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Genetics

Ligand-regulated site-specific recombination

(steroid/estrogen/FLP recombinase/inducible gene expression/genetic engineering)

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ABSTRACT **Site-specific recombination offers a potential way to alter a living genome by design in a precise and stable manner. This potential requires strategies which can be used to regulate the recombination event. We describe a strategy to regulate FLP recombinase activity which relies on expressing FLP as a fusion protein with steroid hormone receptor ligand binding domains (LBDs). In the absence of a ligand cognate to the LBD, the recombinase activity of the fusion protein is extremely low. Upon ligand administration, recombinase activity is rapidly induced. These results outline the basis for inducible expression or disruption strategies based on inducible recombination. Additionally, we have exploited the conditional nature of FLP–LBD fusion proteins to direct integration of a plasmid into a specific genomic site at frequencies approaching the frequency of random integration.**

Site-specific recombinases of the integrase family catalyze excisions, insertions, inversions, or translocations of DNA between their recognition target sites. The orientation of these sites relative to each other and the configuration of the surrounding DNA determine the product(s) obtained (1, 2). Several integrase family members, including FLP and Cre recombinases, carry all functions required for the recombination reaction in a single polypeptide chain (3–5). Consequently, these recombinases are particularly amenable to applications in living systems. They are active when introduced into cells from a broad range of organisms, including *Escherichia coli*, yeasts, and mammals (2, 3, 6–12). A variety of applications based on the introduction of both the recombinase and appropriately disposed recombination target sites have been successful. Full exploitation of site-specific recombination to alter genotype in living systems requires strategies to regulate the recombination event. In previous studies, regulation has been accomplished by controlled expression of the recombinase gene (2) or by introduction of recombinase mRNA or protein (13, 14). Here we describe a strategy based on regulating the activity, rather than the expression, of a site-specific recombinase.

The molecular cloning of cDNAs for members of the steroid receptor superfamily revealed that the protein domain responsible for binding steroid, the ligand-binding domain (LBD), also inactivates the other functions of the receptor in the absence of bound steroid (15–17). Inactivation is probably mediated by association of the nonligand-bound LBD with an abundant protein complex containing Hsp90 (18–20). The LBD has been fused to several transcription factors and oncogenes, imparting the properties of inactivation in the absence of, and activation by, cognate ligands (21). To test whether LBD properties could be employed to regulate a site-specific recombinase, we made fusion proteins between FLP recombinase and LBDs from the estrogen, glucocorticoid, or androgen receptor. We show that recombination mediated by FLP–LBD fusion proteins is strictly regulated in the absence and efficiently induced in the presence of ligand administration. The strict regulation permits the establishment of stable cell lines carrying both the expressed fusion protein and the unrearranged recombination substrate, and recombination is rapidly induced upon ligand administration. Furthermore, the presence of expressed FLP–LBD in cells transfected with plasmids containing recombinase target sites permits site-specific integration at efficiencies approaching that of random integration.

MATERIALS AND METHODS

Cell Culture. All cells were cultured in Dulbecco's modified Eagle's medium without phenol red containing 10% charcoal-stripped fetal calf serum, 2 mM glutamine, and 100 international units each of penicillin and streptomycin per ml. Fetal calf serum was stripped by stirring 500 ml with 25 g of Norit A (Serva) for 30 min, followed by centrifugation at 13,000 × *g* for 20 min and filtration twice through filters with 0.2-μm diameter pores.

