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Regulated gene insertion by steroid-induced ΦC31 integrase

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ABSTRACT

Nonviral integration systems are widely used genetic tools in transgenesis and play increasingly important roles in strategies for therapeutic gene transfer. Methods to efficiently regulate the activity of transposases and site-specific recombinases have important implications for their spatiotemporal regulation in live transgenic animals as well as for studies of their applicability as safe vectors for genetic therapy. In this report, strategies for post-translational induction of a variety of gene-inserting proteins are investigated. An engineered hormone-binding domain, derived from the human progesterone receptor, hPR891, and specifically recognized by the synthetic steroid mifepristone, is fused to the *Sleeping Beauty*, *Frog Prince*, *piggyBac* and *Tol2* transposases as well as to the Flp and ΦC31 recombinases. By analyzing mifepristone-directed inducibility of gene insertion in cultured human cells, efficient posttranslational regulation of the Flp recombinase and the ΦC31 integrase is documented. In addition, fusion of the ΦC31 integrase with the ER^{T2} modified estrogen receptor hormone-binding domain results in a protein, which is inducible by a factor of 22-fold and retains 75% of the activity of the wild-type protein. These inducible ΦC31 integrase systems are important new tools in transgenesis and in safety studies of the ΦC31 integrase for gene therapy applications.

INTRODUCTION

Nonviral integration systems, including DNA transposons and site-specific recombinases (SSRs), are widely used genetic tools for genome mutagenesis and transgenesis (1–3). Desirable properties such as simple and inexpensive production, together with the lack of induction of immunological responses and improved safety, have made nonviral integration systems become widespread alternatives to viral integration systems for therapeutic gene transfer applications (4,5).

DNA transposons are mobile DNA elements that can move from one genetic location to another through a cut and paste mechanism. Transposon-based vector systems consists of a donor plasmid harboring the transgene of interest placed between terminal inverted repeats (IRs) of the transposon and a helper plasmid carrying the transposase-coding sequence under the control of a promoter. Codelivery of donor and helper plasmids will facilitate integration of the transgene into the genome of cells (6). *Sleeping Beauty* (*SB*) and *Frog prince* (*FP*), members of the Tc1/*mariner* family of transposable elements, were constructed from inactive transposon elements residing in the genomes of salmonid fish and the Northern leopard frog (*Rana pipiens*), respectively (7,8) and both transpose efficiently in vertebrate cells. Recently, two natural transposable elements, *piggyBac* (*PB*) isolated from the genome of the cabbage looper moth *Trichoplusia ni*, and *Tol2*, a *hAT*-like transposable element isolated from the genome of the Japanese medaka fish *Oryzias latipes*, have shown to have activity in mammalian cells (9–14). *PB* has been used previously for genetic analysis in a wide variety of insects (11), whereas *Tol2* has been used mainly for germline transgenesis in zebrafish (13).

SSRs can induce deletion, insertion or inversion of DNA sequences by breaking and joining DNA molecules at specific sites (1). The tyrosine recombinase Flp, originating from the yeast *Saccharomyces cerevisiae*, mediates recombination between two 34 bp homotypic *Flp recombination target* (FRT) sequences. Flp has, along with its family member the Cre recombinase, been used in numerous gene modification studies in mammalian animals and cells (15–17). Another SSR, the serine recombinase ΦC31 integrase, encoded by a phage of *Streptomyces lividans* (18), recognizes the 34- and 39-heterotypic sequences *attB* and *attP*. Recombination results in generation of two hybrid sites, *attL* and *attR*, and the reaction is

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therefore unidirectional (19). The ΦC31 integrase mediates site-specific integration of a plasmid containing an *attB* sequence into specific chromosomal sequences, called pseudo *attP* sites, in human and mouse cells (20). The total number of pseudo *attP* sites present in the mammalian genome is estimated to be in the range of 100–1000 sites, and the ability of the ΦC31 integrase to integrate DNA into such sites in unmodified mammalian genomes has made this recombinase system become a promising vector system for somatic gene therapy approaches (21,22).

