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Regulation of Cre recombinase activity by the synthetic steroid RU 486

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ABSTRACT

To create a strategy for inducible gene targeting we developed a Cre–lox recombination system which responds to the synthetic steroid RU 486. Several fusions between Cre recombinase and the hormone binding domain (HBD) of a mutated human progesterone receptor, which binds RU 486 but not progesterone, were constructed. When tested in transient expression assays recombination activities of all fusion proteins were responsive to RU 486, but not to the endogenous steroid progesterone. However, the observed induction of recombination activity by the synthetic steroid varied between the different fusion proteins. The fusion with the highest activity in the presence of RU 486 combined with low background activity in the absence of the steroid was tested after stable expression in fibroblast and embryonal stem (ES) cells. We could demonstrate that its recombination activity was highly dependent on RU 486. Since the RU 486 doses required to activate recombination were considerably lower than doses displaying anti-progesterone effects in mice, this system could be used as a valuable tool for inducible gene targeting.

INTRODUCTION

Gene disruption by homologous recombination has recently gained importance in the study of gene function (1). However, this approach has several inherent problems, such as possible early lethality, the inability to study the role of a gene in a specific tissue and the inability to do so at a given point in time. The use of a recombinase expressed in a tissue-specific manner can partially overcome these limitations (2). Temporally regulated recombination should permit studies in a single mouse before and after inactivation of the appropriate gene. To develop a system for inducible gene targeting we combined the Cre–lox system with the regulative properties of a mutant human progesterone receptor (hPR891) that responds to a synthetic steroid (RU 486) but not to endogenous steroid (3).

The *cre* gene of coliphage P1 encodes a 38 kDa site-specific recombinase of the integrase family. It efficiently promotes intra- and intermolecular recombination at specific 34 bp sequences termed loxP sites (4). Depending on the orientation of the loxP sites, intramolecular recombination between two sites will lead to reversible excision or inversion of the intervening DNA sequence (5). Intermolecular recombination can result in integration of loxP-containing circular DNA (6) or, if two loxP sites are on different chromosomes, in chromosomal translocations (7,8). Cre-mediated recombination is not restricted to prokaryotes and has been reported in yeast (9), plants (10), mammalian cells (11) and mice (12,13).

Steroid receptors are modular proteins organized into structurally and functionally defined domains (14). Fusing the C-terminal hormone binding domain (HBD) onto other transcription factors or tyrosine kinases confers hormone-dependent activities on these proteins (15–18). Recently it was shown that HBDs from estrogen, androgen or glucocorticoid receptors confer ligand-dependent, site-specific recombination. (19,20). These observations outline the basis for direct, ligand-responsive temporal regulation of site-specific recombination. To explore the potential of this strategy we fused the progesterone HBD to the Cre recombinase. Rather than use the wild-type progesterone HBD, a mutant human progesterone receptor (hPR891) containing a 42 amino acid C-terminal deletion was used. The hPR891 HBD was selected because it is unable to bind progesterone or other endogenous hormones (3). However, it still binds the synthetic steroid RU 486, which has well-characterized anti-progesterone and anti-glucocorticoid properties (21). Previous studies with hPR891 showed that when fused to another transcription factor the ligand binding domain of hPR891 confers RU 486 responsiveness (22). By fusing the hPR891 HBD onto Cre we sought to create a ligand-regulated recombinase that can be used in mice, since it should be insensitive to the presence of endogenous steroids. To find an optimal Cre–hPR891HBD fusion a series of fusion proteins were constructed and tested in green monkey CV-1 and mouse embryonal stem (ES) cells.

MATERIALS AND METHODS

Cell culture

CV1 cells were cultured in Dulbecco's modified Eagle's medium containing 10% fetal calf serum and 100 U/ml penicillin and streptomycin. R1 ES cells (23) were grown in Dulbecco's modified Eagle's medium supplemented with 15% fetal calf serum, 100 µM non-essential amino acids, 1 µM β-mercaptoethanol and leukemia

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inhibitory factor (LIF) (ESGRO™; Gibco-BRL) at 37°C in a humidity-saturated 9% CO_2 atmosphere. The cells were cultured on confluent feeder layers mitotically inactivated by treatment with mitomycin C, except when cells were grown in the presence of neomycin, when they were plated onto gelatin-coated plates.

Plasmid constructions

The plasmid containing NLS (nuclear localization sequence)–Cre was constructed by inserting the Cre recombinase-containing fragment from pMC1 (pIC1) (24) into pHD2 (G.Müller, F.Weih and G.Schütz, unpublished data). The plasmids containing NLS–lacZ and alkaline phosphatase were obtained by inserting fragments containing the open reading frame (ORF) of NLS–lacZ (kindly provided by M.Pontoglio) and of placental alkaline phosphatase (kindly provided by J.Sharpe) into pHD2. The target vector was constructed by inserting the loxP-flanked *neo* cassette from ploxPneo-1 (provided by A.Nagy) into pHD2-lacZ between the promoter and the *lacZ* gene.