Transfections. Transfections for transient expression utilized *N*-[1-(2,3-dioleoyloxy)propyl]-*N*,*N*,*N*-trimethylammonium methyl sulfate (DOTAP; Boehringer Mannheim) according to the manufacturer's instructions with 5 μg of plasmids pOG44, pFRTβGal, p44HER1, p44HGR1, or p44HAR1, as indicated, and 10^5 E25B2B2 cells grown in 3.5-cm dishes. Stable derivatives of 293 cells (ATCC CRL-1573; American Type Culture Collection) were generated by electroporation. Cells were harvested by trypsinization and centrifugation, washed one time in phosphate-buffered saline (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 per ml. Then, 500 μl of cell suspension was mixed with 2 μg of linearized or 5 μg of circular plasmid DNA in 0.4-cm cuvettes and electroporated at 300 V and 960 μF by using a Bio-Rad Gene Pulser. The cells were then cultured for 24 h before being selected [400 μg of Geneticin (G418; GIBCO) and 400 units of hygromycin (Calbiochem) per ml].

Cell Lines. E25B2B2 cells were derived by calcium phosphate cotransfection of E25B2 cells (ref. 9; Stratagene) with 19 μg of pOG44 and 1 μg of pOG45, followed by selection for G418 resistance. A site-specific genomic integrant was identified by a lack of β-galactosidase expression and by Southern analysis. Derivatives of 293 cells containing randomly integrated pNEOβGAL were created by transfecting cells with *Apa* I-linearized pNEOβGAL. Four clones that were assessed by Southern analysis to contain independent, single-copy integrations of the pNEOβGAL transgene were selected for transfection with pHFE1. Cell lines resistant to hygromycin and G418 were generated by transfection with *Xmn* I linearized pHFE1. Cell lines P1.2B, P1.4B, and Q3.2B were made by plating 100 P1.2, P1.4, or Q3.2 cells onto 10-cm dishes and culturing them in the presence of 100 nM estradiol. Several

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Abbreviations: LBD, ligand-binding domain; EBD, estrogen-binding domain; FRT, FLP recombinase recognition target.

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isolated colonies were picked for each and further testing confirmed that all had excised the neomycin-resistance gene.

Assays. β-Galactosidase expression was detected *in situ* by the method of Sanes *et al.* (22). β-Galactosidase-expression levels in cell extracts were determined by the method of Herbomel *et al.* (23). Southern analysis was performed as described (24).

RESULTS

Transient Expression of FLP–LBD Fusion Proteins. Previous studies with LBD fusion proteins have demonstrated that various transcription factors and oncoproteins can be regulated by steroid hormones (21). To test whether the LBD fusion-protein strategy could also regulate a site-specific recombinase, we used a genomic recombination assay (9). In this assay, the open reading frame of the *E. coli* β-galactosidase (*lacZ*) gene is interrupted by an expression cassette for neomycin resistance, flanked by directly repeated FLP recombinase recognition targets (FRTs; Fig. 1). FLP-mediated recombination excises the neomycin resistance gene from its chromosomal locus, thereby reconstituting the open reading frame of the *lacZ* gene. In transient-expression experiments with no added ligand, the FLP–LBD fusions displayed approximately 1% of the activity of unmodified FLP on the genomic FLP excision target. Addition of the cognate ligand restored recombinase activity to up to 50% that of unmodified FLP enzyme (Fig 2).

Stable Expression of FLP–Estrogen-Binding Domain (EBD) Fusion Proteins. Stable cell lines containing both the unrecombined pNEOβGAL and FLP–LBD expression plasmid were established in two steps. First, the recombination target pNEOβGAL (Fig. 1) was introduced into 293 cells by electroporation and selected by G418 resistance. After expansion of 16 randomly picked colonies, Southern blotting was employed to select 4 clones which contained a single copy of pNEOβGAL. These four clones, P1, R3, R8, and Q3, were then transfected with pHFE1, a plasmid which confers hygromycin B resistance and expresses a FLP–EBD fusion protein (Fig. 1). The EBD present in pHFE1 is the Gly-400 to Val point mutant which shows decreased sensitivity to estrogens at 37°C (25). The use of the Gly-400 → Val EBD was important for the experiments described here because the culture medium contained sufficient residual estrogens to induce the FLP–wild-type EBD recombinase to significant levels of recombination (unpublished data).

