing, a distinct cryptic splice donor at the 3' end of the Cre gene was destroyed, the only one which was still present in the optimized hCre gene. The mutation also introduces a conservative amino acid substitution at this position, namely V336A. This mutated form of hCre, designated Cre*, when fused to any PR-LBD, exhibits negligible background activity, whereas maximum activity is not affected and still reaches more than 40% of authentic Cre. The resulting range of inducibility is more than 200-fold, whereas previously published constructs displayed factors of not more than 40-fold (Kellendonk *et al*., 1996; Kellendonk *et al*., 1999). A side by side comparison of the new Cre*PR system with the previously described CrePR1 system under identical assay conditions revealed a more than 50-fold reduction in residual activity of Cre*PR leading to a dramatically expanded range of inducibility (Wunderlich *et al*., 2001).

4.3 The Cre*PR system in ES cells

To test the Cre*PR system in ES cells, the Cre*PR transgene was inserted into the HPRT locus and into the ROSA26 locus by knock-in. The targetings were performed in ES cells which already contained a loxP-flanked construct at the ROSA26 locus or the HPRT locus, thus allowing to test quantitatively the Cre*PR system first in ES cells and subsequently in chimeric mice.

In a first approach for a knock-in into the HPRT locus, I used an ES cell line which contained RRDR as an indicator for Cre activity in the ROSA26 locus. The promoters EF1α and CAGGS were used to drive the ubiquitous expression of Cre*PR as a single copy gene from the HPRT locus of RRDR ES cells. Since both promoters are ubiquitously active in mice, transgenes should also be expressed in ES cells (Hanaoka *et al*., 1991; Sato *et al*., 2001). However, I was unable to achieve Cre-mediated inducible recombination of the DsRed-stop cassette in EF1αCre*PR clones. Indeed, no protein synthesis of Cre*PR was detectable by Western blot. This is consistent with the previous observation that the EF1α promoter is active in ES cells only at a very low level (Hanaoka *et al*., 1991).

Though controversially discussed, the EF1α gene is subjected to translational downregulation mediated by a cis acting element in the 5'UTR of its mRNA (Zhu *et al*., 2001). This cis acting element is an oligopyrimidine rich tract (TOP) close to the CAP site of mRNAs of many translational initiation factors and elongation factors, which is bound by inhibitory proteins such as La (Cardinali *et al*., 2003) in cells that are growth-arrested (Avni *et al*., 1997; Biberman *et al*., 1997; reviewed in Wilkie *et al*., 2003). Since ES cells are dividing continuously, the EF1α gene product should not be downregulated. However, an EF1α CAT transgene was downregulated in undifferentiated ES cells, but was expressed in ES cell derived neuronal precursors (Hanaoka *et al*., 1991).

Remarkably, when these ES cells were used to generate mice, the CAT transgene was expressed in most tissues of the mice. Therefore, I tried to differentiate the ES cells into neuronal precursors *in vitro*, in order to investigate the properties of EF1αCre*PR (Strickland *et al*., 1980; Coleman *et al*., 1996). Again, I did not detect protein expression of Cre*PR in the potential neuronal precursors, and hence no induction of Cre activity.

There are two alternative explanations for the latter observation. First, the HPRT locus in EF1αCre*PR ES cells is always present as a single copy gene, whereas the ES cells used in Hanaoka *et al*. (1991) contained multiple copies of the EF1αCAT transgene. Second, it is possible that the neuronal precursor cells were not differentiated to the neuronal lineage. In the neuronal differentiation protocol according to Strickland *et al*. (1980), the ES cell-derived neuronal precursors were identified by morphological criteria which was also applied by Hanaoka *et al*. (1991) and also in my experiment. Later on, Okabe and coworkers (1996) proved the neuronal differentiation of ES cells by control stainings against the nestin gene product. However, the evidence for nestin expression in these cells is of limited usage for distinguishing between neuronal precursor cells and ES cells, since nestin is also expressed in ES cells (Lenka *et al*., 2002). At present, it is attempted to identify differentiated neuronal precursors derived from ES cells by coupling the morphology of the cells with the downregulation of an Oct-4 EGFP transgene stably integrated into the genome of ES cells, indicating their differentiation since Oct-4 is only expressed in non-differentiated ES cells.