CrePR1–CrePR7 fusion genes were obtained by means of PCR, as described by Horton *et al*. (25). To construct the CrePR1 and CrePR2 fusions the Cre fragment was amplified by PCR from pIC1 using an N-terminal oligomer A (AAG CAA CTC ATC GAT TGA TTT ACT G) and a C-terminal oligomer B (AAC TTT TTA TCG CCA TCT TCC AGC AGG C). The hPR891 HBD fragment was amplified by PCR from pGL-VP (22) with oligonucleotides C (GAT GGC GAT AAA AAG TTC AAT AAA GTC A) and D (CGG CTC GAG CTC TAG AGT CAG CAG TAC AGA TGA AGT TGT). The overlapping PCR fragments obtained were used in a fusion PCR with oligonucleotides A and D as described by Horton *et al*. (25). The resulting fragment was then subcloned into *Cla*I and *Xho*I sites of pMC1 (or pIC2 for CrePR2). The whole CrePR1 ORF was finally cloned into the *Sma*I/*Xho*I sites of pHD2. For stable expression a *Bgl*II–*Xho*I fragment, including the CrePR1 ORF, was inserted into *Sca*I/*Xho*I-digested pZeoSV (Invitrogen) plasmid.

CrePR3–CrePR9 fusion genes were obtained by a similar method using for CrePR3 GTG AAT CTA TCG CCA TCT TCC AGC AGG C as oligomer B and CAT GGC GAT AGA TTC ACT TTT TCA CCA G as oligomer C, for CrePR5 TTG ATC AGA TCG CCA TCT TCC AGC AGG C as oligomer B and GAT GGC GAT CTG ATC AAC CTG TTA ATG A as oligomer C. To clone CrePR9, in which the HBD is positioned N-terminal, the hPR891 NLS–HBD sequence was obtained by PCR with oligomers E (GGA AGA TCT TCC ACC ATG CCC AAG AAG AAG AGG AAG GTG AGA TTC ACT TTT TCA CCA GGT CAA G) and F (TAA ATT GGA GCA GTA CAG ATG AAG TTG T), the Cre sequence by PCR with oligomers G (CTG TAC TGC TCC AAT TTA CTG ACC GTA) and H (AGA AGA TAA TCG CGA ACA TCT TCA G). Fusion PCR with oligomers E and H ended with a *Bgl*II–*Nru*I fragment, which was inserted into pCre1. Subcloning this fragment into pNLS-Cre yielded CrePR7.

Transient transfection experiments and enzymatic assays

CV1-5B cells were plated into 6 cm dishes, grown for 24 h and then transfected with 1 µg NLS–Cre or an equimolar amount of the different test DNAs. In each transfection 1 µg pHD2-AP (encoding the placental alkaline phosphatase gene) was included as an internal control. Transfection was performed by calcium phosphate precipitation as previously described (26). Cells were washed in phosphate-buffered saline (PBS) 12 h after transfection and 100 nM RU 486 or 1 µM progesterone was added to the medium. Cells were harvested 24 h later and fixed in PBS containing 1% formaldehyde (Merck) and 0.2% glutaraldehyde (Serva). They were stained overnight with a solution containing 4 mM potassium hexacyanoferrate (III), 4 mM potassium hexacyanoferrate (II), 2 mM $MgCl_2$ and 0.4 mg/ml X-gal (Biomol). After heat inactivation of endogenous alkaline phosphatase (AP) activity (30 min, 65°C) cells were stained for AP activity with fast red tablets (Boehringer Mannheim). Red and blue cells of an area of 120 × 2 mm were then counted. β-Galactosidase activity from cytoplasmic extracts was measured in the presence of orthonitrophenyl-β-D-galactoside as described (27).

Stable transfection in cell lines

Stably transfected subclones of the CV1 cell line (28) were obtained by the electroporation of 10^7 cells in 0.8 ml PBS containing linearized plasmid DNA (10 µg CrePR plasmids or 4 µg target vector). A BioRad Gene Pulser was set at 960 µF and 300 V. Cells were grown for 24 h prior to starting selection with 250 µg/ml Zeocin (Invitrogen) or 400 µg/ml G418 in the medium.

Electroporation of R1 ES cells was after trypsinization and resuspension in PBS at a concentration of 1×10^7/ml. For each individual transfection 0.8 ml cells (5.6×10^7) were mixed with 20 µg linearized DNA in an electroporation cuvette with a 0.4 cm electrode gap. Cells were electroporated with a BioRad Gene Pulser set at 240 V, 500 µF. R1 cells were first electroporated with *Sca*I-linearized pPGKpaX1 plasmid (target construct; Fig. 1B). The cells were plated onto three 10 cm gelatinized plates. After 24 h cells were fed with medium additionally supplemented with puromycin at 1 µg/ml final concentration. After selection with puromycin for 10 days colonies were picked for expansion and a second round of transfection with the CrePR expression plasmid.

Two independent puromycin-resistant clones were electroporated with *Aat*II-linearized pNPK-CrePR1 plasmid (expression construct) and treated as above except that selection with both G418 (200 µg/ml) and puromycin was applied. Southern analyses of the stably transfected clones were performed by standard procedures (27).

