Nucleic Acids Research, 1998, Vol. 26, No. 13 **3263–3269**

Inducible expression based on regulated recombination: a single vector strategy for stable expression in cultured cells

Pierre-Olivier Angrand, Catherine P. Woodroofe, Fränk Buchholz and A. Francis Stewart*

Gene Expression Program, EMBL, Meyerhofstrasse 1, D-69117 Heidelberg, Germany

Received February 23, 1998; Revised and Accepted April 10, 1998

ABSTRACT

When fused to the ligand binding domain (LBD) of steroid hormone nuclear receptors, site-specific recombinases (SSRs) acquire a ligand-dependent activity. Here, we describe the use of SSR–LBD fusion proteins in an inducible expression system, introduced into cells in a single step. A single transgene contains a constitutively active, bi-directional enhancer/promoter, which directs expression, on one side, of an SSR–LBD fusion protein gene and, on the other, a selectable marker/inducible gene cassette. The selectable marker, the puromycin acetyltransferase (*pac*) gene, is used for stable genomic integration of the transgene and is flanked by recombination target sites. The inducible gene is not expressed because the *pac* gene lies between it and the promoter. Activation of the SSR–LBD by a ligand induces recombination and the *pac* gene is excised. The inducible gene is thus positioned next to the promoter and so is expressed. This describes a ligand-inducible expression strategy that relies on regulated recombination rather than regulated transcription. By inducible expression of diptheria toxin, evidence that this system permits inducible expression of very toxic proteins is presented. The combination of the complete regulatory circuit and inducible gene in one transgene relates expression of the selectable marker gene to expression from the bi-directional enhancer/promoter. We exploit this relationship to show that graded increases in selection pressure can be used to select for clones with different induction properties.

INTRODUCTION

Site-specific recombinases (SSRs) are enzymes from bacterial and yeast elements that nick and ligate DNA at specific targets inducing recombination. The best characterized SSRs are two members of the integrase family, Cre recombinase from the bacteriophage P1, which recognises a 34 bp sequence called *loxP* (1), and FLP recombinase from the *Saccharomyces cerevisiae* 2μ circle, which recognizes the 34 bp *FRT* site (2). Each recognition target site consists of two 13 bp inverted repeats flanking an 8 bp non-palindromic core region. The position and orientation of these targets relative to each other determine the product(s) of recombination, which can be excisions, insertions, inversions or translocations of DNA molecules (3). Cre and FLP recombinases carry all functions required for the recombination event in a single polypeptide chain. Consequently, both Cre and FLP have been used for genome engineering in bacteria, yeasts, plants, flies, mammalian cells and mice (4–12). However, full exploitation of site-specific recombination to modify the genome of living organisms requires strategies to control the desired recombination event. In most cases, regulation has been achieved by selective introduction of the recombinase gene, mRNA or protein or by controlled expression of the recombinase encoding gene using transcriptionally regulated promoters (3,13,14). More recently, a strategy based on the regulation of SSR activity, rather than expression, has been described (15). This strategy relies on fusing a ligand binding domain (LBD) from steroid receptors onto the SSR, thereby creating recombinases that require a ligand for activity. We, and others, have shown that LBDs can regulate FLP and Cre recombinase activities in different mammalian cells, such as 293, CV-1 and ES cells (15–17), hematopoietic cells from chicken embryos (18) and mice (19,20). Here, we develop the use of FLP LBDs for ligand-inducible expression systems which are introduced into cells in a single step.

MATERIALS AND METHODS

DNA techniques

Small scale plasmid DNA preparation was performed according to Maniatis *et al.* (21). Large scale preparation of plasmid DNA was done with the Qiagen Plasmid Kit (Qiagen). Restriction endonucleases and T4 DNA ligase were purchased from New England Biolabs. Plasmids were grown in *Escherichia coli* strain XL1-blue [F′::Tn*10* *proA*$^{+}$*B*$^{+}$ *lacI*^{q} Δ(*lacZ*)*M15*/*recA1* *endA1* *gyrA96* (Nal^{r}) *thi* *hsdR17* (r_k − m_{k+}) *supE44* *relA1* *lac*]. Complete nucleotide sequences and restriction maps of the plasmids used are available on our World Wide Web site http://www.embl-heidelberg/ExternalInfo/stewart/plasmids.html

*To whom correspondence should be addressed. Tel: +49 6221 387 562; Fax: +49 6221 387 518; Email: stewart@embl-heidelberg.de

Cell culture

293 cells (ATCC CRL-1573; American Type Culture Collection) were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum (PAA), 2 mM L-glutamine, 1 U/ml penicillin and 1 µg/ml streptomycin at 37°C in a humidity-saturated 5% CO_2 atmosphere. Raloxifene and mibolerone (NLP-024 Dupont; NEM Research) were used at 100 nM final concentration. Puromycin was from Sigma.

Electroporation

Stable derivatives of 293 cells were generated by electroporation. Cells were trypsinized, washed in PBS (137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4, 1.4 mM KH_2PO_4) and resuspended in PBS at 10^7 cells/ml. Then, 500 µl cell suspension were mixed with 5 µg *Xmn*I-linearized plasmid in 0.4 cm electrode gap cuvettes and electroporated at 300 V, 960 µF using a BioRad Gene Pulser. Electroporated cells were plated into two 10 cm dishes, one tenth into one dish and nine tenths into the second one, and selection pressure (1 µg/ml puromycin or greater as specified) was applied the following day.