In contrast to the EF1αCre*PR approach, I succeeded to achieve ligand inducible recombination of the RRDR indicator in the absence of background recombination when Cre*PR in the HPRT locus was driven by the CAGGS promoter. However, the inducibility of Cre*PR by the ligand RU486 is odd in so far as it is not dependent on the dose of ligand and, even at a given concentration of ligand, maximal Cre-mediated deletion in 81% of the ES cells can only be achieved upon daily administration of fresh ligand for 22 days. The latter observation cannot be satisfactorily explained in terms of the well known short half life of RU486 in medium (Deraedt *et al*., 1985a). One possibility for the delayed activation of Cre*PR is that Cre-mediated recombination is dependent on the cell cycle (Loonstra *et al*., 2001; Jo *et al*., 2003). Regular trypsinization keeps ES cells in an undifferentiated stage and synchronizes their cell cycle (Held *et al*., 1989). However, trypsinized and non-trypsinized cells did not reveal any significant difference in Cre-mediated deletion efficiencies of the DsRed-stop cassette of the RRDR indicator. A second possibility is an impaired availability of RU486. Indeed, it is known that, in man, RU486 is bound in the serum to α-1-acid-glycoprotein, thus limiting its tissue bio-availability. Since the RU486-induced Cre-mediated recombination in ES cells was performed in the presence of 15% FCS, it is reasonable to assume such a binding of RU486 to unknown serum components. This possibility is currently being tested by using serum free medium to induce Cre*PR activity in targeted ES cells.

In a third approach, Cre*PR was targeted into the ROSA26 locus. An ES cell clone from U.Strobel was used which contained a conditional allele for an artifical LMP1-CD40 fusion protein in the HPRT locus. Administration of the ligand RU486 to activate Cre*PR did not reveal any excision of the loxP-flanked stop cassette of the LMP1-CD40 allele. Moreover, treatment with 2 µM HTNC was not able to induce any deletion of the conditional LMP1-CD40 allele, although deletion efficiencies of up to 97% are normally achieved with HTNC in ES cells (Peitz *et al*., 2002). The observation that neither ROSACre*PR nor HTNC could excise the loxP-flanked sequence in the HPRT locus is not yet understood. A possible defect in the Cre*PR was not the reason for the negative outcome, since the Cre*PR was verified by sequencing after PCR amplification of the construct from ES cells and the Cre*PR was expressed from the ROSA26 locus, as revealed by Western blot analysis. Therefore, it is possible that there was a defect in the LMP1-CD40 construct. For instance, one of the loxP sites can be mutated or the restriction digest used to detect

the excised allele is not indicative to distinguish between loxP-flanked allele and the excised allele. Another possibility is that the loxP sites of conditional alleles in the HPRT locus may not be accessible for Cre, since the loxP sites may be incorporated into unfavourable chromatin environment. Indeed, such a situation was recently described to occur, when inducible Cre systems were used to delete loxP-flanked targets inserted into the ROSA26 locus (Vooijs *et al*., 2001).

4.4 The Cre*PR system in mice

Several chimeric mice derived from CAGGS Cre*PR; RRDR ES cells were used to perform preliminary *in vivo* experiments, in which the DsRed-stop cassette of RRDR was inducibly excised by Cre*PR after administration of RU486 in a broad range of the organs, as detected by a PCR based analysis. In particular, it is worth mentioning that this system did not reveal any significant background activity of Cre*PR.

The CAGGS Cre*PR transgene is linked to the HPRT locus on the X chromosome. For the *in vivo* analysis of CAGGS Cre*PR, male mice were generated that carry both the CAGGS Cre*PR and the loxP-flanked target gene. The RAGE-EG reporter, which indicates Cre-mediated recombination by eGFP expression (Constien *et al.*, 2001), was used to analyze the inducibility of the CAGGS Cre*PR. No background recombination was detected in untreated mice indicating the tightness of the Cre*PR system. However, when four week old mice were injected with RU486, excision of the RAGE conditional allele was achieved only in liver, kidney and brain with an efficiency of approximately 13% quantified by Southern blot. In Org31710 injected mice, CAGGS Cre*PR could mediate recombination only in liver. The restriction of inducible recombination to a few organs and the low deletion efficiencies in these organs were unexpected, as deletion occurred in most organs of the CAGGS Cre*PR/RRDR chimeras upon administration of RU486.

No RU486 inducible activation of Cre*PR was detected in ROSACre*PR mice intercrossed with several different loxP-flanked alleles such as the loxP-flanked gene segment of the DNA polymerase-β (Gu *et al*., 1994), the RAGE-EG reporter (Constien *et al.*, 2001), and the RRDR indicator.