RESULTS

An assay for Cre-mediated recombination

To test the activity of the different fusion proteins we constructed a target vector and stably integrated it into CV-1 cells (Fig. 1A). This vector contains an ORF of the *Escherichia coli* β-galactosidase gene which is not expressed, since it is separated from the promoter sequence by a neomycin resistance gene flanked by two loxP sites. In the presence of active Cre recombination between the loxP sites excises the neomycin resistance gene and its polyadenylation signal, leading to expression of the *E.coli* β-galactosidase gene. Cells which undergo recombination will turn blue after staining with X-gal. We analyzed 24 G418-resistant clones obtained after transfection of the target vector into CV-1 cells. Of the 16 clones that showed Cre-dependent β-galactosidase expression, one clone (5B) was chosen for further work since it contained only one integrated vector copy (data not shown).

We first compared Cre and NLS–Cre on 5B cells by transient transfection. NLS–Cre has the SV 40 large T NLS at its N-terminus, a modification previously employed to increase the activity of Cre

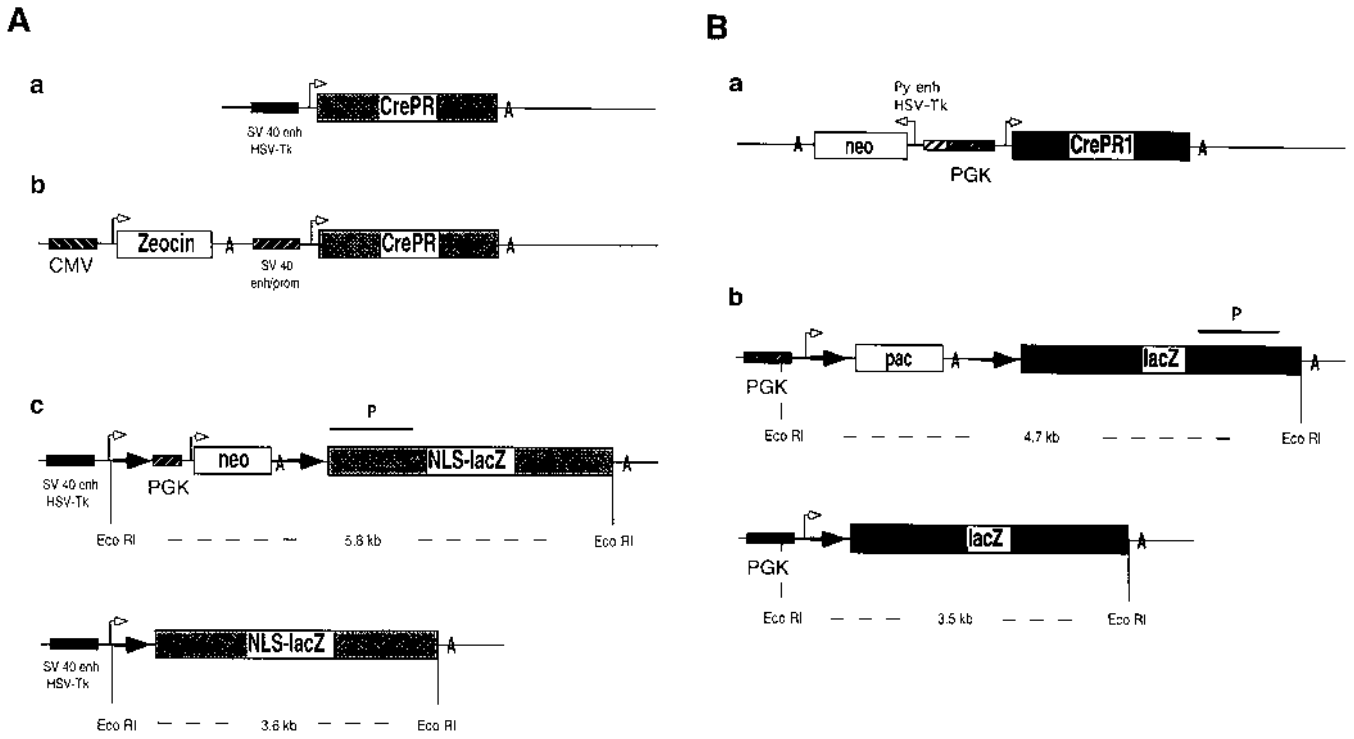

Figure 1. (**A**) Cre recombinase expression vectors and Cre target construct used for expression in CV1 cells. (**a**) Scheme of expression vectors used for transient transfection assays shown in Figure 2. All Cre-derived proteins (Cre, NLS–Cre and CrePR1–CrePR9; Fig. 2), as well as placental alkaline phosphatase and *E.coli* modified β-galactosidase (NLS–lacZ; Fig. 2), were expressed under the control of two SV40 early enhancers fused upstream of the –105 HSV-1 Tk promoter. (**b**) Scheme of expression vector used for stable expression in CV1 cells. Stable expression of the CrePR1, CrePR3 and CrePR9 transgenes was controlled by the SV40 early enhancer/promoter (pZeoSV; Invitrogen). The Zeocin resistance gene used for selection was driven by the CMV immediate-early promotor. (**c**) Target construct used to visualize Cre-mediated recombination. In the upper part is drawn the construct before recombination, in the lower part the construct after recombination. The ORF of the *E.coli* β-galactosidase gene, which is modified by an integrated nuclear localization signal (NLS–lacZ), is separated from the controlling promotor by a PGK promotor-driven neomycin resistance gene. A SV40 polyadenylation signal (A) downstream of the neomycin ORF prevents expression of the β-galactosidase protein. Cre-mediated recombination between two loxP sites flanking the neomycin expression cassette (indicated by closed arrows) will direct excision of the cassette, thus leading to expression of the β-galactosidase protein, which is then controlled by the SV40/HSV Tk promotor of pHD2 described in (a). Digestion of non-recombined DNA with *Eco*RI produces a 5.8 kb fragment, which can be detected by a probe within the *lacZ* gene. After recombination the size of the corresponding fragment is 3.6 kb. (**B**) Cre recombinase expression