X-Gal staining

Cells were fixed in PBS containing 1% formaldehyde (Merck), 0.2% glutaraldehyde (Sigma) and *lacZ* expression was detected by *in situ* X-Gal (5-bromo-4-chloro-3-β-D-galactopyranoside; Biomol) staining. Cells were incubated overnight in PBS with 5 mM potassium ferricyanide (Sigma), 5 mM potassium ferrocyanide (Sigma), 2 mM $MgCl_2$, 1 mg/ml X-Gal.

Methylene blue staining

The medium was removed from the dishes and cells were fixed *in situ* by addition of ethanol for 5 min. The solution was then replaced with 1% methylene blue in ethanol for 10 min.

Southern analyses

Cells were lysed with 1% SDS in 20 mM Tris–HCl, pH 8, 50 mM EDTA and incubated for 16 h at 37°C in the presence of 200 µg/ml proteinase K (Boehringer Mannheim). Genomic DNA was isolated from the lysate by phenol extraction. Southern blots were prepared as described (21) and hybridized with riboprobes. Hybridization and washes were carried out according to Church and Gilbert (22). Recombination products were visualized by autoradiography and quantified by PhosphorImager analysis. Percent recombination was calculated as the counts corresponding to the recombined product divided by counts corresponding to recombined plus unrecombined bands.

RESULTS

Strategy and vectors

We adapted FLP–LBD regulation to develop an inducible expression system for application in cells in culture (Fig. 1A). A bi-directional enhancer/promoter unit was created by positioning the human cytomegalovirus (CMV) early enhancer/promoter region next to a duplicated simian virus 40 (SV40) early enhancer and herpes simplex virus Tk promoter. The SV40/Tk enhancer/promoter drives expression of the FLP–LBD fusion protein gene.

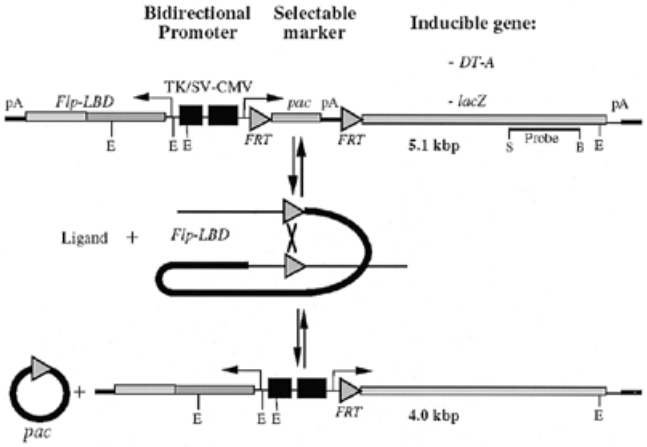

Figure 1. The single vector, FLP–LBD-inducible expression system. The vector used for stable integration (first line), the recombination intermediate (second line) and the recombination products, including the excised circle (third line), are shown. The vector contains a bi-directional enhancer/promoter made from Tk, SV40 and CMV elements that drive constitutive expression of both a FLP–LBD fusion protein gene and the selectable marker (*pac*) used for stable integration of the vector into the genome. The selectable marker gene includes a polyadenylation signal (pA) which blocks expression of the downstream inducible gene. The selectable marker gene is flanked by two FRTs (triangles) in direct orientation. The inducible genes used here are the *lacZ* gene, which is shown in the diagram, and the diptheria toxin A chain gene (*DT-A*), which is not shown. Addition of ligand activates the FLP–LBD fusion protein to excise the selectable maker and thereby positions the inducible gene next to the CMV promoter. The positions of the probe and *Eco*RI restriction sites used for Southern analysis are also shown. CMV, human cytomegalovirus early enhancer/promoter; Tk/SV, hybrid HSV-Tk promoter/duplicated SV40 early enhancer; *pac*, puromycin acetyltransferase gene; *Flp–LBD*, FLP recombinase–steroid receptor ligand binding domain fusion protein gene.

We chose two LBDs that are insensitive to ligands present in fetal calf serum. In p22LFA2 and p22LFA3, the human androgen receptor (amino acids 624–919; see 15) was used. In p22LFE1, the human estrogen receptor (amino acids 251–595; see 15), containing the mutation G521R, homologous to the murine mutant G525R (23,24), was used. This mutation renders FLP–EBD^{521R} refractory to 17β-estradiol but inducible by synthetic ligands such as 4-hydroxytamoxifen and raloxifene. In both cases, phenol red-containing medium can be used and stripping of steroid hormones from fetal calf serum is not necessary.

The CMV enhancer/promoter drives expression of the puromycin acetyltransferase gene (*pac*; 25), which was placed between two FLP recombination targets (FRTs). Expression of the downstream, inducible gene (in this study either the *lacZ* or *DT-A* genes) is occluded by the presence of the SV40 early polyadenylation signal, which was also placed between the FRTs. The presence of an efficient polyadenylation signal is essential to this strategy to block polycistronic expression (data not shown). To select for integration of the constructs, the *pac* gene was chosen because sensitive cells die within 48 h of treatment.