Among several attempts to further improve nonviral integration systems, mutational analysis of the *SB* transposase and the Flp recombinase have given rise to several hyperactive mutants with increased activity in vertebrate cells (23–26), whereas a ΦC31 integrase with increased specificity for a single pseudo *attP* site has been created by DNA shuffling (27). In addition, *SB* transposase fusion variants with altered functional properties have been generated recently in attempts to increase the site-specificity of the integration systems (28,29). Alternative ways to improve these systems further include development of strategies for inducible recombination and gene insertion. Regulation at the level of expression by using inducible promoters is an obvious possibility, although this approach is often hampered by leaky expression. Regulation of the activity at the posttranslational level will allow temporal control of the transposition or recombination reaction, and this will be an advantage in studies where transposition or recombination is required at a certain point or within a certain time span. Inducible recombinase systems have previously been developed by fusion of the recombinase to the regulatory domain of a steroid receptor (30,31). Cre and Flp recombinases fused to the modified estrogen receptor hormone-binding domain, ER^{T2}, demonstrated hormone-dependent recombination with high activity in the presence of the receptor ligand, 4-hydroxytamoxifen (4-OHT) and low activity when 4-OHT was absent (32,33). By using mice transgenic for the Cre-ER^{T2} gene driven by tissue-specific promoters, tamoxifen-induced spatiotemporally regulated transgene expression has been reported in a range of cell types, including adipocytes (34), melanocytes (35), gut epithelial cells (36), hepatocytes (37), astroglial cells (38,39) and nociceptive neurons of dorsal root ganglia (40). An inducible Cre recombinase has also been obtained by fusion to the hormone-binding domain of the mutant human progesterone receptor hPR891 (41). The hPR891 contains a 42 amino acid C-terminal deletion, which makes the receptor bind specifically to the synthetic steroid mifepristone (RU486) and not to progesterone or other endogenous hormones (42). Mifepristone-regulated activity of this fusion has been utilized for spatiotemporal control of gene expression in brain (43) and skin (44).

In this study, we used the hormone-binding domain (here referred to as 'MiBD' for 'Mifepristone-binding domain') of hPR891 to generate fusion proteins with the *SB*, *FP*, *PB* and *Tol2* transposases and the Flp and ΦC31 recombinases in an attempt to develop inducible nonviral integration systems. We demonstrate that engineered high-efficiency fusion variants of the Flp and ΦC31 recombinases mediate site-directed gene insertion in the presence of mifepristone and show low levels of activity in the absence of the drug. Finally, we show that fusion of another hormone-binding domain, the ER^{T2} domain, to the ΦC31 integrase also results in the creation of an inducible integrase. We expect that these new inducible ΦC31 integrase systems will be of great utility in transgenesis and as a tool for studies of safety features of the ΦC31 system for therapeutic gene transfer.

MATERIALS AND METHODS

Plasmid construction

The Flp coding region [corresponding to enhanced Flp variant, Flpx9, derived from ref. (26)] was PCR-amplified from pHD.SB.Flp (45) and inserted into $pBSKS^{-}$.CMV-hAAT (46), generating pCMV-Flpx9. The pFRT-hygro.PGK-puro (pLV/FRT-hygro) was previously described in (Moldt,B., Staunstrup,N.H., Jakobsen,M., Yanez-Munoz,R.J. and Mikkelsen,J.G., unpublished data). To generate pCMV-Flpx9.MiBD and pCMV-MiBD.Flpx9, two in-frame fusion constructs were made by overlap extension PCR of a 753-bp MiBD PCR fragment amplified from pT7bhPRB-891 (42) and a 1272-bp Flpx9 PCR fragment amplified from pCMV-Flpx9, and the two fusion constructs were subsequently cloned into NheI-digested pCMV-Flpx9. A short linker with the sequence 5′-GCCGGC-3′ connects the MiBD and Flpx9 sequences in the fusion constructs. The pUC.CMV-HSB3 was generated by ligation of a 1023-bp HSB3 PCR fragment, amplified from pCMV-HSB3 (25), into SacII-digested pCMV-SB (47). In pUC.CMV-HSB3 the HSB3 sequence is flanked by a NotI site and a PacI site. The two plasmids pCMV-HSB3.L1.MiBD and pCMV-MiBD.L1.HSB3 contain fusion constructs made by in-frame PCR ligations of a 753-bp MiBD PCR fragment amplified from pT7bhPRB-891 and a 1023-bp HSB3 PCR fragment amplified from pCMV-HSB3. The two fusion constructs were digested with NotI and PacI and cloned into double-digested pUC.CMV-HSB3. A short linker (Linker 1) with the sequence 5′-CCCGGGGGAGGAACTAGT-3′ connects the MIBD and HSB3 sequences in the fusion constructs. The pSBT/PGK-puro plasmid, previously referred to as pTpuro, was previously described (48). The pCMV-FP plasmid was constructed by inserting the *FP* transposase open reading frame, PCR-amplified from pFV-FP (8), into SacII-digested pCMV-SB. To generate pCMV-FP.L1.MiBD the 1023-bp *FP* transposase gene from pCMV-FP was amplified by PCR and cloned in-frame into NotI/XmaI-digested pCMV-HSB3.L1.MiBD. The pCMV-MiBD.L1.FP plasmid was made by in-frame ligation of the *FP* gene into SpeI/PacI-digested pCMV-MiBD.L1.HSB3. To construct pFPT/PGK-puro, an overlap PCR product containing the left *FP* IR, amplified from pFP-neo (8) and the PGK-puro cassette, amplified from pSBT/PGK-puro, was inserted into KpnI/NotI-digested pSBT/PGK-puro. The right *FP* IR sequence, amplified by PCR from pFP-neo, was inserted into the resulting plasmid digested with NotI and SalI to generate pFPT/PGK-puro. The pCMV-PB and