Why is Cre*PR activity not inducible in the ROSACre*PR strain and only at low levels in the CAGGS Cre*PR strain? There are three possible explanations and two of them I can exclude experimentally.

One possibility is that the inducer RU486 is not available at sufficient concentrations *in vivo*. The metabolism of RU486 in organisms is not yet well understood (Deraedt *et al*., 1985b; Babij *et al*., 2003; for review, see e. g. Sakar, 2002). In humans, it was reported that RU486 reached a maximal concentration in the serum 1-2 hours after oral application (Leminen *et al*., 2003), and this did not progressively increase upon application of increasing amounts of RU486 (Heikinheimo & Kekkonen, 1993; Heikinheimo *et al*., 2003). In the serum, RU486 exists in a bound and an unbound state. In the bound state, RU486 is stabilized by interaction with the α-1-acid glycoprotein, the concentration of which is limited. The unbound RU486 is quickly metabolized in the liver by a two step process, i.e. demethylation and hydroxylation, with its metabolites detectable in plasma about 1 hour after oral application. Metabolites bind to the PR with an affinity of only 10 to 20% as compared with the parent RU486. In order to determine the bio-availability of RU486 in the serum of mice, mice were injected with RU486 and sera were isolated after various periods of time. Subsequently, sera were applied to twice targeted ES cells (CAGGS Cre*PR; RRDR) to test the RU486 activity. Sera taken after 1 hour and 4 hours induced high Cre*PR activity as indicated by excision of the DsRed-stop cassette of RRDR-DsRed, thus demonstrating that RU486 is active in serum when applied by *i.p*. injection.

The second possibility is that Cre*PR becomes posttranslationally modified preventing the protein to fulfil its function. This view is supported by the fact that the endogenous PR, for example, can be hyper-phosphorylated *in vivo* at special residues, which has been shown to influence the translocation of the PR from cytoplasm to nucleus (Chauchereau *et al*., 1992). In order to test this possibility for the Cre*PR, I performed experiments with embryonic fibroblasts (MEFs). These MEFs contain the Cre*PR on one allele of ROSA26 and the RRDR indicator on the other allele. Cytoplasmic fractions were separated from the nuclear extracts of RU486 induced and non-induced MEFs and analyzed by Western blot. In the cytoplasmic fraction of non-induced MEFs, Cre*PR was abundant, whereas almost no Cre*PR protein was detectable in the cytosol of induced MEFs. To exclude a possible degradation of the Cre*PR after ligand binding, controls were included in this experiment, in which the complete cell lysates of induced and non-induced MEFs were loaded on the Western. In both controls, the Cre*PR protein was present in equal amounts. This experimental approach indicates that the Cre*PR protein

translocates to the nucleus upon RU486 binding in MEFs, but failed to recombine the DsRed-stop cassette of RRDR-DsRed for unknown reasons. The possibility that Cre*PR is not imported in the nucleus, but is retained at the nuclear pore complexes and therefore cannot excise the DsRed-stop cassette seems to be rather unlikely. Since the non-ionic detergent NP-40 used for isolation of the cytosolic fraction is known to solubilize the nuclear envelope (Wunderlich *et al.*, 1976), the cytosol should contain that Cre*PR which possibly sticks to the nuclear pore complexes. However, this is not consistent with my findings. Therefore, I conclude from this experiment that Cre*PR can indeed translocate to the nucleus upon RU486 binding, but, recombination of the DsRed-stop cassette did not take place, presumably due to the low expression level of Cre*PR.

The third possible explanation is that the Cre*PR protein is not expressed at sufficient levels to induce Cre-mediated recombination. In order to examine this possibility, Western blot analysis of lysates from all major organs of CAGGS Cre*PR mice was performed which all displayed a specific band of Cre*PR. The highest expression levels were detected in liver being one of the three organs in which deletion of the loxP-flanked region of the RAGE-EG reporter occurred after administration of RU486 and Org31710. These data suggest that high levels of Cre*PR have to accumulate in cells to achieve RU486 inducible recombination.

Experimental conditions to induce high levels of Cre*PR activity have to be still further investigated. However, the fact that even under experimental conditions of high protein levels, the Cre*PR system does not exhibit any significant background activity is of major relevance for other experimental approaches. For instance, a stringently controllable Cre system without any background activity is required for Cre-mediated activation of cancer promoting genes (Lakso *et al*., 1992; Meuwissen *et al*., 2001; Politi *et al*., 2003). In such experiments, background activity will result in immortalization and proliferation of cancer cells and, thus, in undesired tumor formation.