In both p22LFE1 and p22LFA2, the F70L mutant *FLP* gene was used. This mutation decreases FLP activity (26). In p22LFA3 the wild-type *FLP* was used.

Properties of the FLP–LBD inducible expression system

Stable cell lines containing the FLP–LBD-mediated inducible expression system were generated in a single step. 293 cells were electroporated with *Xmn*I-linearized plasmids. After selection with puromycin (1 μg/ml final concentration), puromycin-resistant colonies were picked randomly for expansion and analysis. Picked clones were individually tested *in situ* in 24-well dishes for ligand-induced, FLP–LBD-mediated recombination according to two criteria: (i) FLP–LBD-mediated induction of *lacZ* expression was tested by *in situ* X-Gal staining of cells; (ii) FLP–LBD-mediated excision of the *pac* gene was tested by the restoration of puromycin sensitivity. Since puromycin kills sensitive cells very quickly, this test can be easily performed during expansion of picked clones to identify ligand-inducible positives.

Figure 2A shows an example of six individual clones (1–6) picked after stable transfection with the p22LFA3 construct and grown under four conditions in a 24-well dish. The first two rows show the six clones cultured without or with the inducing ligand, mibolerone, and then stained for *lacZ* expression. Three of the six puromycin-resistant clones (2, 4 and 6) showed strong expression of the *lacZ* gene in the presence of mibolerone, however, clones 2 and 6 also showed some *lacZ* expression in the absence of mibolerone. The third and fourth rows show a test for excision recombination of the puromycin resistance gene. The *lacZ*-inducible clones 2, 4 and 6 also showed sensitivity to puromycin when cultured with mibolerone. Methylene blue staining was used here to stain residual organic material for the purpose of photography. Ligand-induced puromycin sensitivity was more evident from direct examination of the culture dishes, by changes in cell morphology and increased cell death, at least 24 h earlier than the methylene blue staining results shown here.

Figure 2A shows part of a comparative experiment where all three constructs, p22LFE1, p22LFA2 and p22LFA3, were used to generate puromycin-resistant, stable clones. In this experiment, nine of 12 p22LFE1, three of 10 p22LFA2 and five of 12 p22LFA3 clones showed ligand-inducible *lacZ* expression and loss of puromycin resistance. For most of the clones, 5 days growth in the absence of ligand gave <1% of [lacZ$^+$] blue cells and >95% of cells revealed β-galactosidase activity when grown in the presence of the inducing ligand. An example is shown in Figure 2B.

As a further test, Southern blot analysis was performed on puromycin-resistant 293 clones showing ligand-dependent *lacZ* inducibility, grown for 3 days in the presence or absence of ligand. Genomic DNA was extracted, digested with *Eco*RI and hybridized to a *lacZ* probe. As indicated in Figure 1, the expected size of the *Eco*RI fragments before and after FLP-mediated recombination are 5.1 and 4.0 kb respectively. Figure 2C shows the results from six clones. Ligand treatment revealed an *Eco*RI fragment of 4.0 kbp length, indicating that *lacZ* inducibility and puromycin sensitivity in the presence of ligand were indeed due to a FLP–LBD-mediated recombination event.