pCMV-Tol2 plasmids were made by PCR amplification of the 1785-bp *PB* transposase gene from p3X3P-DsRed1 and the 1950-bp *Tol2* gene from pCAGGS-T2TP (12) and subsequent ligation of the PCR fragments into NotI/PacI-digested pUC.CMV-HSB3. The four plasmids pCMV-PB.L1.MiBD, pCMV-MiBD.L1.PB, pCMV-Tol2.L1.MiBD and pCMV-MiBD.L1.Tol2 were constructed by ligation of the *PB* and *Tol2* transposase genes into NotI/XmaI-digested pCMV-HSB3.L1.MiBD and SpeI/PacI-digested pCMV-MiBD.L1.HSB3. To construct pPBT/PGK-puro a PGK-puro cassette was amplified from pSBT/PGK-puro by PCR, digested with KpnI and NotI and cloned into double-digested pXL-BacII (49). The pTol2T/PGK-puro plasmid was made by cloning a NheI/BsrGI-digested PGK-puro cassette into double-digested pT2AL200R150 (13). To generate pCMV-ΦC31, the 1842-bp ΦC31 integrase gene was amplified by PCR from pCMV-Int (18) and ligated into NotI/PacI-digested pUC.CMV-HSB3. The pCMV-ΦC31.L2.MiBD plasmid was made by ligation of the ΦC31 integrase gene into NotI/SpeI-digested pCMV-HSB3.L1.MiBD. The pCMV-MiBD.L2.ΦC31 plasmid contains a MiBD-ΦC31 fusion construct that was made by in-frame PCR ligation of a MiBD PCR fragment amplified from pT7bhPRB-891 (42) and a ΦC31 PCR fragment amplified from pCMV-Int. To generate pCMV-MiBD.L2.ΦC31, the MiBD-ΦC31 fusion construct was ligated into NotI/PacI-digested pUC.CMV-HSB3. Both the pCMV-ΦC31.L2.MiBD plasmid and the pCMV-MiBD.L2.ΦC31 plasmid have a short linker (Linker 2), with the sequence 5′-ACTAGT-3′, between the ΦC31 integrase and the MiBD sequences. To generate pAttB/PGK-puro, the 293-bp *attB* sequence was amplified by PCR using p11 (22) as template and cloned into EcoRI-digested pSBT/PGK-puro in which the left IR sequence was removed upon insertion of the *attB* sequence. The pCMV-MiBD.L2.HSB3 was made by insertion of the PCR-amplified HSB3 sequence into SpeI/PacI-digested pCMV-MiBD.L2.ΦC31. The pCMV-MiBD.L1.ΦC31 plasmid contains a MiBD-ΦC31 fusion construct with the linker 5′-CCCGGGGGAGGAA CTAGT-3′. The fusion construct was made by in-frame PCR ligation of a MiBD PCR fragment and a ΦC31 PCR fragment. To generate pCMV-MiBD.L1.ΦC31 the MiBD-ΦC31 fusion construct was ligated into NotI/PacI-digested pCMV-ΦC31.L2.MiBD. The pCMV-ΦC31.L2.ER^{T2} plasmid was made by insertion of an ER^{T2} PCR fragment, amplified from pCre-ER^{T2} (30), into SpeI/PacI-digested pCMV-ΦC31.L2.MiBD. The plasmid pCMV-mΦC31 (pCMV-mINT) has been described previously (22). All produced DNA constructs were verified by restriction digestion and DNA sequencing.

Integration analyses

HeLa (human cervical cancer cells), HEK-293 (human embryonal kidney cells), Hep1A (mouse hepatoma cells) and Flp-In T-Rex HEK-293 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, 1% penicillin–streptomycin and 1% glutamine. For treatment with mifepristone, the medium was further supplemented with 500 nM mifepristone (Sigma-Aldrich, St Louis, MO, USA) on the day prior to transfection.