4.5 B cell specific inducible Cre systems

In order to insert Cre*PR into the mCD19 locus, which is specifically expressed in B cells, a new CD19 targeting vector was generated, in which insertion of any transgene can be performed by a single cloning step. This new vector is similar to the

original CD19-cre targeting vector from Rickert *et al*. (1995), but it contains an elongated short arm of homology, which makes a cointegration event of the disrupted endogenous CD19 start codon more likely. With this vector, I succeeded to generate a correctly targeted ES cell clone in the CD19Cre*PR targeting, which additionally carried the restriction site introduced instead of the start AUG. I expected higher expression levels of the Cre*PR in B cells than in the original CD19-cre mouse strain. High expression of Cre is achieved in the latter mouse strain, despite the fact that the endogenous CD19 start codon is still retained in the first exon and the inserted Cre in the second exon is not in frame to that codon. This might be due to the introduction of a Kozak consensus sequence (Kozak, 1987) at the start codon of the Cre gene, which optimizes the initiation of translation (J. Roes, personal communication). The CD19Cre*PR transgene also contains the Kozak consensus sequence and the first AUG of the mRNA is that from the Cre*PR. Indeed, I could verify the B cell specific expression of Cre*PR at the RNA level by RT-PCR and at the protein level by Western blot. Correct integration of Cre*PR into the mCD19 locus was confirmed by flow cytometry. These data demonstrate that the CD19Cre*PR mouse strain reflects the expression pattern of the CD19-cre mouse and, thus, a B cell restricted expression of Cre*PR.

Though Cre*PR was expressed at reasonable levels, I was not successful to induce Cre*PR activity *in vivo* by RU486 administration in mice carrying the CD19Cre*PR and the RRDR indicator. Possible explanations for that were already discussed above and the analysis of the CD19Cre*PR mouse strain is still ongoing. In particular, I currently try to inducibly excise the loxP-flanked gene segment of the insulin receptor (Brüning *et al*., 1998) in B cells using the CD19Cre*PR strain, since this loxP-flanked gene appears to be well accessible for inducible Cre-mediated recombination as revealed by high excision efficiency using the ROSACreERT2 mouse strain (Seibler *et al.*, 2003; L. Koch & J. Brüning, personal communication).

A new B cell specific inducible Cre system is urgently required. To this end, I am currently performing the knock-in of the CreERT2 fusion protein into the CD19 locus. This approach may achieve highly B cell specific tamoxifen inducible Cre activity in the subsequently generated CD19CreERT2 mouse strain with tolerable background activity. The expectation to obtain B cell specific expression of CreERT2 from the CD19 locus is supported by the fact that Cre*PR expression is restricted to B

lymphocytes in the novel CD19Cre*PR mouse strain and that the same targeting strategy will be used to insert the CreERT2 into the second exon of the CD19 gene. Moreover, tamoxifen inducible Cre-mediated recombination in B cells can be achieved either with the EDCre strain (Schwenk et al., 1998) or with the ROSACreERT2 strain (Seibler *et al*., 2003), indicating that the CreERT2 system has the potential to work in B cells.

4.6 Doxycycline inducible systems

The tet-on systems presently used consist of the doxycycline regulatable rtTA transgene and the tet-responsive cassette driving the transgene of interest. A major disadvantage of this system is that mouse strains carrying these two transgenes have to be intercrossed. A still higher breeding effort has to be undertaken with respect to a doxycycline-controllable Cre, since four different alleles have to be combined in one mouse. In order to reduce the number of breeding steps, I constructed a tet-inducible system for Cre, in which both elements of the tet-on system are combined in one allele. In this system, the improved rtTA-M2 transactivator (Urlinger *et al*., 2000) is driven by either the CAGGS promoter or the EF1α promoter and these two transgenes were inserted separately into the HPRT locus of RRDR ES cells.

n order to test these systems first *in vitro*, I did not perform transient transfections, but stably integrated the transgenes. The reason for this is that transient transfections of tet-systems result in spontaneous activation of the CMV minimal promoter, which is part of the tetO7 promoter (Baron *et al*., 1995) and which initiates the non-inducible transcription of the gene of interest, when many copies of the transgene are present in the cell. In my targeting experiments, I noticed that electroporation of the HPRT tet-on targeting vectors in RRDR ES cells resulted also in a transient expression phase. Administration of doxycycline to ES cells targeted with the CAGGS tet-on system resulted in inducible expression of Cre and, thus, in high levels of Cre-mediated excision of the DsRed-stop cassette of RRDR-DsRed. However, ES cells cultured without doxycycline displayed a significant background activity.