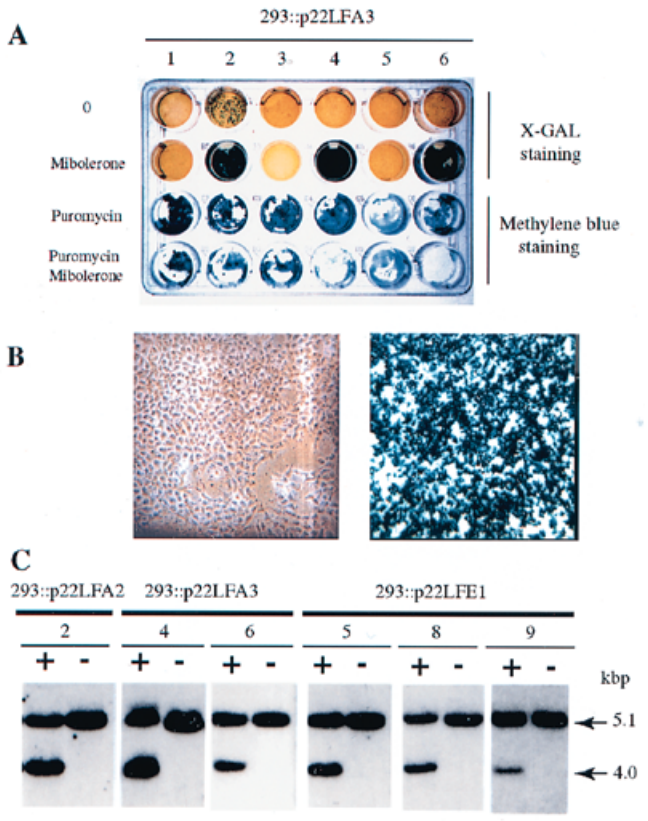

Figure 2. Properties of the FLP–LBD inducible system in stable experiments with 293 cells. (**A**) Test of individual clones stably expressing the p22LFA3 construct in a 24-well dish. Six clones, labelled 1–6, were plated into four rows and grown in different medium conditions for 3 days. From the top to the bottom, the rows show clones grown in medium alone (0), with 100 nM mibolerone, with puromycin and with both puromycin and mibolerone. The top two rows show *lacZ* expression in the absence or presence of the inducible ligand as tested by *in situ* X-Gal staining; the lower two rows show methylene blue staining of organic material. (**B**) Detail at a higher magnification of *lacZ* expression in clone p22LFA3-4. Cells were grown for 5 days in the absence (left) or presence (right) of 100 nM mibolerone before *in situ* X-Gal staining. (**C**) Ligand-induced recombination as assessed by Southern analysis. Representative clones were grown for 72 h in the absence (–) or presence (+) of inducing ligand (100 nM). Genomic DNA was isolated, digested with *Eco*RI and hybridized with the probe as shown in Figure 1. Before recombination, *Eco*RI digestion produces a 5.1 kb fragment recognized by the indicated probe. Upon ligand induction of FLP–LBD-mediated recombination, this fragment is reduced to 4.0 kb. Inducing ligands were mibolerone for clones 293::p22LFA2#2, 293::p22LFA3#4 and 293::p22LFA3#6 and raloxifene for clones 293::p22LFE1#5, 293::p22LFE1#8 and 293::p22LFE1#9.

Inducible expression of the *DT-A* gene

In contrast to other expression systems based on transcriptional regulation, the FLP–LBD-mediated inducible expression strategy is based on a regulated recombination event. Figure 3A diagrammatically presents the difference between transcription- and recombination-based inducible systems. For transcriptional systems, responsiveness often requires some level of promoter activity before induction. For recombination systems, as long as the recombination cassette completely occludes polycistronic expression, no expression before induction is expected, whatever the activity of the promoter. One interesting corollary of this reasoning is that inducible expression of very toxic genes should be readily possible with recombination-based inducible expression. In order to test this, and to evaluate whether the p22LF vectors show the desired properties, the *DT-A* gene was cloned into the p22LFE1 vector, generating the p22EDT1 plasmid (Fig. 3A). DT-A was chosen because it has been reported to be one of the most cytotoxic proteins known (27,28).

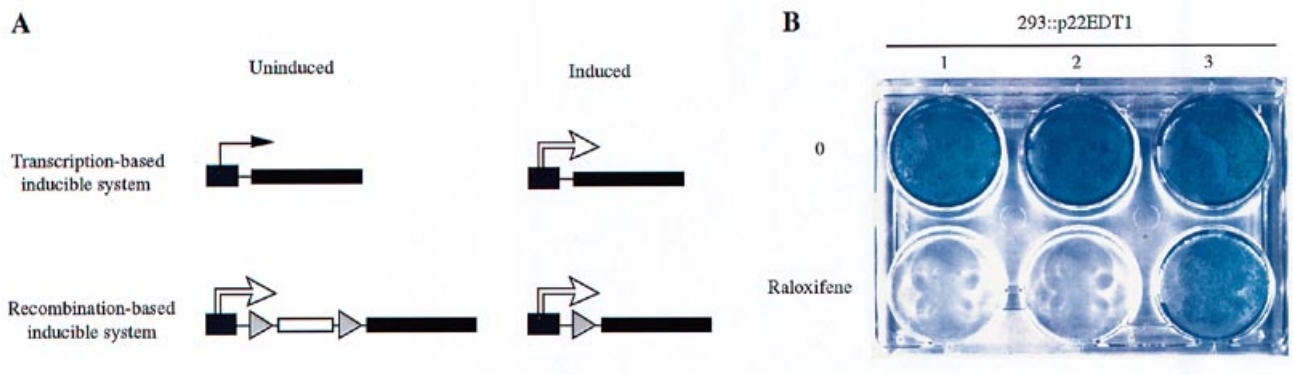

Figure 3. Recombination-based inducible expression applied to a cytotoxic protein. (**A**) Transcriptional and recombinational strategies for inducible expression are depicted. Transcriptional strategies amplify a predisposition to responsiveness, as depicted by the change between thin and broad arrows, whereas recombinational strategies are based on a DNA rearrangement by excision of DNA between directly repeated recombination target sites, depicted as triangles. (**B**) Induction of diptheria toxin A chain expression in stable clones. Three individual clones stably expressing the p22EDT1 construct were grown for 5 days in 6-well dishes in the absence (top) or presence of 100 nM raloxifene (bottom), before staining with methylene blue. Clones 1 and 2 show complete cell death, as evident from the total lack of methylene blue staining. Clone 3 shows partial cell death. The construct, p22EDT1 was made by cloning the *DT-A* gene in place of the *lacZ* gene in p22LFE1.