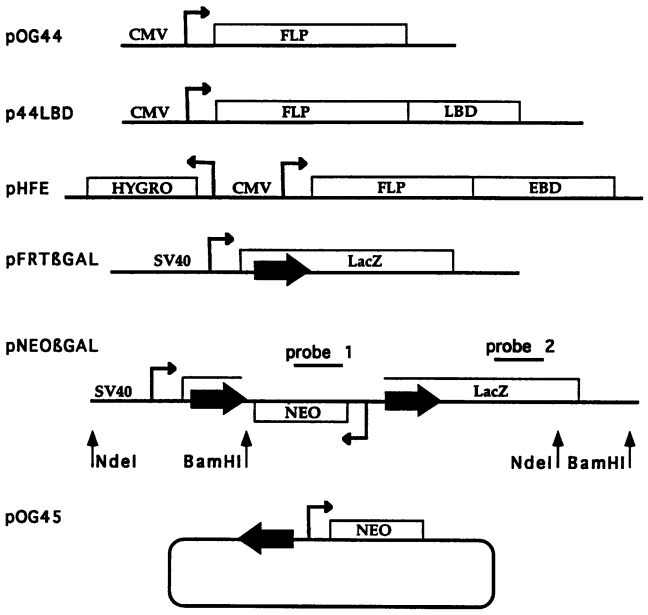

FIG. 1. DNA constructs. FLP expression vectors were derived from pOG44 (ref. 9; Stratagene) by replacing the FLP stop codon with a *Bsi*WI site and fusing LBDs from the human androgen (HAR; amino acids 624–919; ref. 33), estrogen (HER, amino acids 251–595; refs. 15 and 25), or glucocorticoid (HGR, amino acids 487–777; ref. 15) receptor, respectively. The bidirectional CMV.tk cassette and hygromycin-resistance gene were derived from pCNH2 (26). pFRTβGAL, pNEOβGAL, and pOG45 have been described (ref. 9; Stratagene). The FLP recombination targets (FRTs) are depicted as large arrows and transcription start sites are depicted as small arrows. Restriction sites and probes relevant for the Southern analyses of Figs. 3, 4, and 5 are shown on pNEOβGAL.

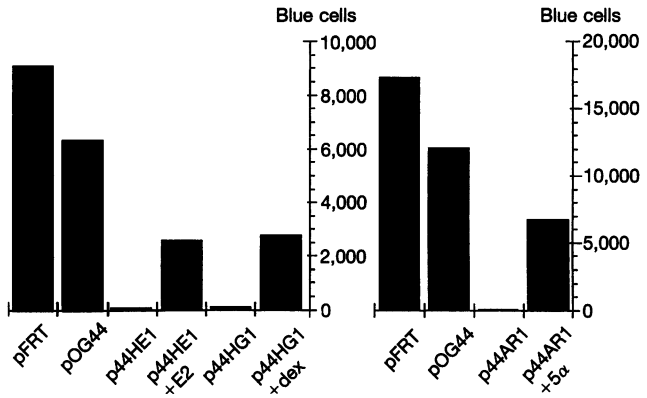

FIG. 2. Ligand-induced genomic recombination in a transient expression assay. E25B2B2 cells, which contain a stably integrated derivative of pNEOβGAL, were transfected with the plasmids indicated and treated or not treated with 100 nM estradiol (E2), dexamethasone (dex), or dihydrotestosterone (5α), as indicated for 48 h. Following staining for β-galactosidase expression, the number of blue-staining cells in 4 cm^2 was counted. Cells staining blue after transfection with pFRTβGAL represent expression from the plasmid and not from the recombined genomic template, thus reflecting transfection efficiency. On the basis of this estimation, most cells transfected with unregulated FLP (pOG44) and up to 50% of the cells transfected with FLP–LBDs in the presence of cognate ligand underwent recombination.