vector and Cre target construct used for expression in ES cells. (**a**) CrePR1 was introduced into a bidirectional expression vector consisting of PGK and a modified polyoma enhancer/HSV Tk promoter. The neomycin resistance gene, polyoma enhancer/HSV Tk promoter was derived from pMC1 (Stratagene). (**b**) The puromycin resistance gene (*pac*) was placed between two loxP sites and cloned downstream of the PGK promoter. The SV40 early polyadenylation signal (A) was included between the lox sites. The entire β-galactosidase gene was placed 3′ of the downstream loxP site. Before recombination *Eco*R1 will produce a 4.7 kb fragment. After recombination this fragment will be reduced to 3.5 kb. Closed arrows indicate loxP sites; open arrows, the location transcription start points; A, a SV40 polyadenylation signal and P the probes used.

in mammalian systems (24). Figure 2 shows that NLS–Cre is indeed more active than unmodified Cre. As a further control, a construct that transiently expresses β-galactosidase without the need for recombination (pHD2-lacZ) was included. Figure 2 indicates that β-galactosidase expression achieved by NLS–Cre-mediated genomic recombination is at least as efficient as that achieved by direct transient expression from pHD2-lacZ. No β-galactosidase activity was observed in 5B cells in the absence of transfected plasmids (data not shown).

Transient transfection assays of CrePR fusion proteins

In order to obtain a CrePR fusion protein which is well regulated by RU 486 several fusions of the Cre recombinase and the hPR891 HBD were constructed. According to results obtained from previous HBD fusion protein studies, the preferred fusion construct has the HBD as close as possible to the sequence encoding the required activity (18). Mutational analysis of the bacteriophage Cre recombinase showed that mutations distributed over the entire protein can lead to completely or severely reduced activity (29). Therefore, hPR891 HBDs fused to both N- and C-temini of Cre were generated. Four different HBD fusions to the C-terminus of Cre were tested, (CrePR1–CrePR3 and CrePR5; Fig. 2). They differ in the length of the PR D domain or the presence of a synthetic SV40 large T NLS at the N-terminus of Cre. The D domain of steroid receptors is proposed to function as a hinge region and plays a role in nuclear localization. A single N-terminal HBD–Cre fusion was constructed (CrePR9; Fig. 2). Finally, a double fusion to both termini of Cre was also tested (CrePR7; Fig. 2).

The constructs were transfected into 5B cells and their activity was tested in the presence of 100 nM RU 486 and in its absence (Fig. 2). CrePR1, which includes the complete D domain, showed ~1% of the activity of the wild-type NLS–Cre recombinase in the absence of RU 486. Treatment with RU 486 increased the activity to 44% (Fig. 2). Shortening (CrePR3) or removing (CrePR5) the D domain did not enhance the efficiency of the system. The background of recombination was comparable with the background