In parallel experiments, 293 cells were electroporated with *Xmn*I-linearized p22EDT1 or p22LFE1. Similar frequencies of puromycin-resistant clones were obtained for both the *DT-A* and the *lacZ* inducible expression vectors; ~900 puromycin-resitant colonies were obtained with the p22EDT1 plasmid and ~600 when the p22LFE1 construct was used. Twelve puromycin-resistant clones from each were further analyzed. The rate of growth of both sets was similar (data not shown). Out of the 12 p22EDT1 clones, 10 died within 4–6 days after addition of 100 nM raloxifene to the culture medium (Fig. 3B). As observed for the *lacZ*-based construct (Table 1 and Fig. 4), differences in kinetics of recombination were also found using the *DT-A* gene as the inducible gene (Fig. 3B and data not shown). This experiment shows that the *DT-A* gene was not expressed in the absence of ligand to any detectable level and could be expressed after addition of the inducing ligand to the medium. In agreement with the reasoning underlying Figure 3A, we conclude that a merit of inducible recombination is the effective absence of expression in the uninduced state.

Table 1. Clones isolated at different puromycin concentrations

	Number of clones		Clone class					Single copy
	Total	Inducible	I	II	III	IV	V	
1 µg/ml	400	11/17	7	3	1	0	0	4/5
5 µg/ml	47	12/15	5	3	2	2	0	4/6
10 µg/ml	28	13/14	1	3	1	2	6	6/7
20 µg/ml	1	1/1	0	0	0	0	1	NT

293 cells electroporated with p22LFA3 were plated into medium containing different puromycin concentrations. The number of puromycin-resitant colonies (Total) and the proportion of clones showing ligand-inducible *lacZ* expression (Inducible) are indicated. Based on the number of LacZ expressing cells shown by X-Gal staining in the absence or presence of mibolerone (100 nM), the clones are classified into five different classes. Classes I and II, <0.1% [lacZ$^+$] cells in the absence of ligand; Classes III and IV, between 0.1 and 1% [lacZ$^+$] cells in the absence of ligand; Class V, >1% [lacZ$^+$] cells in the absence of ligand. In the presence of mibolerone, clones from Classes I and III showed between 50 and 90% [lacZ$^+$] cells and clones from Classes II and IV >90%. The frequency of clones containing a single copy of the transgene determined by Southern blot quantifications is indicated. NT, not tested.

Puromycin selection pressure can be used to vary recombination induction kinetics

A second potentially beneficial consequence of the use of recombinational rather than transcriptional mechanisms for inducible expression was explored. As shown above with the *DT-A* gene, expression from p22LF vectors before recombination is essentially zero. After recombination, the inducible gene replaces the selectable marker gene and therefore the expression levels of the selectable marker gene before, and the inducible gene after, are related. We reasoned that increased selection pressure during isolation of stable clones may select for clones with high levels of expression of the selectable marker before recombination and, consequently, high expression levels of the inducible gene after recombination. The use of increased selection pressure may also increase expression of the FLP–LBD protein from the other side of the bi-directional promoter cassette. Therefore, we evaluated both the effect of increased selection pressure on recombination kinetics and *lacZ* expression.

Cells electroporated with the p22LFA3 construct were plated into medium containing different puromycin concentrations. As expected, the number of puromycin-resistant colonies decreased with increased puromycin concentration (Table 1) and no colonies were obtained at puromycin concentrations >20 µg/ml. Ligand-inducible *lacZ* expression properties of individual clones were tested by X-Gal staining after 3 days growth in the absence or presence of 100 nM mibolerone in 24-well dishes. Most of the clones showed ligand-inducible *lacZ* expression (Table 1), however, different levels of *lacZ* expression in the absence of ligand induction were evident. By these properties, the clones were classified into five different classes. Class I corresponds to clones showing no or <0.1% [lacZ$^+$] cells in the absence and between 50 and 90% [lacZ$^+$] cells in the presence of mibolerone. Class II corresponds to clones presenting no or <0.1% [lacZ$^+$] cells in the absence of ligand, but >90% [lacZ$^+$] cells after induction. Classes III and IV group clones showing a background between 0.1 and 1% [lacZ$^+$] cells and between 50 and 90% (Class III) or > 90% (Class IV) [lacZ$^+$] cells when grown in the presence of the inducing ligand. Class V corresponds to clones containing

>1% [lacZ$^+$] cells in the absence of mibolerone. As shown in Table 1, most of the puromycin-resistant clones isolated in the presence of 1 μg/ml puromycin showed very low numbers of [lacZ$^+$] cells in the absence of mibolerone (Classes I and III). When the puromycin concentration was increased to 5 μg/ml, the proportion of clones presenting nearly complete *lacZ* expression upon ligand administration (Classes II and IV) increased. These clones corresponded to the majority of the isolated clones when the puromycin concentration used was 10 μg/ml. However, at this concentration most clones showed high numbers of [lacZ$^+$] cells in the absence of ligand (Class V). Southern blot analyses of recombination time courses initiated by addition of ligand confirmed these observations (Fig. 4A and data not shown). Most of the clones isolated at low levels of puromycin (1 μg/ml) showed slow kinetics of recombination (Fig. 4A and data not shown). Ligand-dependent recombination was faster in the majority of the clones isolated at 5 μg/ml puromycin. At higher levels of puromycin selection (10 μg/ml) the kinetics of recombination in a number of clones was even faster. Southern blot quantifications also showed that the increased number of clones having fast kinetics of recombination in the presence of higher concentration of puromycin was not due to the selection of clones containing higher numbers of transgene copies (Table 1). This demonstrates that puromycin selection pressure is a parameter which facilitates the isolation of clones showing different properties of inducibility. At low levels of puromycin clones with gentle kinetics of recombination and *lacZ* inducibility were isolated, while at higher concentrations of puromycin, clones with fast kinetics of recombination were isolated, presumably by selection for clones expressing higher levels of the *pac* and FLP–LBD genes. Although these data describe a general relationship between increased puromycin selection pressure, increased recombination kinetics and increased background recombination, several clones that show no or very little background and rapid rates of recombination were identified (Fig. 4A and data not shown).