Since the neomycin-resistance gene of pNEOβGAL can be excised by FLP, continued selection for G418 resistance after the introduction of pHFE1 could select against recombination. To evaluate that potential, we divided pools of pHFE1-transfected P1 cells into two and selected for hygromycin resistance in the presence or absence of G418. Approximately 450 colonies were observed under both conditions, indicating that the effect of G418 selection on recombination was negligible or undetectable by this assay. Further tests on isolated clones also failed to detect any differences with respect to recombination between these two selection protocols (unpublished data).

As a further test for any effect of G418 selection on recombination, two randomly selected clones (P1.2 and P1.4) were cultured for 14 days without G418, then for 48 h with 100 nM estradiol or ethanol vehicle. Southern analysis showed no recombination in the absence of either G418 or hormone (Fig. 3*A*, lanes 2 and 5). Staining for β-galactosidase expression, however, did reveal approximately 1 blue cell in 10,000 in the absence of G418 and inducing ligand. This rare event may reflect sensitivity to residual estrogens in the culture medium (25), or it may reflect a nonligand-dependent background of recombination.

Clones P1.2 and P1.4, along with five clones selected to represent three additional genomic integration sites of the recombination substrate, were subjected to time courses of estradiol treatment and Southern analysis (Figs. 3*B* and 4*A*). Two clones did not display recombination (P1.1, R8.1), but remarkably, the kinetics of recombination were very similar in

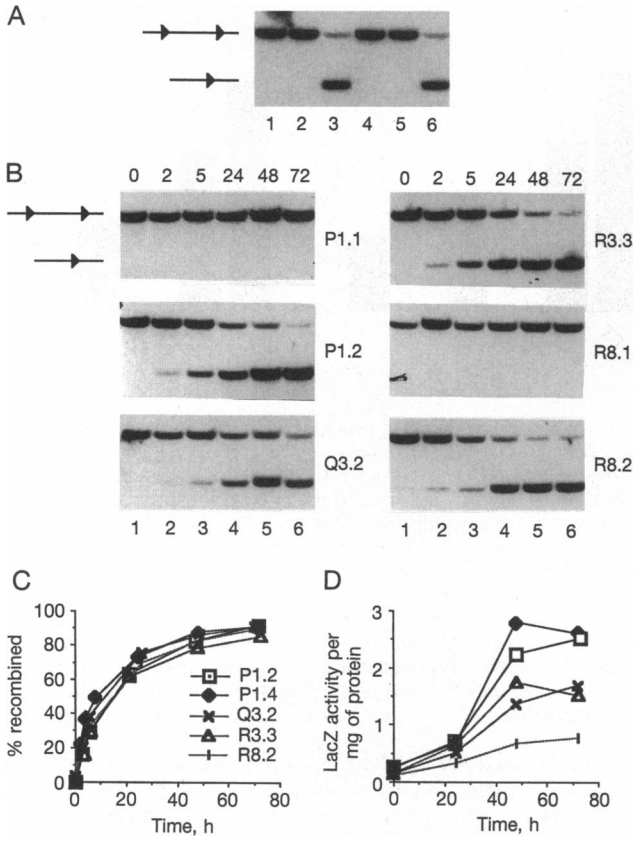

FIG. 3. Ligand dependent recombination in cell lines stably expressing the FLP–EBD^{G400V} fusion protein. (*A*) Southern analysis using probe 2 (see Fig. 1) of *Nde* I restriction digests of genomic DNAs prepared from clone P1.2 (lanes 1–3) or P1.4 (lanes 4–6). The cells were grown for 14 days in the presence (lanes 1 and 4) or absence (lanes 2, 3, 5, and 6) of G418 before being treated with 10^{-7} M estradiol (lanes 3 and 6) or ethanol vehicle (lanes 2 and 5) for 2 days. The expected unrecombined and recombined *Nde* I fragments, depicted on the left, are 4.9 kb and 3.6 kb, respectively. (*B*) Randomly chosen G418- and hygromycin-resistant colonies were expanded and tested for recombination induced by exposure to estradiol for up to 72 hours (lanes 1–6). Time (in hours) in estradiol is given at the top. Southern analysis was performed as described in *A*. (*C*) The time courses of recombination shown in *B* and Fig. 4*A* were quantified by PhosphorImager analysis. The percent recombination for each lane was calculated by dividing the value of the recombined signal by the value of the recombined plus unrecombined signals. (*D*) Recombination-induced β-galactosidase expression was measured during time courses of estradiol treatment of the FLP–EBD^{G400V} clones as indicated. The clone symbol designations are the same as in *C*.