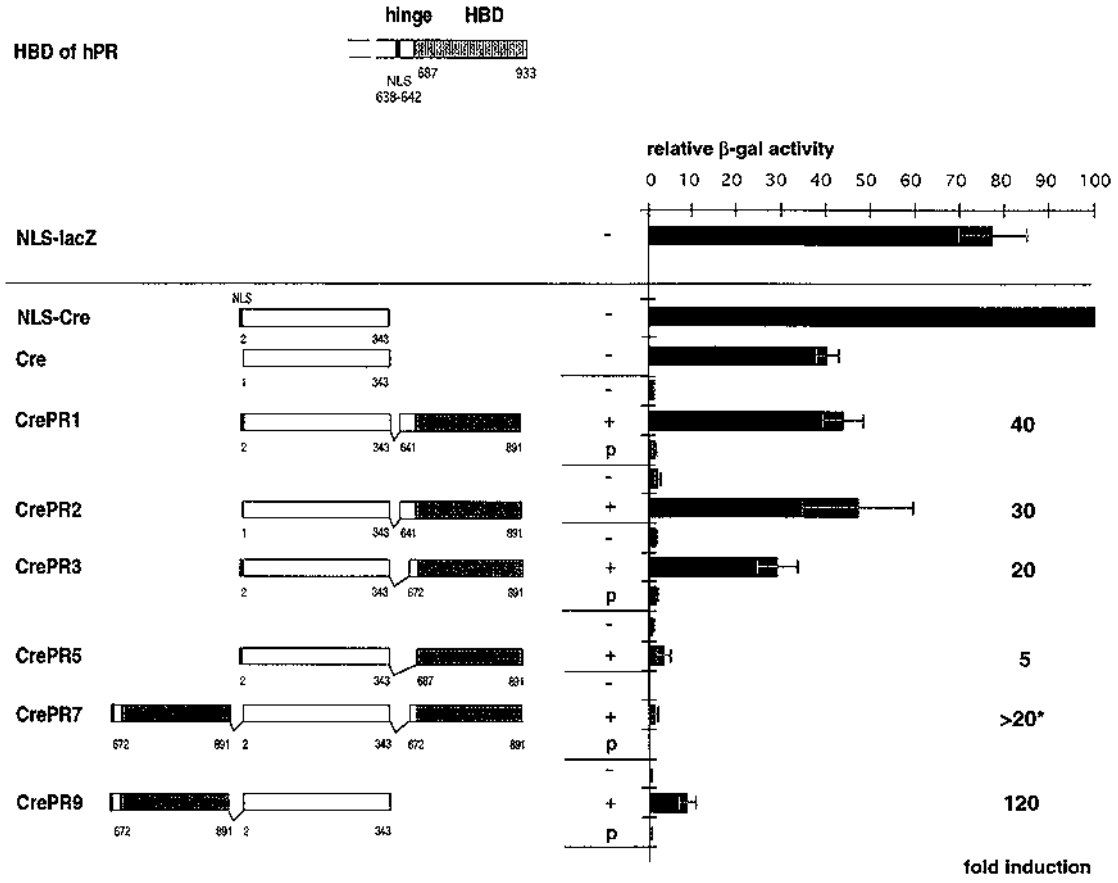

Figure 2. Comparision of different Cre recombinase–hPR981 fusion proteins in transient transfection assays. The different hPR–Cre fusion constructs are shown on the left. The corresponding relative β-galactosidase activities reflecting Cre recombinase activities are indicated on the right. With the exception of Cre and CrePR2, all constructs contain a nuclear localization signal at the N-terminus (black box). The exact fusion points are given by amino acid numbers. The relativeβ-galactosidase activities were measured after transfection of CV1 subclone 5B (containing the target construct) with different Cre or CrePR expression vectors. Cells were cultured in the absence (–) or presence (+) of 100 nM RU 486 or in the presence of 1 µM progesterone (P). The relative activity represents a standardized number of blue cells counted after fixation and staining with X-gal. Each value is the mean of at least six different transfections. To normalize the results for transfection efficiency we used alkaline phosphatase as an internal control. All plates were co-transfected with a plasmid expressing alkaline phosphatase and the presence of alkaline phosphatase was detected by *in situ* histochemistry. The activity of NLS–Cre was arbitrarily set to 100. This corresponds to 600–900 blue cells/2 cm^2. To further assess transfection efficiency a construct expressing NLS–lacZ under the same promotor was transfected in parallel and blue stained cells counted. The structure of the human progesterone receptor C-terminus, including the hinge region (D domain), the ligand binding/dimerization domain (E domain, amino acids 687–933) and the internal nuclear localization sequence (NLS, amino acids 638–642, black box) is shown at the top. *There were no blue cells in plates transfected with CrePR7 and cultured without RU 486. In consequence, the background was set at <1.

exhibited with CrePR1, whereas the induction by RU 486 was significantly lower.

Fusing the hPR891 HBD to the N-terminus of Cre recombinase reduced Cre activity in the presence of the hormone. Interestingly, the observed inducibility was higher, due to a strongly reduced background in the absence of RU 486. Compared with NLS–Cre, cells cultured with or without RU 486 displayed 8.5 or 0.07% activity respectively. Fusing the HBD to both termini of the recombinase completely abolished the background. Adding the anti-hormone restored the activity, but only to 1%.

In transient transfection experiments the presence of a NLS at the N-terminus of wild-type Cre enhances the recombination activity by ~2-fold (Fig. 2; 24). The requirement for a NLS in CrePR constructs was tested by comparing CrePR1 with CrePR2. No differences in activities were detected in the induced or in the uninduced states. This may reflect the fact that HBDs appear to carry nuclear localization functions.

It is expected that all CrePR (hPR891) fusions cannot be activated by progesterone (3). Several of the fusion proteins were tested for this by incubating transfected cells with 1 µM progesterone. None of them responded to progesterone (Fig. 2).

Stable expression of CrePR fusion proteins

To determine the RU 486 inducibility of genomically expressed CrePR fusion proteins stable cell lines were established. Three fusions (CrePR1, CrePR3 and CrePR9) were cloned into pZeoSV (Fig. 1A), electroporated into 5B cells and selected for Zeocin resistance. From each electroporation over 50 clones were expanded and tested for recombination activity by culturing them for 3 days with or without 100 nM RU 486. The cells were then stained with X-gal in order to assay the frequency of recombination.

