RESOURCE ARTICLE

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Dre recombinase, like Cre, is a highly efficient site-specific recombinase in *E. coli*, mammalian cells and mice

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SUMMARY

Tyrosine site-specific recombinases (SSRs) including Cre and FLP are essential tools for DNA and genome engineering. Cre has long been recognized as the best SSR for genome engineering, particularly in mice. Obtaining another SSR that is as good as Cre will be a valuable addition to the genomic toolbox. To this end, we have developed and validated reagents for the Dre-*rox* system. These include an *Escherichia coli*-inducible expression vector based on the temperature-sensitive pSC101 plasmid, a mammalian expression vector based on the CAGGs promoter, a rox-lacZ reporter embryonic stem (ES) cell line based on targeting at the Rosa26 locus, the accompanying Rosa26-rox reporter mouse line, and a CAGGs-Dre deleter mouse line. We also show that a Dre-progesterone receptor shows good ligand-responsive induction properties. Furthermore, we show that there is no crossover recombination between Cre-*rox* or Dre-*loxP*. Hence, we add another set of efficient tools to the genomic toolbox, which will enable the development of more sophisticated mouse models for the analysis of gene function and disease.

INTRODUCTION

Many applications have been found for site-specific recombinases (SSRs) in DNA and genome engineering. Cre recombinase from the coliphage P1 and FLP recombinase from the *Saccharomyces cerevisiae* 2 micron circle have become the most widely used SSRs (Branda and Dymecki, 2004; Glaser et al., 2005). Both belong to the class of tyrosine recombinases because they share a common reaction mechanism, involving nucleophilic attack on the phosphodiester backbone by a tyrosine hydroxyl group, to establish the covalent protein-DNA intermediate during recombination (Grindley et al., 2006).

Cre and FLP mediate recombination between target sites, termed *loxP* and *FRT*, respectively, which are both 34 base pairs (bp) long and are based on 13-bp palindromes separated by 8-bp spacers. Recombination occurs between two *loxP* or *FRT* sites through the spacer regions. By deploying the target sites in different ways, a variety of different outcomes have been achieved. In DNA engineering, notable examples include the fluent conversion of λ phage inserts into plasmids (Elledge et al., 1991), removal of selectable genes (Zhang et al., 1998), linearization of bacterial artificial chromosomes (BACs) (Mullins et al., 1997) and biotin end-labeling (Buchholz and Bishop, 2001). In genome engineering, the most important applications include conditional mutagenesis (Gu et al., 1994; Kuhn et al., 1995; Logie and Stewart, 1995), gene expression switches (O'Gorman et al., 1991; Lakso et al., 1992; Angrand et al., 1998), lineage tracing (Awatramani et al., 2003), chromosomal engineering (Ramirez-Solis et al., 1995; Su et al., 2000; van der Weyden and Bradley, 2006), chromosomal translocations (Smith et al., 1995; Herault et al., 1998; Buchholz et al., 2000; Collins et al., 2000; Liu et al., 2002), recombinase-mediated cassette exchange (RMCE) (Schlake and Bode, 1994; Baer and Bode, 2001; Wallace et al., 2007), removal of selectable genes (Gu et al., 1993) and conversions of multipurpose alleles (Nagy et al., 1998; Testa et al., 2003; Testa et al., 2004; Schnutgen et al., 2005).

The successes of Cre and FLP in genome engineering arise from their simplicity and efficiency. In terms of simplicity, both enzymes do not require any cofactors and their recombination target sites (RTs) are well defined and precisely understood (Senecoff et al., 1988; Lee and Saito, 1998). These RTs are small enough to be introduced very easily during DNA engineering, but large enough to minimize the problems associated with cryptic occurrence in eukaryotic genomes.

The remarkable efficiency properties of Cre recombinase have emerged from many studies. For example, Cre can delete megabase regions and can mediate translocations between non-homologous chromosomes in mammalian genomes at remarkable efficiencies (Su et al., 2000; Wu et al., 2007). However, it was realized some time ago that FLP was less efficient than Cre, partly owing to an unsuitable optimum temperature for mammalian cells (Buchholz et al., 1996), which led to the application of molecular evolution to identify the thermostable variant called FLPe (Buchholz et al., 1998). However, FLPe is still less efficient than Cre, although a recent codon-optimized FLPe, termed FLPo, has bridged some of the remaining gap (Raymond and Soriano, 2007) (K.A., unpublished data).

The weaker properties of FLP, as well as the merits in finding a third efficient recombinase for genome engineering, prompted searches for new tyrosine SSR tools (Araki et al., 1992; Ringrose et al., 1997; Christ et al., 2002). The search also encompassed

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experiments with the serine SSRs, which were largely unfruitful until the large serine recombinases were discovered (Thorpe and Smith, 1998). Two members of this class, phiC31 and phiBT1, have now been shown to work in eukaryotic genome engineering applications (Groth et al., 2000; Belteki et al., 2003; Groth et al., 2004; Keravala and Calos, 2008; Xu et al., 2008). Large serine SSRs bring the advantage that they mediate directional, rather than reversible, recombination. Although the issue has not been published systematically, our experience and the published reports indicate strongly that none of these other SSRs share the remarkable efficiencies of Cre in mammalian genome engineering.