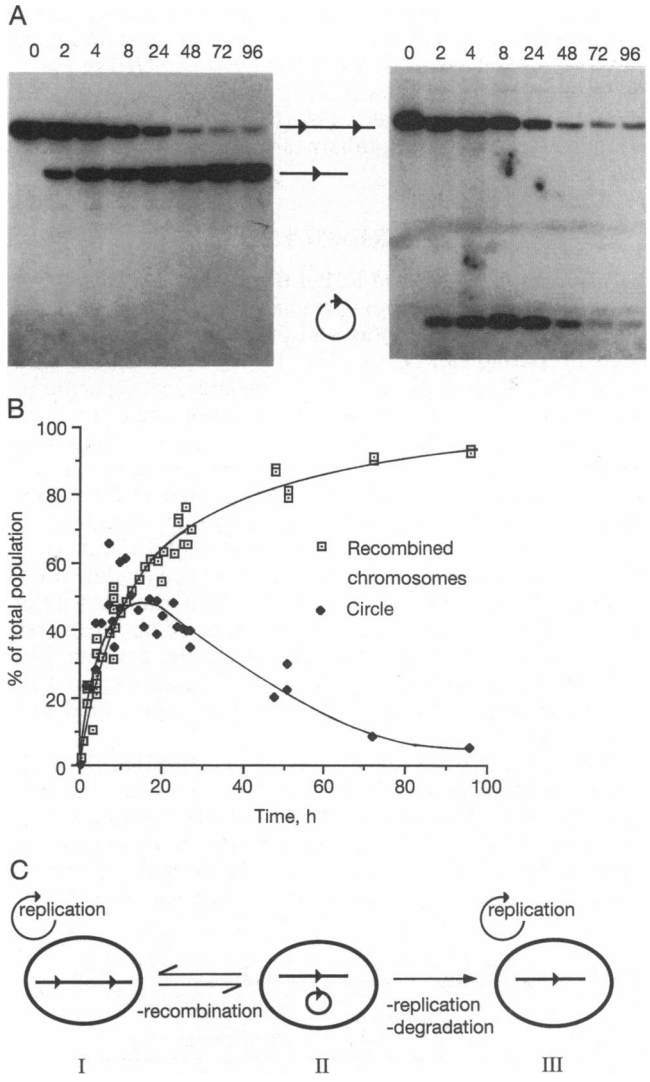

FIG. 4. Kinetics of excision recombination in clone P1.4. (*A*) Southern analyses of a time course of estradiol-induced recombination showing hybridization of *Nde* I-digested genomic DNA with probe 2 (see Fig. 1; *Left*) and hybridization of *Bam*HI-digested genomic DNA with probe 1 (see Fig. 1; *Right*). The unrecombined fragment is 5.2 kb, and the excised circular product of recombination was linearized by *Bam*HI to give a 1.3-kb linear fragment. Time (in hours) in estradiol is given at the top. (*B*) PhosphorImager quantification of three independent time courses of estradiol-induced recombination in clone P1.4. The percentage of circle forms was calculated as circle signal divided by the sum of the recombined and unrecombined signals. (*C*) Excision recombination on a single, chromosomally integrated substrate in living cells. Recombination between directly repeated FRTs (arrowheads) excises the intervening DNA as a circle which carries one FRT, converting cell type I to cell type II. The excised circle is able to reintegrate, converting II to I, until it is lost from the cell through its failure to replicate or its degradation, converting II to III. Both I and III can replicate.