As observed in the transient transfection experiments, stably expressed CrePR9 was much less active than CrePR3, while

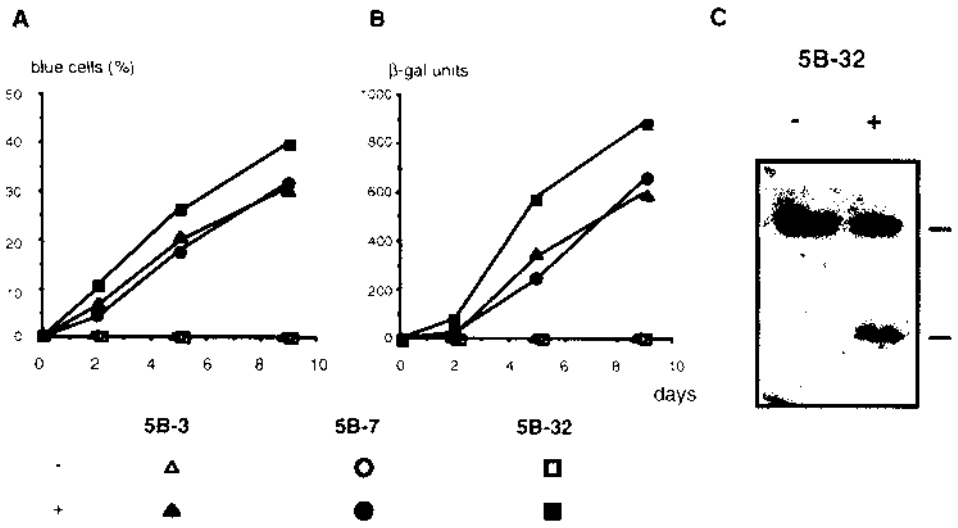

Figure 3. Recombination in cell lines stably expressing CrePR1. (**A**) The percentage of recombined cells determined at different time points. Clones 5B-3, 5B-7 and 5B-32 were grown in the presence of 100 nM (+) or the absence (–) of RU 486. The percentage of blue cells was calculated by counting blue cells after staining with X-gal. At each time point cells were split to avoid confluency. (**B**) β-Galactosidase enzymatic activity of cytoplasmic extracts (ONPG assay) from different cell lines cultured in the presence of 100 nM (+) or absence (–) of RU 486. (**C**) The percentage of cells with a recombination event after 9 days determined by Southern blot. Clone 5B-32 was cultured as above. Genomic DNA was harvested after 9 days, digested with *Eco*RI and hybridized with a *lacZ* probe. The 5.8 and 3.6 kb fragments correspond to a non-recombined and recombined NLS–lacZ gene respectively (Fig. 1).

CrePR3 was less active than CrePR1 (data not shown). Three clones expressing CrePR1 (5B-3, 5B-7 and 5B-32) were selected for further analysis, since they showed a high recombination frequency combined with good RU 486 inducibility.

To determine the recombination rate more precisely we measured the accumulation of recombination events over time. Cells were cultured for 9 days in the presence or absence of RU 486. At different time points the percentage of blue cells was determined after X-gal staining. After 9 days clone 5B-32 displayed the highest background (0.15% blue cells) and the highest recombination activity when induced with RU 486 (40% blue cells). For clones 5B-3 and clone 5B-7 the percentages were 0.13 and 30% and 0.03 and 32% respectively. Thus a 200- to 1000-fold induction of CrePR-mediated recombination by RU 486 was observed (Fig. 3A). The differences in recombination activity may result from different levels of CrePR1 expression in the 5B-derived clones. The results obtained by X-gal staining were confirmed by *in vitro* enzymatic assay measurements (Fig. 3B) and by Southern blot analysis (Fig. 3C). The latter demonstrated the predicted excision of the loxP-flanked neomycin resistance cassette. Quantification of this Southern analysis using a phosphorimager showed 45% recombination for clone 5B-32 after 9 days exposure to RU 486. This value is very similar to the percentage of blue staining cells (40%), thus confirming that *in situ* staining for β-galactosidase activity is a faithful reflection of recombination.

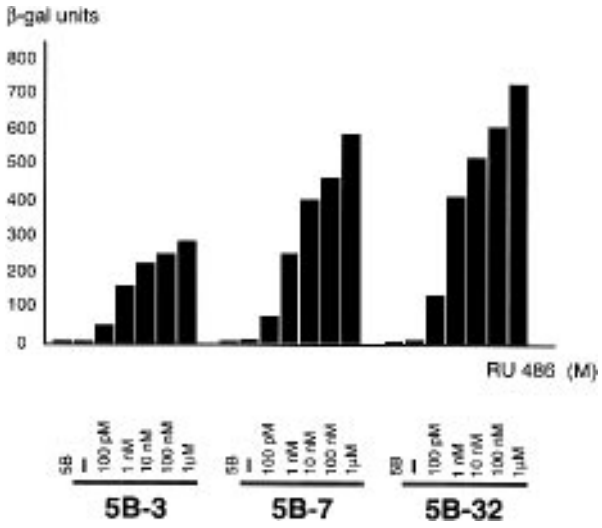

Figure 4. CrePR fusion proteins respond to very low doses of RU 486. Clones 5B-3, 5B-7 and 5B-32 were grown for 5 days in the presence of different concentrations of RU 486 (as indicated). The amount of β-galactosidase present in the cells was measured by enzymatic reaction (ONPG assay). The background activity observed in the 5B cell line, which only has the target construct integrated is indicated (5B), as well as the activity observed in the tested cell lines in the absence of RU 486 (–).

CrePR fusions can be activated at low concentrations of RU 486

Since RU 486 has anti-progesterone and anti-glucocorticoid activities (21,30), the use of CrePR fusions for gene targeting in animals should be performed using the lowest possible concentration of RU 486. To estimate the required RU 486 concentration which can activate the CrePR fusions we determined recombination activity by measuring enzymatic β-galactosidase activity after culturing 5B-3, 5B-7 or 5B-32 cells in the presence of variable amounts of the steroid. As shown in Figure 4, substantial activation is obtained with 1 nM RU 486. Induction with 100 pM RU486 is observable, although it is weak (Fig. 4).