HeLa cells were plated at 2×10^5 cells/well in 6-well dishes 1 day before cotransfection with (i) a donor plasmid, containing a PGK-puro cassette situated in a transposon or together with an *attB* sequence and (ii) a helper plasmid encoding the different transposases or the ΦC31 integrase. Transfections were carried out using FuGene-6 (Roche, Basel, Switzerland) according to the manufacturer's instructions using 3 μl of reagent per 2 μg of DNA. One microgram of helper plasmid plus 1 μg substrate plasmid was used in each transfection. In negative control experiments, pcDNA3.1D/V5.TOPO (Invitrogen, Carlsbad, CA, USA) was transfected instead of the helper plasmid. In the dose–response assay and the final comparison of pCMV-ΦC31.L2.MiBD and pCMV-ΦC31.L2.ER^{T2}, 1.5 μg of each helper and substrate plasmid was transfected using 6 μl FuGene-6. One day after transfection, fresh medium was added to the cells. Two days after transfection, cells were split to varying densities and plated in 10-cm dishes. Three days after transfection, selection medium containing 1 μg/ml puromycin (Sigma-Aldrich) was added to the cells. After 10 days of selection, colonies of cells were stained with methylene blue, air-dried and counted. For analysis of the mifepristone dose–response transfected cells were incubated in medium containing from 0 to 2 μM mifepristone.

The activity of the Flp recombinase fusion proteins was quantified as integration events in cultured Flp-In T-REx 293 cells (Invitrogen). Cells were seeded at 5.5×10^5 cells/dish in 10-cm dishes the day before cotransfection with pFRT-hygro.PGK-puro and different Flp recombinase-expressing plasmids. One hour before transfection, fresh medium was added to the cells. Transfection was performed using calcium-phosphate coprecipitation, and in each transfection 1 μg pFRT-hygro.PGK-puro plus 10 μg recombinase plasmid was used. Three hours post-transfection, the medium was refreshed. Two days after transfection, cells were diluted (1:50 and 1:100) and seeded in 10-cm dishes. Three days after transfection, selection medium containing 1 μg/ml puromycin (Sigma-Aldrich) or 200 μg/ml hygromycin B (Invitrogen) was added to the cells, and after 10 days of selection colonies were stained in 0.6% methylene blue, air-dried and counted.

β-galactosidase assays

The recombinogenic activity of Flp variants was measured by monitoring expression of β-galactosidase in HEK-293 and Hep1A cells cotransfected (by calcium-phosphate treatment) with 17 μg Flp-encoding plasmid DNA and 2 μg of the reporter construct, pneoβgal (Stratagene, San Diego, CA, USA). Cells were seeded at 1×10^4 cells/cm^2 in 10-cm dishes on the day prior to transfection. The concentration of β-galactosidase in cell lysates was determined using a βgal ELISA kit (Roche).

Detection of integration at pseudo *attP* site 19q13.31

The ability of the ΦC31.MiBD fusion protein to recognize the pseudo *attP* site, 19q13.31, was investigated using cultured HEK-293 cells. HEK-293 cells were plated at

2×10^5 cells/well in 6-well dishes on the day before transfection. A total of 1.5 μg of pAttB/PGK-puro together with 1.5 μg pCMV-ΦC31 or pCMV-ΦC31.MiBD were transfected into the cells using FuGene-6 reagent, with 6 μl reagent per 3 μg DNA. One day posttransfection fresh medium was added to the cells. The cells transfected with pCMV-ΦC31.MiBD plasmid received medium supplemented with 500 nM mifepristone. Two days posttransfection, cells were diluted 1/50 and seeded in 10-cm dishes. Three days posttransfection, selection was applied using medium containing 1 μg/ml puromycin. After 10 days of selection, puromycin-resistant colonies were trypsinized and collected in pools. Ten pools were grown to confluency and genomic DNA was extracted. A two-step nested PCR was performed on genomic DNA to detect integration in the pseudo site 19q13.31. Vector- and locus-specific primers were utilized in both PCR steps. The vector-specific primer for the first PCR was 5′-CAGGTACCGTCGACGATGTA-3′ and for the second PCR 5′-CGATGTAGGTCACGGTCTCGA-3′. Four genomic primers were designed using the genome database using the UCSC genome browser. First- and second-PCR primers were designed for both the plus strand and the minus strand, in order to detect plasmid integration in both orientations. The plus strand primers were 5′-CATGAGGTGACTGCAGCTTGA-3′ for the first PCR and 5′-GATAAGGAATGTGCTGTGCTT-3′ for the second PCR. The minus strand primers were 5′-GCTGCATACTGGGCTACACGT-3′ for the first PCR and 5′-ATATAGGTTTCAATGAACCTT-3′ for the second PCR. Primers were designed to give bands at a size of 350 bp. In the first PCR reactions, 100–400 ng of genomic DNA was used. Two microliters of the first-round PCR products were then used as template in the second-round PCR. In both PCR steps, 40 cycles and an annealing temperature of 57°C were used. Products of the second PCR products were subjected to agarose gel electrophoresis, and bands of the expected size were excised, purified using Gel Extraction Kit (Omega Bio-Tek, Doraville, GA, USA) and sequenced using the primer 5′-CGATGTAGGTCACGGTCTCGA-3′.