the five clones that displayed recombination (Fig. 3*C*). Each of the recombination substrate integration sites is represented in the inducible clones. In parallel time courses we observed that all five recombination-competent clones showed β-galactosidase inducibility, albeit to differing levels, reflecting the position effect exerted on expression levels of different genomic loci (Fig. 3*D*). Consequently, we observe that recombination does not reflect the position effects that influence expression levels. Note, however, that the recombination substrate was (*i*) selected to be within genomic regions that are permissive for gene expression and (*ii*) situated within a transcribed region of the transgene.

Kinetics of Excision Recombination. To study the recombination reaction in greater detail, a time course was performed on clone P1.4 and analyzed by probing Southern blots to visualize either the integrated *lacZ* gene or the excised neomycin-resistance gene (Fig. 4*A*). The excision reaction was initially rapid and reached approximately 50% completion by 10 h. The average initial rate of recombination measured from Figs. 3*B* and 4*A* is 8% per hour or 2.15 events per cell cycle (27 h). FLP recombination is reversible and ideally will reach equilibrium at 50% if the substrate and products are equivalent (1, 3–5, 27, 28). In excision recombination, the substrates and products differ, in that the forward reaction (excision) is intramolecular and the reverse (reintegration) is intermolecular. Moreover, in a living system, the excised product, unlike the chromosomal product and the substrate, may not replicate and can be degraded. We reason that the progress of excision recombination beyond the equilibrium between forward and

reverse reactions will reflect the parameters that decrease reintegration, such as physical separation of the products or loss of the excised DNA. The model shown in Fig. 4*C* accounts for the effects of reintegration, degradation of the excised DNA, and replication of chromosomal DNA. The kinetics of recombination observed in Figs. 3 and 4 do not reflect loss of saturating ligand concentration, since periodic readdition of estradiol had no additional effect (unpublished data). Similarly, Western analysis revealed no correlation between the recombination kinetics and expression levels of the FLP–EBD fusion protein in the five recombination-competent clones (Fig. 3*B*), arguing that all five clones contained saturating levels of recombinase activity throughout the time course of estradiol exposure (unpublished data).

Site-Specific Integration. The kinetics of excision recombination (Figs. 3*C* and 4*B*) imply the frequent reintegration of the excised region. We therefore undertook experiments to assess the frequency of FLP–EBD-mediated site-specific integration of introduced plasmids carrying an FRT. Recombinases can integrate transfected DNA into a specific site in mammalian cells (9, 13, 29, 30). The ability of endogenous FLP–EBD to site-specifically integrate transfected DNA was assessed by transfecting pOG45 (Fig. 1) into *neo*^{S}*lacZ*$^+$ derivatives of clones P1.2, P1.4, or Q3.2. Since pOG45 contains a complete neomycin-resistance gene, including its own promoter and polyadenylylation signal, these transfection experiments compared the frequencies of site-specific and random integration. For site-specific integration, recombinase activity must be regulated to disfavor the reexcision of integrants. We therefore regulated recombinase activity by adding estradiol, either immediately after transfection or 2 h before, to potentially preload the genomic FRT with the FLP–EBD fusion protein. The estradiol was then removed by washing the cells, either 22 or 28 h after transfection, whereupon selection for G418 resistance was imposed.