Hence, the recent publication of a Cre-like SSR, Dre, presented the attractive possibility that it could be a tool that is as remarkable as Cre. Dre was identified in a search through P1-like phages for a Cre-like enzyme that had diverged sufficiently to recognize an RT that is distinct from *loxP* (Sauer and McDermott, 2004). The Dre RT was termed *rox*. The Dre-*rox* system was tested in *Escherichia coli*, as well as by transient expression in Chinese hamster ovary (CHO) cells, with good results. Unfortunately, the Dre-*rox* materials reported by Sauer and McDermott (Sauer and McDermott, 2004) were unavailable for use and independent verification. Rather than simply repeat these experiments, we decided to build Dre expression constructs according to our decisions regarding the best design.

RESULTS

Validation of Dre-*rox* reagents in *E. coli*

To evaluate the data of Sauer and McDermott (Sauer and McDermott, 2004), we obtained an aliquot of the original D6 phage from June Scott (Emory University, GA) and built an expression construct in the temperature-sensitive plasmid pSC101 (Hashimoto-Gotoh and Sekiguchi, 1977) under the arabinose-inducible BAD promoter (Guzman et al., 1995). We also designed and ordered a mammalian codon-optimized version from GeneArt (supplementary material Fig. S1), and cloned it into the BAD-pSC101 plasmid. We built a plasmid to serve as a *rox* reporter in *E. coli* that was also a Rosa26-targeting construct (Fig. 1). Complete recombination between the *rox* sites was achieved after arabinose-induced expression of Dre from pSC101-BAD-Dre in *E. coli*. Notably, no recombination was observed without arabinose induction, indicating that the combination of the BAD promoter in the low-copy pSC101 plasmid delivered excellent on-off regulation of Dre in *E. coli* (Fig. 1B; and results not shown). This outcome was notable because we have been previously unable to achieve complete repression of Cre in *E. coli* under various promoters (e.g. Buchholz et al., 1996). Therefore, we constructed the equivalent pSC101-BAD-Cre expression plasmid and found that this configuration also served to keep Cre repressed when uninduced (Fig. 1B; and results not shown).

Using these Dre and Cre expression plasmids, we found that Dre did not provoke any recombination between *loxP* sites and that Cre did not provoke any recombination between *rox* sites (Fig. 1B; and results not shown). We did not observe any difference between the original or codon-optimized Dre in these tests (results not shown). These results confirm those of Sauer and McDermott, and validate the performance of our new reagents.

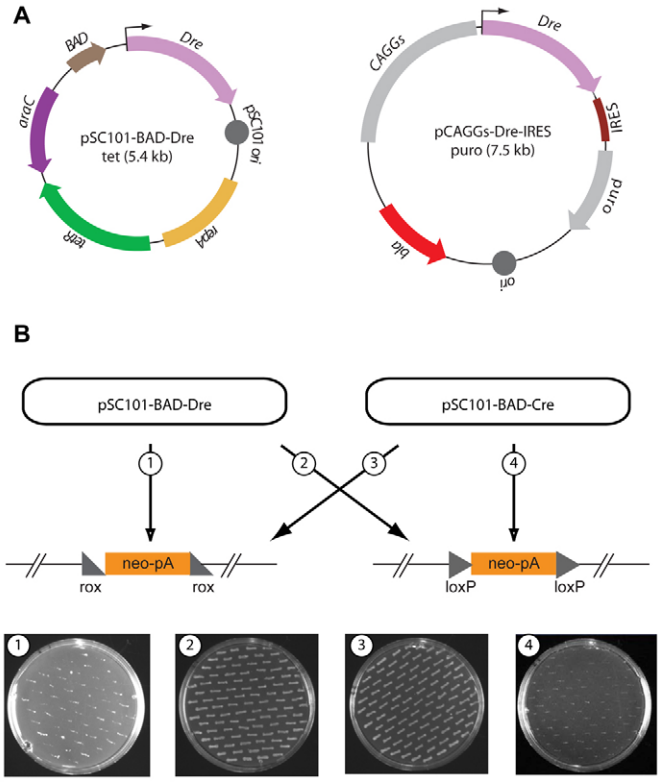

Fig. 1. Plasmids and prokaryotic assay. (A) Plasmid diagrams showing pSC101-BAD-Dre and pCAGGs-Dre-IRES-puro. BAD, arabinose-inducible promoter; *repA*, encodes the replication protein for the pSC101 origin; *tetR*, tetracycline resistance gene; *araC*, encodes the regulator of the BAD promoter; CAGGs, the composite cytomegalovirus (CMV) enhancer/β-actin promoter; IRES, the internal ribosome entry site from encephalomyocarditis virus (EMCV); *puro*, puromycin resistance gene; *bla*, ampicillin resistance gene. (B) Test for complete recombination in *E. coli*. Either pSC101-Dre or pSC101-Cre were introduced into cells containing either a *rox* or *loxP* reporter, as indicated, followed by streaking individual *E. coli* colonies onto kanamycin plates. (1) Dre recombines completely with the *rox* reporter so that no growth was observed under selection for kanamycin resistance, which was conveyed by the neomycin (*neo*) gene. (2) Dre did not affect the growth of cells containing the *loxP* reporter. (3) As for plate 2, except that the Cre expression plasmid and the *rox* reporter were used. (4) As for plate 1, except that the Cre expression plasmid and the *loxP* reporter were used.