Increased puromycin selection pressure on established clones also increases recombination kinetics

Since increased puromycin concentration during selection of stable clones increased inducible recombination kinetics, we asked whether established clones could also respond to increased concentrations of puromycin during culture. Clones isolated in the presence of 1 μg/ml puromycin were grown for three passages in the presence of 5 μg/ml puromycin-containing medium and recombination kinetics in the presence of the inducers was followed by Southern blot analysis. As shown in Figure 4B and C, the clones showed faster rates of recombination after three passages in the presence of 5 μg/ml puromycin. In the case of clone 293::LFA3#4, this treatment had dramatic effects on recombination efficiency, leading to nearly 100% recombination after 72 h mibolerone induction. Notably, background recombination in the absence of ligand was not increased in this case. These results indicate that the properties of the FLP–LBD-based inducible system were not fixed by the initial integration event and could be modulated by growth in the presence of higher concentrations of puromycin.

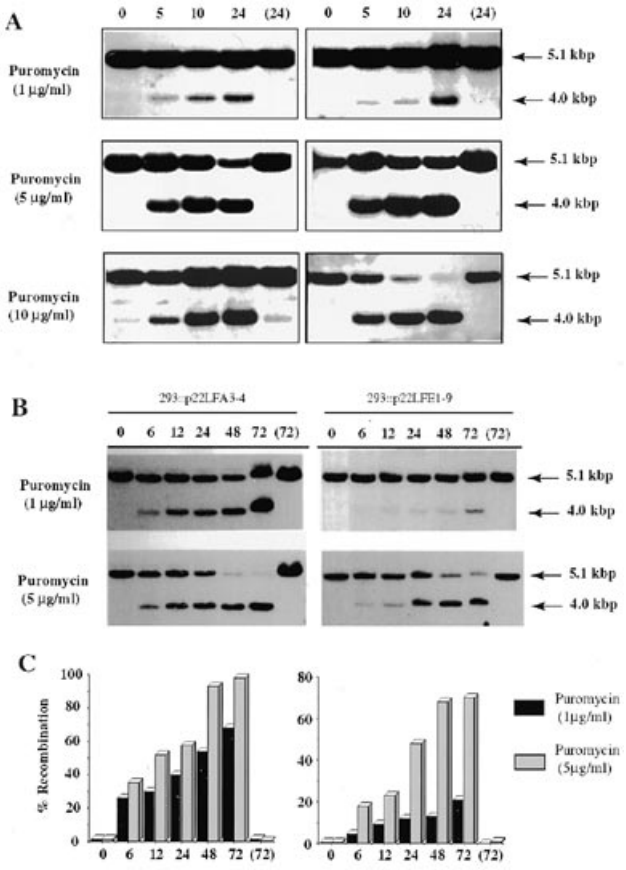

Figure 4. Puromycin selection pressure alters ligand-induced recombination kinetics. (**A**) Southern analysis of ligand-induced recombination time courses of p22LFA3 clones isolated under 1, 5 or 10 μg/ml puromycin selection pressure. Six independent clones are shown. (**B**) Southern analysis of ligand-induced recombination time courses of two clones, 293::p22LFA3#4 (left) and 293::p22LFE1#9 (right) initially selected and expanded under 1 μg/ml puromycin selection pressure. Both clones were then passaged three times in 5 μg/ml puromycin-containing medium to compare recombination kinetics before (top) and after (bottom) 5 μg/ml puromycin treatment. Cells were grown for 72 h without ligand [(72)] or with ligand for 0, 6, 12, 24, 48 and 72 h (as indicated) in the absence of puromycin. Inducing ligands were mibolerone (100 nM) for clone 293::p22LFA3#4 and raloxifene (100 nM) for clone 293::p22LFE1#9. (**C**) Quantification by phosphoimager analysis of percent of recombination observed in the Southern analysis of (B).