Inducible recombination in ES cells

Based on the above studies, we selected CrePR1 as the best fusion protein for use in mice. To this end we stably introduced a

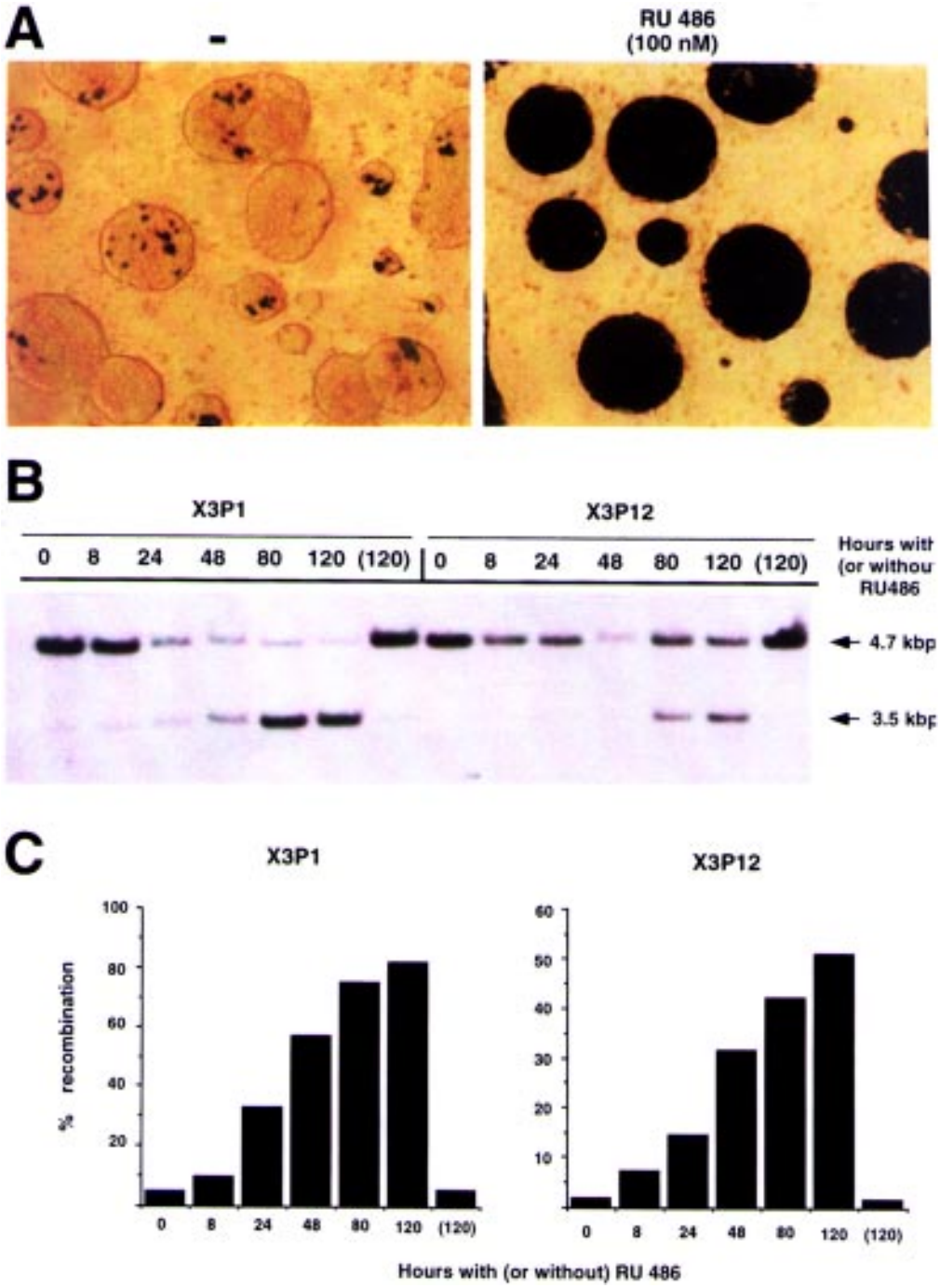

Figure 5. RU 486-inducible recombination in ES cells. (**A**) A representative clone showing RU 486-inducible β-galactosidase expression after X-gal staining. The clone was cultured for 3 days in the absence (–) or presence (+) of RU 486. (**B**) The time course of RU 486-induced recombination as assessed by Southern analysis. Genomic DNA was isolated from cells and cultured with RU 486 for the times shown above the figure. Zero (0) samples were harvested at the beginning of the study, whereas 120 samples were cultured for the duration of the study in the absence of RU 486. Genomic DNA was digested with*Eco*RI and hybridized with a probe to the *lacZ* coding region. Before recombination the *Eco*RI fragment is 4.7 kb, after recombination 3.5 kb. (**C**) Quantification by phosphorimager analysis of percent recombination observed in the Southern analysis of (B). Percent recombination is calculated as recombined counts divided by unrecombined plus recombined counts multiplied by 100.