Generation of CAGGs-Dre ES cells and mice

The CAGGs-Dre-IRES-puro construct was built by modifying pCAGGs-FLPe-IRES-puro (Schaft et al., 2001). pCAGGs-Dre-IRES-puro was linearized with *Spe*I and electroporated into R1 ES cells. Cells were selected with 1 μg/ml puromycin and 23 colonies were picked and screened by Southern analysis to find clones with single-copy integrations. After injection into 8-cell embryos (Poueymirou et al., 2007), germline transmission for one of these single-copy clones was obtained.

Generation of a Rosa26-Dre reporter ES cell and mouse line

We next established a Rosa26-rox reporter ES cell line by gene targeting in R1 ES cells (Fig. 2A). We used the Rosa26 targeting strategy of Soriano (Soriano, 1999). The Rosa-rox-neo-rox-lacZ construct was electroporated into R1 ES cells, and G418-resistant

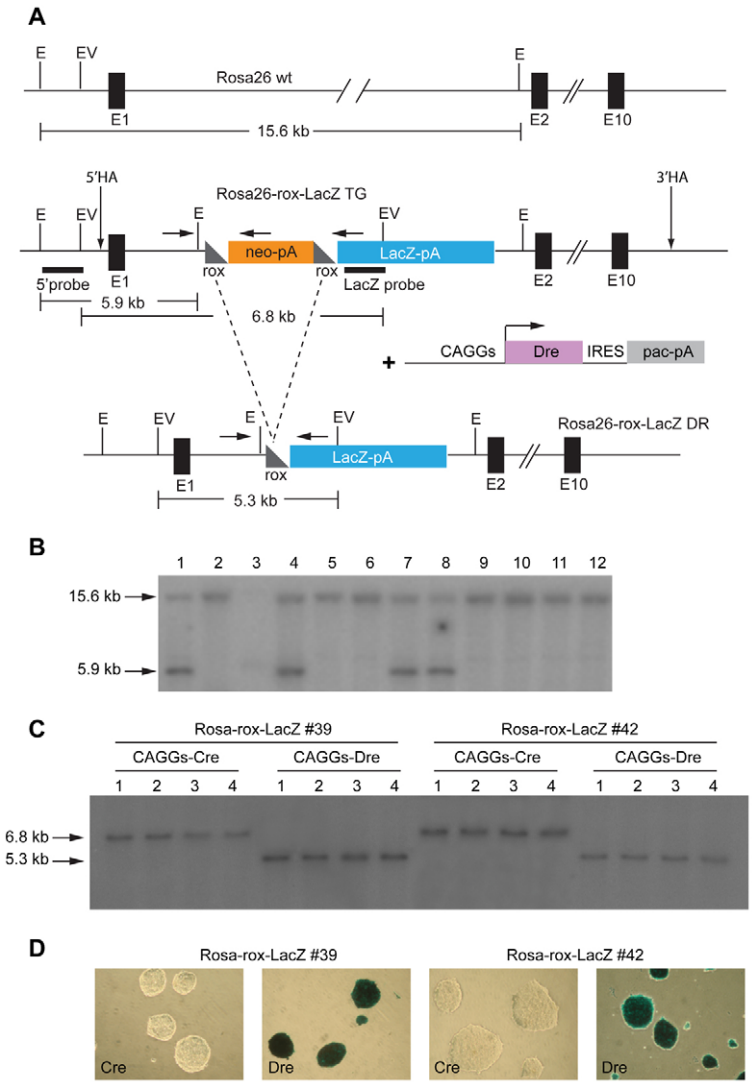

Fig. 2. Targeting of the Dre reporter to the Rosa26 locus and Dre recombination in ES cells. (A) The Rosa26 locus is illustrated before (above) and after (middle) targeting. The extents of the 5′ and 3′ homology arms (5′HA, 3′HA) are indicated by arrows. Targeting of a *rox*-flanked splice acceptor-neomycin resistance gene with polyadenylation signal (neo-pA) followed by the *lacZ* gene was detected by Southern analysis using the 5′ probe and *Eco*RI (E) digestion. After Dre recombination (below), the *lacZ* probe was used for Southern analysis with *Eco*RV (EV) digestion. The horizontal arrows represent the primers used for detecting recombination events. Exons are represented by black rectangles (E1, E2, E10). (B) Southern hybridization of DNA prepared from resistant ES colonies (1-12), digested with *Eco*RI and hybridized with the 5′ probe. The wild-type (wt) allele is 15.6 kb and the targeted allele is 5.9 kb. (C) Southern hybridization of DNA prepared from double stable ES colonies transfected with either the Cre or Dre recombinase. The DNA was digested with *Eco*RV and hybridized with a *lacZ* probe. The unrecombined allele is 6.8 kb and the recombined allele is 5.3 kb. (D) Representative β-galactosidase (LacZ) staining of Rosa-rox reporter colonies (either colony 39 or 42) that have been stably transfected with Cre or Dre recombinases.

colonies were screened by Southern analysis. The targeting efficiency was 30% (Fig. 2B). Two correctly targeted clones were electroporated with either the CAGGs-Dre-IRES-puro or the CAGGs-Cre-IRES-puro construct, and constitutive Dre or Cre expression was selected with puromycin. We picked 12 clones from each electroporation and stained them for β-galactosidase expression. We found complete homogenous staining of all colonies transfected with Dre, but no staining whatsoever of colonies transfected with Cre (Fig. 2C,D). Similarly, Southern analysis showed complete Dre recombination in the Dre-stable clones and no recombination in any clone that contained Cre recombinase.