Site-specific integration will disrupt the *lacZ* gene, so that white, G418-resistant colonies will represent site specific integrants and blue, G418-resistant colonies will represent random integrants. We therefore determined the ratios of site-specific to random integration by staining plates of G418-resistant colonies for β-galactosidase expression. Table 1 shows the results of a single experiment encompassing five different protocols of estradiol treatment. All colonies obtained from transfections that were not treated with estradiol stained blue. For each estradiol protocol, the frequency of white colonies observed varied consistently according to the host cell line (P1.4 > P1.2 > Q3.2). Since P1.4 and P1.2 share the same genomic FRT integration site, we are not able to attribute this difference to genomic position effects. Within each host cell line, the frequency of white colonies observed varied consistently according to the estradiol treatment protocol (2 h pretreatment > no pretreatment; 22 h posttransfection > 28 h). To confirm that the white colonies did represent site-specific integrations, 48 randomly chosen G418-resistant clones from P1.4B, protocol −/28, were isolated and cultured with or without estradiol to test for FLP–EBD reexcision of site-specific integrants. In agreement with the frequencies observed by staining primary colonies (9.4%), six isolated clones showed β-galactosidase reexpression only in the presence of estradiol, and Southern analysis confirmed that they contained site-specific integrants (Fig. 5*A*). The Southern blot was stripped and reprobed with probe 1 (see Fig. 1) to determine whether the site-specific integrants also contained a randomly integrated neomycin-resistance gene. As shown in Fig. 5*B*, they did not. However, the control blue colony (4B5) showed a randomly integrated neomycin-resistance gene that unexpectedly recombined in the presence of estradiol (lanes 15 and 16). Although it is likely that the efficiency of site-specific integration will vary depending on the locus being targeted

Table 1. Ratio of site-specific to random integration

Estradiol treatment pre/post transfection, h	Recipient cell line					
	P1.4B		P1.2B		Q3.2B	
	w/b	%	w/b	%	w/b	%
−/−	0/160	0	0/248	0	0/230	0
−/22	17/104	14.0	16/98	14.0	10/101	9.0
−/28	16/155	9.4	11/226	4.6	7/220	3.1
2/22	54/169	24.2	35/190	15.6	25/204	10.9
2/28	44/220	16.7	29/311	8.5	11/170	6.1

Three cell lines expressing FLP–EBD^{G400V}, either treated or not treated (−) with 100 nM estradiol for 2 h before being harvested as indicated, were electroporated with 5 μg of pOG45 and then treated with 100 nM estradiol. After 22 or 28 h as indicated, the cells were washed three times in culture medium and then replated in G418-containing medium lacking estradiol. G418-resistant colonies were stained for β-galactosidase expression and scored as being white, indicative of site-specific integration, or blue, indicative of random integration. The total number of white (w) and blue (b) G418-resistant colonies on each plate is given, as is the number of white colonies expressed as a percentage of the total number of colonies.