recombination reporter into ES cells (Fig. 1B) and then selected a clone (X3P) that contained a single integrated copy (data not shown). CrePR1 was then stably introduced into X3P cells using a PGK expression vector carrying a neomycin resistance gene (Fig. 1B). Of 96 neomycin-resistant clones selected 83 showed RU486-inducible recombination, as assessed by staining for β-galactosidase expression (data not shown). However, all clones also showed variable levels of staining in the absence of induction, indicative of a background level of ligand-independent recombination. This can be seen in Figure 5A, which shows

β-galactosidase staining of a representative clone. Strong induction by RU 486 to nearly complete recombination is also apparent, as is the fact that exposure to RU 486 does not induce morphological changes in ES cells. Figure 5B shows Southern analysis of the time courses of RU486 administration. Quantification (Fig. 5C) revealed that uninduced recombination in clone X3P1 was 4.7% at the start of the incubation and 5.5% after 120 h, whereas in X3P12 it was 2.0% at the start and 2.0% at the end. RU486 treatment of X3P1 resulted in over 80% recombination within the time course of the study, whereas X3P12 was ~50% recombined after 120 h.

DISCUSSION

To develop a system in which gene targeting could be induced when desired we combined the use of the Cre–lox system with the regulative properties of the mutated human progesterone receptor hPR891. We therefore fused the hPR891 HBD domain to the phage P1 Cre recombinase. General observations on fusions between steroid hormone binding domains and heterologous proteins (18) showed that the closer the HBD is fused to an active domain the stronger is its inhibition. Since active regions were described in both the N- and the C-termini of Cre, we designed several fusion proteins which differed in the position of the HBD or the length of the hinge region (D domain). Their activities were compared in transient transfection experiments with the activity of a NLS-containing Cre (NLS–Cre). The highest activity in combination with good inducibility was observed when the HBD, including most of the D domain, was fused to the C-terminal of Cre (CrePR1). An N-terminal fusion was less active, although it displayed higher induction due to a lower background. A double fusion (CrePR7) resulted in the tightest regulation, but also showed a very low activity when induced with the anti-hormone.

The presence of a NLS enhances the activity of Cre ~2-fold. This is consistent with previous observations (24). However, the presence of a NLS had no influence on CrePR1 activity. Although the previously described hPR NLS is almost completely removed in CrePR1, a further mechanism for nuclear localization could be mediated by the hPR HBD, which is not dependent on activation of the DNA binding domain (31).

The most promising fusions (CrePR1, CrePR3 and CrePR9) were tested by stable expression in a CV1-derived cell line (5B). Both clones CrePR3 and CrePR9 showed poor to moderate inducibilities. Clone CrePR1 showed better RU 486 induction properties, however, the recombination rate was still low (3–4.5%/day; Fig. 3). We believe that the low recombination rate is probably due to low expression of the CrePR1 construct in the CV1 clones, since its expression, as determined by RNase protection experiments, was very weak (data not shown).

In contrast, activation of CrePR1 in ES cells was more efficient (Fig. 5), suggesting that inducible gene targeting in cells or animals is feasible. A recombination rate of 10–30% cells/day (Fig. 5) might be too slow for gene disruption in embryogenesis, where rapid recombination in all cells is required, however, enhanced Cre expression levels should enhance the rate of recombination. The use of strong and specifically activated promoters are presently being tested to investigate control at the level of expression and protein activity.

The Cre–hPR891 system presents several advantages for use in mice. First, it does not respond to any endogenous hormone, even in the presence of 1 μM progesterone, where we could not detect any activation. Second, RU 486 has been shown to be rapidly and widely distributed after oral or i.v. administration (32). Finally, the abortifacient properties and toxicity of RU 486 require higher doses than that required for induction of CrePR1 (Fig. 4; 33,30). *In vivo* studies in rats demonstrate that RU 486 administered at doses of 400 μg/kg or lower cannot induce abortion or prevent nidation (30). Mice are less sensitive to RU 486 and need 3-fold higher doses to induce abortion or prevent nidation (30). We show here that CrePR(891) is inducible at a concentration of 1 nM. As a mouse serum concentration of 1 nM corresponds to an orally administrated dose of ~5 μg/kg (22), the system should work in the mouse with 100- to 1000-fold lower concentrations of RU 486 than needed for abortion or anti-nidation. Toxicological studies on RU 486 showed no teratogenic or mutagenic effects in rats and mice or during embryogenesis (33).

Previous work has shown that HBDs from estrogen, glucocorticoid and androgen receptors can be fused to FLP recombinase to generate ligand-inducible recombinase–HBD fusion proteins (19). Also, work suggesting that a fusion between Cre and the estrogen receptor is estrogen inducible has been published (20). We demonstrate here that Cre is strongly regulated by RU 486 when it is fused to the hPR891 HBD. Others have reported the use of inducible Cre recombinase under the control of the interferon-responsive Mx promoter (34). The strategy presented here has an important advantage. Induction of the CrePR fusion relies on the use of a synthetic steroid that binds to a mutated HBD. Thus interference by endogenous mechanisms is limited.

The high inducibility of the reported system, combined with the described advantages of the inducing agent, suggests it as a useful system for inducible gene targeting in cells or animals. Introduction of a CrePR fusion into animals containing a gene locus which can be inactivated via recombination between two loxP sites is under way.

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