Dre recombination in mice

For the generation of the Rosa-rox reporter mouse, two targeted ES clones were injected into 8-cell stage embryos, and one of them resulted in germline transmission. To test whether the CAGGs-Dre transgenic line functions as a Dre deleter, we first backcrossed it to obtain homozygosity, then we crossed it to the Rosa-rox reporter and isolated embryos at embryonic day E9.5. All embryos carrying the deleter and the reporter transgenes showed complete recombination, as determined by β-galactosidase staining (Fig. 3B). To ascertain that Cre does not recognize the *rox* sites in a deleter experiment, we crossed a phosphoglycerate kinase (PGK)-Cre deleter (Lallemand et al., 1998) with the Rosa-rox reporter and isolated embryos at E9.5. β-galactosidase staining showed that Cre did not recombine with the *rox* sites (Fig. 3B).

The results of the β-galactosidase staining were confirmed by PCR genotyping of DNA that was prepared from whole embryos (Fig. 3C). Dre recombination was complete in all three embryos that contained the Dre transgene and the Rosa-rox reporter. By contrast, no Cre recombination was detected in any embryo containing the Cre transgene and Rosa-rox reporter. We also crossed our CAGGs-Dre mouse line to the Cre reporter ZEG (Novak et al., 2000), in which GFP is expressed upon recombination. We did not find any GFP expression in E12.5 embryos, whereas GFP was visible in the control mating of the PGK-Cre deleter with the ZEG reporter line, as expected (Fig. 3A). These results were confirmed by the PCR test used above and by a different PCR test, using a primer that anneals in the Rosa26 sequence and a primer

that anneals in the *neo* gene, which also showed complete Cre-*loxP* or Dre-*rox* recombination, but no Cre-*rox* or Dre-*loxP* recombination (data not shown).

In summary, all embryos carrying both the Dre deleter and *rox* reporter transgenes showed complete recombination, as did the Cre deleter and *loxP* reporter embryos. No recombination whatsoever was detected in Dre-*loxP* or Cre-*rox* embryos.

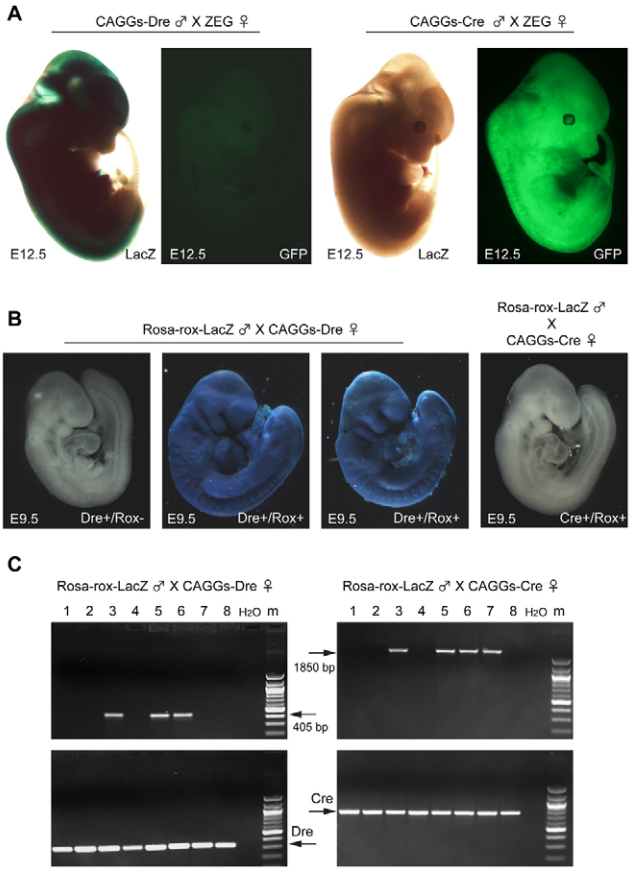

Fig. 3. Dre recombination in mice. (A) β-galactosidase (LacZ) staining and green fluorescent protein (GFP) epifluorescence, as indicated, of E12.5 embryos from a CAGGs-Dre × ZEG cross or a CAGGs-Cre × ZEG cross. The ZEG reporter line contains the *lacZ* gene flanked by *loxP* sites and followed by GFP. Dre cannot recombine with the ZEG reporter and the embryos are positive for β-galactosidase staining and negative for GFP. By contrast, Cre can recombine with the ZEG reporter and the embryos lose the β-galactosidase staining and express GFP. (B) β-galactosidase staining of E9.5 embryos from a Rosa-rox-lacZ reporter × CAGGs-Dre cross or a Rosa-rox-lacZ reporter × CAGGs-Cre cross. *lacZ* is expressed upon Dre recombination, as shown in the second and third panels from the left. Cre cannot recombine with the *rox* sites (right panel). The genotype of the embryos is indicated. (C) PCR was used to confirm recombination. The PCR was performed in a litter of eight embryos from a Rosa-rox-lacZ reporter × CAGGs-Dre cross and in another litter of eight embryos from a Rosa-rox-lacZ reporter × CAGGs-Cre cross. The Rosa-lacZ primer combination generates a 405-bp band upon recombination of the *rox* sites and a 1850-bp band in the unrecombined state. Complete recombination was observed in three embryos that contain the Rosa-rox reporter (as shown by the presence of the 405-bp band and the absence of the 1850-bp band). By contrast, no recombination was observed with Cre (as shown by the absence of the 405-bp band and the presence of the1850-bp band; right panels).