This background activity can be explained in two ways. First, the expression level of the rtTA driven by the CAGGS promoter could be too high, thus causing the ‘leaky’ expression of the Cre from tetO7. Though this possibility cannot be excluded,

another explanation seems to be more likely. This possibility specifically concerns the CAGGS promoter which consists of the chicken β-actin promoter coupled to the CMV enhancer (Niwa *et al*., 1991). Since enhancers are acting over long distances (reviewed in Ogata *et al.*, 2003) and both elements, i.e. the CMV enhancer and the CMV minimal promoter of the tetO7 are located in the same construct in close vicinity, it is possible that these two elements can act together and activate the 'leaky' Cre transcription. This view is supported by the finding that the tet-responsive cassette has to be integrated into a site of the genome that is distant from any enhancer activity to avoid the spontaneous activation of the tetO7 promoter (Schönig *et al*., 2003). Indeed, an integration site with this feature is the HPRT locus I have used (Melton *et al*., 1984).

In contrast to the CAGGS tet-on system, the EF1α tet-on system displayed no significant background activity. After doxycycline administration, however, Cre-mediated excision of the DsRed-stop cassette of RRDR-DsRed was incomplete and only low levels of Cre protein were detected by Western blot. This can be explained by the low level activity of the EF1α promoter in ES cells, as already discussed before. However, this low level activity appeared to be sufficient to express the rtTA-M2, which after doxycycline binding activated expression of Cre from the tetO7 promoter, at least in some ES cells. The results obtained with the EF1α tet-on system *in vitro* are promising enough to investigate the performance of this system *in vivo*. This work is in progress.

5.1 SUMMARY

Inducible gene targeting based on the Cre/loxP system is a novel technique allowing conditional gene modification in mice. Precondition of this method is the regulation of Cre activity by inducible genetic switches. This study aimed at improving inducible systems for the regulation of Cre activity at both the transcriptional and the posttranslational level, which should be tested first *in vitro* and subsequently in newly generated transgenic mice. In order to detect Cre activity, a special double indicator containing DsRed and eGFP was constructed and targeted into the ROSA26 locus of HM-1 ES cells. This indicator should constitutively express the DsRed gene flanked by loxP sites. Cre-mediated recombination excises the DsRed gene and turns on the eGFP gene, as evidenced by a change in fluorescence from red to green. Though the double indicator worked properly in transiently transfected fibroblasts, the targeting strategy used for the integration of the indicator into the ROSA26 locus presumably disrupted a putative stem loop motif of the 5'UTR resulting in a silencing of the indicator at the protein level. However, the ES cell line with the integrated double indicator is suited for targetings of inducible Cre constructs into the HPRT locus, since inducibilities are detectable by Southern blot analysis. The Cre*PR system was one inducible system I have established. In contrast to the classical CrePR1 system, the background activity of Cre*PR was almost abolished by introducing a point mutation into the 3'end of the Cre part, thus preventing aberrant splicing of the Cre*PR encoding mRNA. Also, the PR-LBD of the Cre*PR system was improved, compared to the CrePR1, to obtain a higher affinity towards the inducer RU486. The Cre*PR system, driven by either the EF1α promoter or the CAGGS promoter, was then targeted into the HPRT locus. The CAGGS Cre*PR worked in ES cells as expected. These targeted ES cells were subsequently used to generate transgenic mice. In these mice, high protein levels of Cre*PR have to be obtained in order to achieve inducible Cre-mediated excision of loxP-flanked gene segments. In addition, I generated a novel B cell specific Cre*PR mouse strain by targeting Cre*PR into the mCD19 locus. In another approach to inducibly regulate Cre, a tet-on system was established consisting of the rtTA-M2 transactivator and the tet-responsive Cre cassette, which was targeted into the HPRT locus of the double indicator ES cell line. This system revealed a tight regulation of Cre expression in a doxycycline dependent manner.