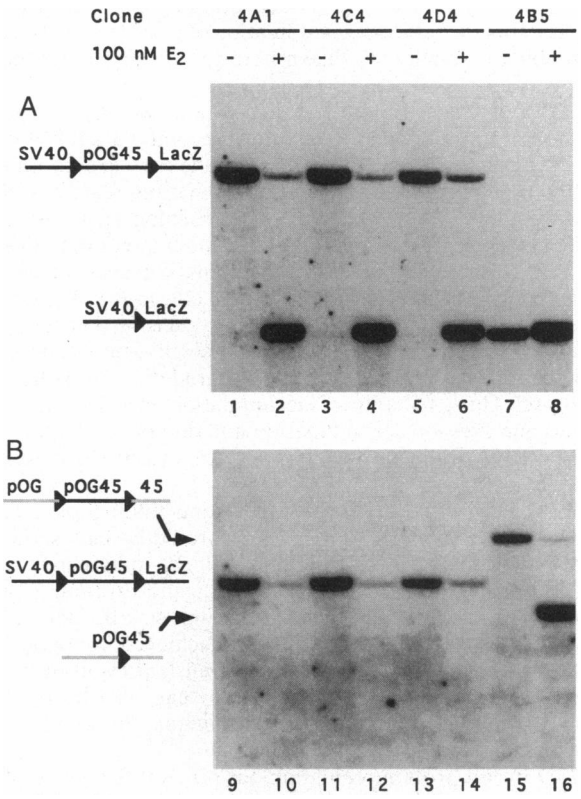

FIG. 5. Southern analysis of three independent clones derived from site-specific integration of pOG45 into P1.4B cells. Three of the six clones that displayed estradiol induction of β-galactosidase expression (Table 1; 4A1, lanes 1, 2, 9, and 10; 4C4, lanes 3, 4, 11, and 12; 4D4, lanes 5, 6, 13, and 14) and one clone that constitutively expressed β-galactosidase (4B5, lanes 7, 8, 15, and 16) were cultured in the absence (−) or presence (+) of 100 nM estradiol for 64 h before genomic DNA was prepared and digested with *Nde* I. (*A*) Southern analysis using probe 2 (see Fig. 1). pOG45 is 4.2 kb, and site-specific integration predicts a 7.8-kb *Nde* I fragment which, after excision, produces the 3.6-kb *Nde* I fragment. (*B*) The filter was stripped and reprobed with probe 1 (see Fig. 1) to look for randomly integrated copies of pOG45. *Nde* I does not cut within the excised circle and, therefore, remaining excised circles in the estradiol-treated samples do not resolve as a single band on this blot. The random integrant in 4B5 (lane 15) was subject to recombination in the presence of estradiol (lane 16). The reduction in size upon estradiol treatment in 4B5 is equal to the size of pOG45, indicating that two copies of pOG45 were tandemly integrated.

(31) and the nature of the DNA construct, our analysis indicates that FLP–EBD-mediated site-specific integration is at least as efficient as homologous recombination at targeting a specific genomic locus.

DISCUSSION

The ability to precisely manipulate living genomes, a prerequisite for understanding the molecular basis of living systems, underlies the promise of reverse genetics in applications such as gene therapy and genetic engineering. Site-specific recombinases can mediate intentional alterations in living genomes. Their application to reverse genetics requires the understanding of their characteristics in living systems (8, 30–32) and strategies to regulate the recombination reactions they mediate. Previous strategies have relied on regulating the recombination reaction by controlling recombinase expression. In the strategy presented here, recombination is regulated by a ligand which regulates recombinase activity. This permits the establishment of cells that contain expressed recombinase and its unrecombined substrate. Therefore, phenotypic changes, such as gene expression, shown here for β-galactosidase, can be induced by exposure to an activating ligand. Our strategy also permits the efficient manipulation of other applications of recombinase activity, as shown here for site-specific integration.

FLP and Cre recombinases are experimentally amenable because they mediate conservative recombination between short target sites and all necessary functions are contained within one polypeptide. The recombination reactions they mediate are reversible and can be used to integrate transfected DNA at frequencies comparable to random integration (Table 1). The reversibility of FLP recombination also influences excision reactions in living cells. In chromosomal-excision substrates, reintegration differs from excision because the excised DNA can be separated from the chromosomal target and lost from the cell, by either degradation or failure to replicate. Thus, the rate of accumulation of cells that have undergone excision recombination and do not reintegrate the excised DNA will be affected by the rate at which the excised DNA is lost.

FLP–LBD fusion proteins couple ligand binding to a readily measurable enzyme activity. Therefore, they can serve in assays to display ligand binding and the parameters that influence ligand binding, accurately. Ligand-titration experiments have shown that ligand binding can be accurately measured as excision recombination achieved (unpublished data). Since FLP–LBDs convert the transient event of ligand binding into fixed DNA changes, they can also be used to record, potentially in an amplified manner, the presence or activity of ligands.

LBD fusion-protein regulation has previously been applied to transcription factors and oncoproteins. Since LBD fusion-protein regulation also works for FLP recombinase, other proteins that directly or indirectly bind DNA, such as components of recombination, replication or repair complexes, nucleases, topoisomerases, and chromatin organizing proteins, could also be regulated by this strategy. Similarly, it may be possible to extend this strategy beyond DNA binding to proteins that bind to sites in other macromolecular complexes, such as the ribosome, membrane surfaces, or the cytoskeleton.

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