Ligand-inducible Dre-progesterone fusion protein

To further develop the potential offered by the Dre-*rox* system, we built an expression construct for a DrePBD (progesterone ligand-binding domain) fusion protein. We used a PBD that had been truncated at the C-terminus to render it insensitive to endogenous progestins but inducible by the synthetic anti-progestin RU486 (Vegeto et al., 1992; Kellendonk et al., 1996; Wunderlich et al., 2001). We chose this improved PBD (PBD*) (Wunderlich et al., 2001), rather than the tamoxifen-inducible, mutant estrogen-binding domain called ERT2 (Feil et al., 1997), because the CreERT2 fusion protein has become used widely. DrePBD* may, possibly, prove to be a useful complement to CreERT2, permitting double ligand experiments. We assessed the potential of the

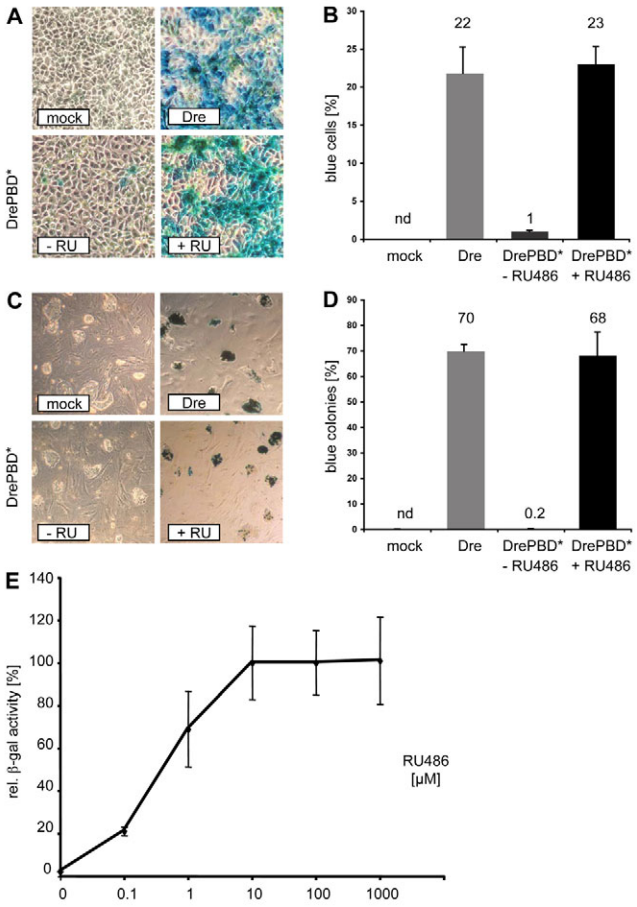

Fig. 4. Induction of DrePBD* by RU486. (A) β-galactosidase staining of rox-lacZ CV1 fibroblast reporter cells transfected with Dre or DrePBD* expression plasmids, as indicated. DrePBD*-treated cells were cultured in the absence (–RU) and presence (+RU) of 100 nM RU486. (B) Quantification of the β-galactosidase staining results depicted in A. For each cell culture condition, 5000 cells were counted and the experiments were done in triplicate (n=3). (C) β-galactosidase staining of rox-lacZ ES reporter cells transfected with Dre and DrePBD* expression plasmids, as indicated. DrePBD*-treated cells were cultured in the absence (–RU) and presence (+RU) of 100 nM RU486. (D) Quantification of the β-galactosidase staining results depicted in C. 450 colonies were counted, n=4. (E) Quantification of β-galactosidase staining results from experiments that were performed as for those in panel A. 5000 cells were counted, n=4. Error bars show standard deviation.

DrePBD* fusion protein to induce recombination in a RU486-dependent manner in both fibroblast and ES reporter cells. Fibroblast reporter cells were transfected with the pNN-DrePBD* expression plasmid and cultured in the presence and absence of the synthetic inducer RU486 (Fig. 4A). We observed almost no β-galactosidase activity in DrePBD* transfectants that were cultured in normal medium, whereas RU486 treatment resulted in widespread activation of β-galactosidase activity. Notably, the maximal activity was similar to that of control cells transfected with an equivalent Dre expression plasmid (Fig. 4B), indicating that C-terminal fusion of Dre with the PBD* does not interfere greatly with recombinase functionality. Assessing the activity of DrePBD* in ES cells resulted in a similar observation. Recombinase activity in DrePBD* transfectants was nearly undetectable but increased upon application of RU486 to levels that were similar to Dre-transfected controls (about 70%) (Fig. 4C,D). A dose-response curve revealed that recombination to permit β-galactosidase expression reflected the expected binding constant of RU486 to the truncated PBD and becomes saturated at between 1 and 10 μM of RU486 (Fig. 4E) (Wunderlich et al., 2001). Consequently, we suggest that DrePBD* provides an efficient and tight system for the inducible control of Dre recombinase activity in mammalian cells and that this fusion protein can serve as a complement to tamoxifen control of CreERT2.