DISCUSSION

Previously described eukaryotic inducible gene expression systems rely on the use of promoters responsive to, for example, heavy metal ions, heat shock, double-stranded RNA or hormones (29). These systems evoke endogenous transcriptional responses. More recently, prokaryotic regulatory circuits, such as lac or Tet repressor/operator/inducer systems (30–32), as well as the *Drosophila* ecdysone receptor system (33), have been applied in mammalian cells. All of these expression systems are based on transcriptional regulation. We have here developed a single vector strategy based on regulated recombination. Fusion of the LBD from a steroid hormone receptor to FLP confers on the recombinase an activity that is dependent on ligand administration. We have used this to regulate FLP activity in order

to develop an inducible expression system based on recombination. In contrast to the quantitative change between the basal and induced states occurring in transcription-based expression systems, recombination induces a qualitative change in expression of the inducible gene (Fig. 3A). This difference presents certain advantages and disadvantages when transcriptional and recombinational strategies are compared. First, transcriptional systems are usually reversible when the inducing agent is removed. The recombinational system described here is not. Second, the cause of background expression before induction differs. In transcriptional systems, the need to position the inducible transgene in chromatin regions permissive to transcriptional induction and the presence of unregulated basal promoter elements often leads to a low level of expression before induction. In the recombinational system described here, uninduced background expression is due to recombination that fully activates expression in a small number of cells, with the majority of cells showing no expression. In part, this background appears to relate to high expression levels of the FLP–LBD fusion, however, the relationship between background and induced rates of recombination is not direct, since clones showing no background and rapid rates of recombination were identified (Fig. 4A). Third, expression levels achieved after induction are determined by different elements. For transcriptional systems, the expression level reflects the duration and strength of transcriptional stimulation, whereas for recombinational systems, the choice of the constitutively active enhancer/promoter, here CMV, is a major determinant.

The differences between recombinational and transcriptional systems recommend selective application of one or the other as appropriate to different circumstances. For example, recombinational induction appears to be well suited to the inducible expression of cytotoxic and, we suggest, dominant negative proteins. Transcriptional systems are better applied when a transient expression period is required.

The recombinational system described here includes two features to facilitate its use. First, the complete regulatory circuit and the inducible gene are present on a single transgene that is introduced in a single step. Second, the dual use of the *pac* gene as selectable marker and the element to block expression of the inducible gene before induction presents a simple initial screen for inducible clones. Puromycin kills senstive cells very rapidly, so recombinational excision of the *pac* gene to restore sensitivity serves as a way to eliminate those clones that show no or slow induction of recombination.

A third experimentally amenable feature of this recombination system emerged from the characterization of its properties. We found that the central role of the selectable marker in the transgene also permits the use of selection pressure to manipulate the kinetics of recombination, and hence expression. We also found that selection pressure influences both recombination properties during initial clonal selection (Fig. 4A) and during culture of established clones (Fig. 4B and C). Whereas the first effect can be explained by reasoning that increased selection pressure identifies a particular subset of genomic integration sites that are particularly favourable, the second effect is more difficult to understand. Since the second effect is apparently not due to transgene amplification or rearrangement (Fig. 4B), we suggest that selection pressure on cultured cells can induce epigenetic responses in gene transcription and/or site-specific recombination. Nevertheless, the utility of this experimental procedure is apparent.

The operational properties of inducible recombination were examined not only to establish the advantages and disadvantages of this system, but also to outline practical steps that can be taken in further implementations. In this regard, it is important to realise that the bi-directional CMV/SV40/Tk enhancer/promoter cassette directs high levels of expression when stably integrated as a single copy in 293 cells, as described here. In other cell types, a different bi-directional promoter or different transfection conditions that lead to multiple genomic integrations may be required. Alternatively, Cre–LBD fusion proteins (16,17,19,20) may be more effective in other circumstances.

Interestingly, we consistently observed that the percentage of total recombination, as measured by Southern blotting, was less than the percentage of cells that acquired puromycin sensitivity, or *lacZ* or *DT-A* expression, as observed *in situ*. Since the site-specific recombinases FLP and Cre mediate conservative, reversible reactions, this difference between apparent recombination and expression is expected when the circular excision product (here the *pac* gene) re-integrates. Thereby, cells that have undergone excision and re-integration events may contain both the inducible expression product and the recombination substrate. As discussed (15), apparent excision recombination kinetics in living cells are based on two parameters. The first is the rate of recombination and the second is the rate of loss of the excised circle from the cell to curtail the re-integration reaction. It is not possible to measure the true rate of recombination here, since this requires separate measurements of the true rates of excision and re-integration or the true rate of loss of the excised circle. Nevertheless, we conclude from our *in situ* observations of recombination-based expression differences that true rates of recombination achieved with the FLP–LBD system are significantly faster than the total apparent recombination rate. Consequently, the FLP–LBD system serves to express the inducible gene uniformly in a clonal cell population with reasonable and, as shown above, adjustable kinetics.

The use of steroid hormones as inducible agents can generate unsuitable effects in those circumstances where an endogenous receptor for the inducing ligand is present. Here, we used two different LDBs, but LBDs from glucocorticoid and progesterone receptors are also able to regulate the activity of site-specific recombinases (15,16) and can be used in the same way. Having several different LBDs and mutant variants permits a rational choice in order to avoid or minimize pleiotropic effects provoked by the inducing ligands.

ACKNOWLEDGEMENTS

We wish to thank Hermann Bujard, Colin Logie, Mark Nichols and Leonie Ringrose for discussions. This work was supported by an EU fellowship to P.-O.A. and by the Volkswagen Stiftung, Program on Conditional Mutagenesis.