5.2 ZUSAMMENFASSUNG

Induzierbare gezielte Mutagenese basierend auf dem Cre/loxP System ist eine neue Methode, die es erlaubt Gene konditional in Mäusen zu verändern. Vorraussetzung dafür ist die Regulierung von Cre durch induzierbare genetische Schalter. Diese Arbeit zielte darauf ab die Schalter für die Regulation von Cre auf transkriptionellem und posttranslationalem Niveau zu verbessern, diese zuerst *in vitro* zu testen und dann in transgenen Mäusen. Um die Cre Aktivität zu detektieren, wurde ein spezielles, doppeltes Reporterkonstrukt für Cre mit DsRed und eGFP konstruiert und in den ROSA26 Locus von HM-1 ES Zellen eingebracht. Dieser Reporter sollte konstitutiv das von loxP Sequenzen flankierte DsRed Gen exprimieren. Cre vermittelte Rekombination schneidet das DsRed Gen aus und stellt das eGFP Gen an, was durch einen Fluoreszenzwechsel von rot zu grün sichtbar wird. Obwohl der Doppel-Reporter in transient transfizierten Fibroblasten funktionierte, zerstörte das gezielte Einbringen des Reporters in den ROSA26 Locus vermutlich ein bisher unbekanntes, mögliches Stamm-Loop-Motiv der 5' untranslatierten Region der ROSA26 mRNA, was zu einem Verlust der Translation führte. Die Reporter ES Zell-Linie wurde jedoch weiterhin genutzt, um induzierbare Cre Konstrukte in den HPRT Locus einzubringen, da man deren Induzierbarkeit über Southern blot nachweisen kann. Das Cre*PR System war eines der induzierbaren Systeme, die ich etabliert habe. Gegenüber dem klassischen CrePR1 System wurde die Hintergrundaktivität des Cre*PR Systems durch Einführen einer Mutation am 3' Ende vom Cre-Anteil fast abgestellt, welche kryptische Spleißereignisse der Cre*PR kodierenden mRNA verhindert. Auch die PR-LBD des Cre*PR Systems wurde gegenüber dem CrePR1 System verbessert, um eine höhere Affinität zum Induktor RU486 zu erreichen. Das Cre*PR System wurde gezielt in den HPRT Locus eingebracht und dann entweder vom CAGGS oder dem EF1α Promoter exprimiert. Das CAGGS Cre*PR Konstrukt zeigte in ES Zellen eine stringent regulierbare Cre-Aktivität und die ES Zellen wurden somit genutzt, um transgene Mäuse herzustellen. In diesen Mäusen mussten hohe Cre*PR Proteinkonzentrationen erreicht werden, um induzierbare Cre vermittelte Rekombination zu erreichen. Ein neuer B Zell spezifischer Cre*PR Maus Stamm wurde durch das gezielte Einbringen des Cre*PR in den mCD19 Locus hergestellt. In einem weiteren Ansatz, Cre induzierbar zu regulieren, wurde ein tet-on System etabliert, welches aus dem rtTA-M2 Transaktivator und der tet-ansprechenden Expressionskassette für Cre besteht, welches weiterhin in den HPRT Locus der Doppelreporter ES Zell-Linie eingebracht wurde. Experimente in ES Zellen zeigten eine stringente Doxyzyklin abhängige Regulation der Cre Expression.

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8. VERSICHERUNG

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Köln, im März 2004 Frank Thomas Wunderlich

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10. LEBENSLAUF

Persönliche Daten:

Name	Frank Thomas Wunderlich
Geburtsdatum	28.07.1972
Geburtsort	Freiburg i. Br.
Adresse	Burgstr.31 41469 Neuss Thomas.Wunderlich@uni-koeln.de
Familienstand	geschieden
Nationalität	deutsch

Schulische Ausbildung:

1979-83	Weiherhof Grundschule, Freiburg
1983-89	Droste Hülshoff Gymnasium, Freiburg
1989-92	Gymnasium Norf, Neuss
1992	Abitur

Zivildienst:

1992-94	Zivildienst im Alexianer Krankenhaus für Psychiatrie

Studium:

1994-96	Grundstudium Diplom Biologie an der Gutenberg Universität Mainz
1996	Vordiplom
1996-98	Hauptstudium Diplom Biologie an der Albertus Magnus Universität Köln
Dez. 1998	Diplomprüfung: Hauptfach Genetik, Nebenfächer Biochemie und Entwicklungsbiologie
1998-99	Diplomarbeit: Modifizierung and Analyse von optimierten Cre Rekombinase Expressionsvektoren zur verbesserten Expression in eukaryontischen Zellen. Bei Prof. K. Rajewsky, Köln.

Promotion:

Seit Feb. 2000	Generation of inducible Cre systems for conditional gene inactivation in mice. Bei Prof. K. Rajewsky, Köln.

10. SUPPLEMENTARY DATA

The compact disc located at this page contains sequence information of the generated expression plasmids and targeting vectors in pdf format.