DISCUSSION

The Dre-*rox* system described by Sauer and McDermott (Sauer and McDermott, 2004) is important because it could be as remarkable as the Cre-*loxP* system, which is the best SSR that has been developed, so far, for genome engineering. In pursuit of this potential, the work described here began shortly after the first Dre-*rox* publication but the reagents were unobtainable for use and independent verification. We then obtained an aliquot of the Dre-*rox* phage, D6, from the original source, June Scott at Emory University. We sequenced the Dre-*rox* region of the D6 phage, and then made expression and reporter vectors for functional testing in *E. coli*. From this work, we can clearly state that all of the data published in Sauer and McDermott are correct.

For functional testing in *E. coli*, we put Dre (and Cre) into the low-copy, temperature-sensitive pSC101 plasmid under the control of the BAD promoter. This combination delivered tight regulation of recombination, with no recombination before induction and complete recombination after induction (data not shown). Because site-specific recombination appears to be a very sensitive reporter of gene expression, we conclude that the pSC101-BAD configuration, which is reported here for Dre, is excellent for regulated gene expression in *E. coli*.

We developed ubiquitous mouse lines for the Dre-*rox* system based on the CAGGs promoter and the Rosa26 locus. As well as validating these lines, we also tested whether the Dre deleter would recombine with *loxP* or whether a Cre deleter would recombine with the Rosa26-rox reporter. No cross recombination of any kind was detected. Together with the *E. coli* results, we are very confident that the Cre and Dre systems are completely independent of each other. We also report the availability of the expression plasmid, pCAGGs-Dre, which includes an IRES-puromycin resistance cassette so that transient or stable expression can be selected (Schaft et al., 2001).

The availability of another high-performance tyrosine recombinase provides new options for genome engineering and strategies. The probable roles for Dre-*rox* should take account of existing reagents and strategies. First, conditional mutagenesis in the mouse is based on Cre-*loxP*, with many mouse lines available. Although this decision could be different for rats now that rat ES cells have been established (Buehr et al., 2008), it is unlikely that the decision will be remade for the mouse even if a better system for conditional mutagenesis is found in the near future. Second, the FLP-*FRT* system is used mainly to perform working tasks such as removal of the selectable cassette from gene-targeting events (Schaft et al., 2001; Testa et al., 2004). Although the FLP recombinase has been improved significantly though molecular evolution and codon optimization (Buchholz et al., 1998; Raymond and Soriano, 2007), it still appears to be less efficient than Cre in mammalian cells (K.A., unpublished observations). Hence, we suggest that the application of FLPo-*FRT* should remain focused on simple working tasks such as the removal of selectable cassettes.

Third, the large serine recombinases such as phiC31 and phiBT1 (Xu et al., 2008) present new options for genome engineering and strategies. In contrast to tyrosine SSRs such as Cre and FLP, the large serine SSRs possess the advantage of directional recombination based on sequence differences between their two substrate RTs. Recombination between these two different substrate RTs generates two different product RTs. As shown by Belteki et al., the two substrate RTs are not equivalent in practice and this asymmetry needs to be incorporated into experimental design (Belteki et al., 2003). This asymmetry was also reflected in an unexpected inefficiency of deletional recombination after genomic integration of the two substrate RTs. The RT asymmetry can be exploited advantageously to insert transgenes into genomic sites, and the large serine SSRs are emerging as excellent tools for site-specific transgene integration (Groth et al., 2000; Belteki et al., 2003; Groth et al., 2004; Bischof et al., 2007). Although some concern surrounds the propensities of the large serine recombinases to promote cryptic recombination in mammalian genomes (Groth et al., 2000) or DNA damage at RTs via non-homologous end-joining (Malla et al., 2005), their utility for genomic integration appears to complement the strengths and weaknesses of Cre and FLP.

In the light of these considerations, we suggest that Dre can complement the existing scheme of SSR specializations by application to complementary tasks that require a highly efficient SSR, such as conditional mutagenesis of alternatively spliced exons. In an extension of the 'knockout first' strategy (Testa et al., 2004), we suggest that FLPo-*FRT* be used to remove the selectable cassette that is used for targeting, Cre-*loxP* be used to remove one alternative exon, and that Dre-*rox* can be used to remove the other alternative exon. A variation of this application involves the use of Cre-*loxP* to remove one protein domain and Dre-*rox* to remove a different protein domain so that protein function can be dissected more precisely. These applications could be combined with the use of tamoxifen and CreERT2 with RU486 and DrePBD*. For another example, it should be straightforward to establish an on/off gene expression system whereby tamoxifen turns a gene on and RU486 turns it off. To date, the ability to turn a gene on and off has been cited as an advantage of tetracycline regulation, so adding this capacity to SSR gene switches may be useful.

Further applications for Dre-*rox* include genome engineering, such as large inversions, to generate balancer chromosomes or chromosomal translocations (van der Weyden and Bradley, 2006). In these cases, the use of Dre will permit the later use of Cre for conditional mutagenesis. We also point out that the adoption of a standardized scheme of SSR specializations will facilitate material exchange and experimental interactions. As a result, it will be useful to discuss these aspects within the research community to arrive at a standard consensus.