REFERENCES

1 Sternberg,N. and Hamilton,D. (1981) *J. Mol. Biol.*, **150**, 467–486.
2 Falco,S.C., Li,Y., Broach,J.R. and Botstein,D. (1982) *Cell*, **29**, 573–584.
3 Kilby,N.J., Snaith,M.R. and Murray,J.A.H. (1993) *Trends Genet.*, **9**, 413–421.
4 Cox,M.M. (1983) *Proc. Natl. Acad. Sci. USA*, **80**, 4223–4227.
5 Sauer,B. (1987) *Mol. Cell. Biol.*, **7**, 2087–2096.

6 Sauer,B. and Henderson,N. (1988) *Proc. Natl. Acad. Sci. USA*, **85**, 5166–5170.
7 Golic,K.G. and Lindquist,S. (1989) *Cell*, **59**, 499–509.
8 Odell,J., Caimi,P., Sauer,B. and Russell,S. (1990) *Mol. Gen. Genet.*, **223**, 369–378.
9 Dale,E.C. and Ow,D.W. (1990) *Gene*, **91**, 79–85.
10 O'Gorman,S., Fox,D.T. and Wahl,G.M. (1991) *Science*, **251**, 1351–1355.
11 Lakso,M., Sauer,B., Mosinger,B.,Jr, Lee,E. J., Manning,R.W., Yu,S.H., Mulder,K. L. and Westphal,H. (1992) *Proc. Natl. Acad. Sci. USA*, **89**, 6232–6236.
12 Orban,P.C., Chui,D. and Marth,J.D. (1992) *Proc. Natl. Acad. Sci. USA*, **89**, 6861–6865.
13 Konsolaki,M., Sanicola,M., Kozlova,T., Liu,V., Arca,B., Savakis,C., Gelbart,W.M. and Kafatos,F.C. (1992) *New Biol.*, **4**, 551–557.
14 Kühn,R., Schwenk,F., Aguet,M. and Rajewsky,K. (1995) *Science*, **269**, 1427–1429.
15 Logie,C. and Stewart,A.F. (1995) *Proc. Natl. Acad. Sci. USA*, **92**, 5940–5944.
16 Kellendonk,C., Tronche,F., Monaghan,A.-P., Angrand,P.-O., Stewart,A.F. and Schütz,G. (1996) *Nucleic Acids Res.*, **24**, 1404–1411.
17 Zhang,Y., Riesterer,C., Ayrall,A.-M., Sablitzky,F., Littlewood,T.D. and Reth,M. (1996) *Nucleic Acids Res.*, **24**, 543–548.
18 Rossi,F., McNagny,K.M., Logie,C., Stewart,A.F. and Graf,T. (1996) *Curr. Biol.*, **6**, 866–872.
19 Feil,R., Brocard,J., Mascrez,B., LeMeur,M., Metzger,D. and Chambon,P. (1996) *Proc. Natl. Acad. Sci. USA*, **93**, 10887–10890.
20 Schwenk,F., Kühn,R., Angrand,P.-O., Rajewsky,K. and Stewart,A.F. (1998) *Nucleic Acids Res.*, **26**, 1427–1432.
21 Maniatis,T., Frisch,E.F. and Sambrook,E.F. (1982) *Molecular Cloning: A Laboratory Manual*. Cold Spring Harbor Laboratory Press., Cold Spring Harbor, NY.
22 Church,G. and Gilbert,W. (1984) *Proc. Natl. Acad. Sci. USA*, **81**, 1991–1995.
23 Danielian,P.S., White,R., Hoare,S.A., Fawell,S.E. and Parker,M.G. (1993) *Mol. Endocrinol.*, **7**, 232–240.
24 Littlewood,T.D., Hancock,D.C., Danielian,P.S., Parker,M.G. and Evan,G.I. (1995) *Nucleic Acids Res.*, **23**, 1686–1690.
25 de la Luna,S. and Ortín,J. (1992) *Methods Enzymol.*, **216**, 376–385.
26 Buchholz,F., Ringrose,L., Angrand,P.-O., Rossi,F. and Stewart,A.F. (1996) *Nucleic Acids Res.*, **24**, 4256–4262.
27 Yamaizumi,M., Mekada,E., Uchida,T. and Okada,Y. (1978) *Cell*, **15**, 245–250.
28 Breitman,M.L., Clapoff,S., Rossant,J., Tsui,L.-C., Glode,L.M., Maxwell,I.H. and Bernstein,A. (1987) *Science*, **238**, 1563–1565.
29 Yarranton,G.T. (1992) *Curr. Opin. Biotechnol.*, **3**, 506–511.
30 Hu,M.C.-T. and Davidson,N. (1987) *Cell*, **48**, 555–566.
31 Labow,M.A., Baim,S.B., Shenk,T. and Levine,A.J. (1990) *Mol. Cell. Biol.*, **10**, 3343–3356.
32 Gossen,M. and Bujard,H. (1992) *Proc. Natl. Acad. Sci. USA*, **89**, 5547–5551.
33 No,D., Yao,T.-P. and Evans,R.M. (1996) *Proc. Natl. Acad. Sci. USA*, **93**, 3346–3351.