Although Cre-*lox* has proven to be remarkably powerful, certain problems have been identified (Schmidt et al., 2000; Loonstra et al., 2001; Schmidt-Supprian and Rajewsky, 2007). The main problem is toxicity caused by Cre recombinase. The molecular basis of this problem is still not known, but it is likely to be either recombination between cryptic *lox* sites in the genome or DNA damage promoted by incomplete recombination. Whether Dre, or indeed FLP, promote the same problems to a greater or lesser degree remains to be determined. If it emerged that Cre was idiosyncratically more problematic than the alternatives, then there may be good reason to restructure the SSR role specializations recommended here.

A patent on Dre recombinase has been assigned to the Stowers Institute, inventors Sauer and McDermott; United States Patent 7422889. We have contacted the Stowers Institute regarding this patent and they have assured us that all Dre-*rox* reagents are freely available for the research community (see Box 1). We have not attempted to seek any intellectual property coverage for any of the materials described here. Therefore, the distribution and utilization of Dre-*rox* reagents for research will not be encumbered. The mice will be available soon through EMMA (European Mutant Mouse Archive).

Box 1. Official statement from the Stowers Institute

'The Stowers Institute considers the sharing of research materials to be an essential aspect of scientific citizenship. In the specific case of dre-rox technology, the Institute owns certain intellectual property, including an issued patent and a pending patent application covering, among other things, "Dre recombinase and recombinase systems employing Dre recombinase." The Institute will allow non-profit organizations to make or use material covered by the Institute's dre-rox intellectual property for non-commercial purposes under terms no more onerous than those contained in the Uniform Biological Material Transfer Agreement. The Institute's policy is to offer for-profit organizations a non-exclusive license (without reach-through royalties) to make or use material covered by the Institute's dre-rox intellectual property for internal research purposes. For other uses by for-profit organizations, the Institute will evaluate requests on a case-by-case basis.'

METHODS

A patent on Dre recombinase has been assigned to the Stowers Institute, inventors Sauer and McDermott; United States Patent 7422889.

Plasmids and *E. coli* experiments

Plasmids were constructed using standard or recombineering methods that are available on request. The mammalian Dre expression vectors used the codon-improved version of Dre (supplementary material Fig. S1). The SSR assay was performed in *E. coli* by transforming cells that carried either pUCRosa26-rox-lacZ or pUCRosa26-loxP-lacZ with pSC101-BAD-Dre or pSC101-BAD-Cre, and culturing overnight at 30°C in the presence of ampicillin and tetracycline. Fresh inoculums from overnight cultures of the double plasmid combinations were cultured in L-broth plus antibiotics for 2 hours, followed by the addition of L-arabinose to 0.2%. After a further hour at 37°C, the cells were pelleted and the fresh L-broth plus ampicillin, minus tetracycline and L-arabinose, was added and cultured for 4 hours at 37°C. Then, a miniprep was made, transformed into fresh cells and plated. Single colonies were taken and streaked onto plates as shown in Fig. 1B.

Cell culture and transfection

Mouse R1 ES cells were cultured on mitomycin C-inactivated mouse embryonic fibroblasts (MEFs) using the following ES medium [DMEM, 15% fetal calf serum (FCS), 2 mM L-glutamine, 1 mM sodium pyruvate, 1% penicillin/streptomycin, 100 μM non-essential amino acids (all from Invitrogen), 100 μM β-mercaptoethanol (Sigma)] containing leukemia inhibitory factor (LIF). ES cells (10^7) were electroporated using a Bio-Rad electroporator (250 V, 500 μF) with 40 μg of the linearized constructs (CAGGs-Dre-IRES-puro or the Rosa-rox-neo-rox-lacZ targeting construct) and selected with 1 μg/ml puromycin (Sigma) or with 200 μg/ml G418 (Invitrogen), respectively. Colonies were screened by Southern hybridization using *Eco*RI and a Dre probe for the CAGGs-Dre-IRES-puro construct, and using *Eco*RI and an external Rosa26 5′ probe for correct targeting events of the Rosa-rox-neo-rox-lacZ construct. To generate Dre reporter cells, vervet monkey fibroblasts (CV1 cells) were electroporated with linearized rox-neo-rox-lacZ reporter construct. We started selection at 48 hours after transfection with 250 μg/ml of G418. To obtain stable clones, we picked surviving cell clusters after 12 days and expanded these clones in the presence of 200 μg/ml of G418. Clones were screened for single integration by Southern hybridization.

Southern blots

Genomic DNA was extracted from cells by proteinase K digestion and isopropanol precipitation. DNA was restricted overnight with the indicated enzyme, separated on agarose gels and blotted to nylon membranes (Pall). Probes were made with ^{32}P by random priming (Roche Diagnostics).

Generation of mice

All experiments involving mice were performed according to German law in the Transgenic Core Facility (TCF), Dresden. CAGGs-Dre-IRES-puro or targeted Rosa26 mouse lines were generated by injection of ES cells into 8-cell stage embryos. All manipulations were done in the TCF of the MPI-CBG, Dresden. Chimeras were crossed to C57BL/6 mice and were screened by Southern blot for germline transmission. The established lines were genotyped by PCR using the following primers: Dre3: 5′-TGCTGTTCCCTCCTATCCAC-3′ and Dre4: 5′-CGGAG-TCCATCAGCCTAGAG-3′ for the CAGGs-Dre-IRES-puro line; and Rosarox1: 5′-TGGAAATGTTACCAAGGAACT-3′ and Rosaroxneo1: 5′-TGACAGGAGATCCTGCCCCGGCACT-3′ for the Rosa-rox reporter line (716 bp). Dre recombination was confirmed using the primers Rosarox1 and RosaroxLZ1: 5′-AACGACGGCCAGTGCCAAGCTACT-3′. Dre recombination generated a 405-bp fragment and, if no recombination occurred, a 1850-bp fragment was generated.

RESOURCE IMPACT

Background

The mouse is the premier venue for modeling human disease because of the unrivalled technology for manipulating its genome. Embryonic stem (ES) cells, pronuclear injection in fertilized oocytes, and transpositional mutagenesis allow for sophisticated manipulation of the murine germ line. Potentially the most powerful and precise strategy for disease modeling is conditional mutagenesis, in which a specific mutation is made in a somatic cell to produce cell type-specific disease states. Conditional mutagenesis relies on the site-specific recombinase (SSR) Cre to mediate a very precise recombination event between its 34-base pair *loxP* recombination target sites. The *loxP* sites have to be placed into the mouse genome at a chosen site, usually by gene targeting in ES cells. Upon exposure to Cre, these sites will be precisely recombined without any other recombination event in the rest of the genome. This remarkable precision is what makes conditional mutagenesis such a valuable technique. Among all SSRs, Cre has proven to be the most effective at mediating recombination in large genomes such as the mouse. However, owing to the complexities of alternative splicing, gene product interactions and genetic redundancy, it will be useful to have the means to mutate a given gene in more than one way, or to combine more than one conditional mutation. To achieve this goal, new tools that perform as well as Cre are required.

Results

The authors show that an SSR that is related closely to Cre, termed Dre, has similar efficiencies to Cre when applied in the mouse. Using recombination tests in *E. coli*, mammalian cells and the mouse, they show that Dre recombinase produced very specific recombination to *rox* sites, even in the presence of *loxP*. Thus, there is no crosstalk between Cre and Dre recombination. They also produced ubiquitous mouse lines for the Dre-*rox* system and propose using this technique to build more complex models by combining it with the existing Cre and FLP methods of genetic manipulation.

Implications and future directions

These results present a second option for efficient conditional mutagenesis in the mouse. Whereas Cre will remain the main instrument for conditional mutagenesis, the advent of Dre permits the development of more sophisticated strategies of combinatorial site-specific recombination and conditional mutagenesis. The use of the two SSRs will simplify the use of conditional mutagenesis to analyze alternative splice forms. Given that most mammalian genes are alternatively spliced, this application could play a significant role in future mouse modeling.

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β-galactosidase staining of embryos and cells

Dissected embryos (E9.5) were fixed for 30 minutes in 4% PFA on ice, then washed three times with PBS and incubated with the staining solution (PBS containing 2 mM $MgCl_2$, 0.02% NP40, 0.01% Na-deoxycholate, 0.25 mg/ml spermidine, 2.1 mg/ml K-ferrocyanide, 1.6 mg/ml K-ferricyanide and 1 mg/ml X-Gal) at 37°C in the dark. Cells were rinsed twice with PBS and fixed with 2% formaldehyde and 0.1% glutaraldehyde for 2 minutes, washed three times with PBS, and stained at 37°C in the dark with PBS containing 2 mM $MgCl_2$, 2.1 mg/ml K-ferrocyanide, 1.6 mg/ml K-ferricyanide and 1 mg/ml X-Gal.

Inducible Dre recombination employing DrePBD*

CV1 Dre-reporter cells were cultured in DMEM with 10% FCS, 2 mM L-glutamine, 1 mM sodium pyruvate and 100 μM non-essential amino acids (all from Invitrogen). To test the recombinase activity of DrePBD*, 250,000 cells were plated per 6-well plate, grown for 18-24 hours, and then transfected with 2 μg of pNNDrePR650-914 using Fugene HD (Roche), according to the manufacturer's protocol. At 16 hours post-transfection, medium with or without 100 nM of RU486 was added to the cells. At 72 hours after transfection, cells were fixed in PBS containing 0.25% glutaraldehyde and 20 mM $MgCl_2$. Cells were stained overnight with an X-Gal staining solution at 37°C. In each well, blue and wild-type cells from three different areas were counted to calculate the value of efficiency. To test DrePBD* recombinase activity in murine ES cells, 50,000 Rosa-krox-lacZ ES cells were seeded per 6-well plate on mitotically inactivated mouse fibroblasts. ES cells were cultivated for 2-4 hours before they were transfected as described for CV1 cells. RU486 treatment and fixation were carried out as described above. Mosaic ES cell colonies containing more than five positively stained cells were counted as positive.

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COMPETING INTERESTS

The authors declare no competing financial interests.

AUTHOR CONTRIBUTIONS

K.A., F.B., F.E. and A.F.S. conceived and designed the experiments and wrote the manuscript; J.F. and S.H. built the DNA constructs and performed the experiments in *E. coli*; K.A., S.W. and K.D. performed the experiments in ES cells and mice; C.P. and F.E. made the Dre-PBD* constructs and performed the corresponding experiments.

SUPPLEMENTARY MATERIAL

Supplementary material for this article is available at
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