Statistical analysis

Statistical analyses were performed using unpaired *t*-tests, except when different variances were detected by an *F*-test, in which case statistical analyses were performed using the Mann–Whitney U-test.

RESULTS

Mifepristone promotes posttranslational induction of a fusion variant of the Flp recombinase

Previous studies of tyrosine recombinases, like Cre and Flp, C-terminally fused to genetically engineered steroid-binding domains have demonstrated robust hormone-induced recombination activities (30,31,41). Hence, Cre and Flp recombinases fused to the ER^{T2} estrogen receptor hormone-binding domain are highly responsive to 4-OHT (30,32), whereas the activity of Cre fused to part of the human progesterone receptor can be regulated by mifepristone. To explore further the inducibility of tyrosine recombinases, we initially designed a variant of the Flp recombinase [based on the enhanced Flpx9 variant created by Buchholz *et al*. (26)], containing a C-terminal MiBD (Figure 1A). The activity of this variant, expressed from pCMV-Flpx9.MiBD, was first analyzed by transient recombination assays in which pCMV-Flpx9.MiBD was cotransfected with the reporter plasmid pneoβgal (Figure 1B). Upon Flp recombination, the neo expression cassette, situated within the βgal reporter gene, was removed from the construct, allowing expression of a functional βgal-FRT fusion variant (50). We first tested the sensitivity of the transient reporter assay by transfecting HEK-293 cells with 2 μg pneoβgal and increasing amounts of pCMV-Flpx9. A linear increase in the β-gal concentration measured in the cell lysate was detected using in the range from 8 to 24 μg of pCMV-Flpx9.MiBD (data not shown). In subsequent experiments, 17 μg of Flp-encoding plasmid and 2 μg pneoβgal was transfected into human HEK-293 cells and Hep1A mouse liver cells, respectively. In both cell lines, the activity of Flpx9.MiBD fusion variant was strongly enhanced in the presence of mifepristone. The activity of the drug-induced Flpx9.MiBD was in both cell lines close to 50% of the activity of the unfused Flpx9 variant (Figure 1C). Moreover, in the absence of mifepristone the fusion variant was inactive and resulted in levels of β-gal production close to the background measured when pneoβgal was cotransfected with pUC19 (negative control). Throughout this and subsequent experiments, mifepristone in control experiments did not affect the activity of the wild-type recombinases (data not shown), indicating that the drug did not directly influence the transfected cells or the recombination processes catalyzed by uninducible recombinases.

Based on our findings, we investigated whether the mifepristone-responsive Flpx9.MiBD variant could facilitate drug-inducible gene insertion. To test the overall flexibility of regulated Flp variants, we also generated an alternative fusion variant, MiBD.Flpx9, in which MiBD had been fused to the N-terminus of Flp. Constructs encoding the different Flp variants were cotransfected with pFRT-hygro.PGK-puro into HEK-293-derived cells containing an FRT docking vector (Figure 1D). In this set-up, only site-directed plasmid insertion mediated by Flp-directed recombination between the FRT site on pFRT-hygro.PGK-puro and the FRT site within the genomic docking vector would reconstitute a functional hygromycin B resistance expression cassette, allowing selection for Flp-mediated insertion events. In addition, by including a PGK-driven puromycin resistance gene on the plasmid we could compare the efficiency of plasmid insertion by Flp-mediated and random insertion, respectively. As measured by the number of hygromycin B-resistant colonies the activity of Flpx9 and mifepristone-induced Flpx9-MiBD did not differ statistically ($P = 0.128$) (Figure 1E). However, by selecting transfected cells with puromycin the activity of the induced protein was significantly lower than the wild-type Flpx9 ($P = 0.005$). In this setup, we measured mifepristone-induced insertion at a level approaching 60% of the unfused Flp (Figure 1F). We also detected a high overall

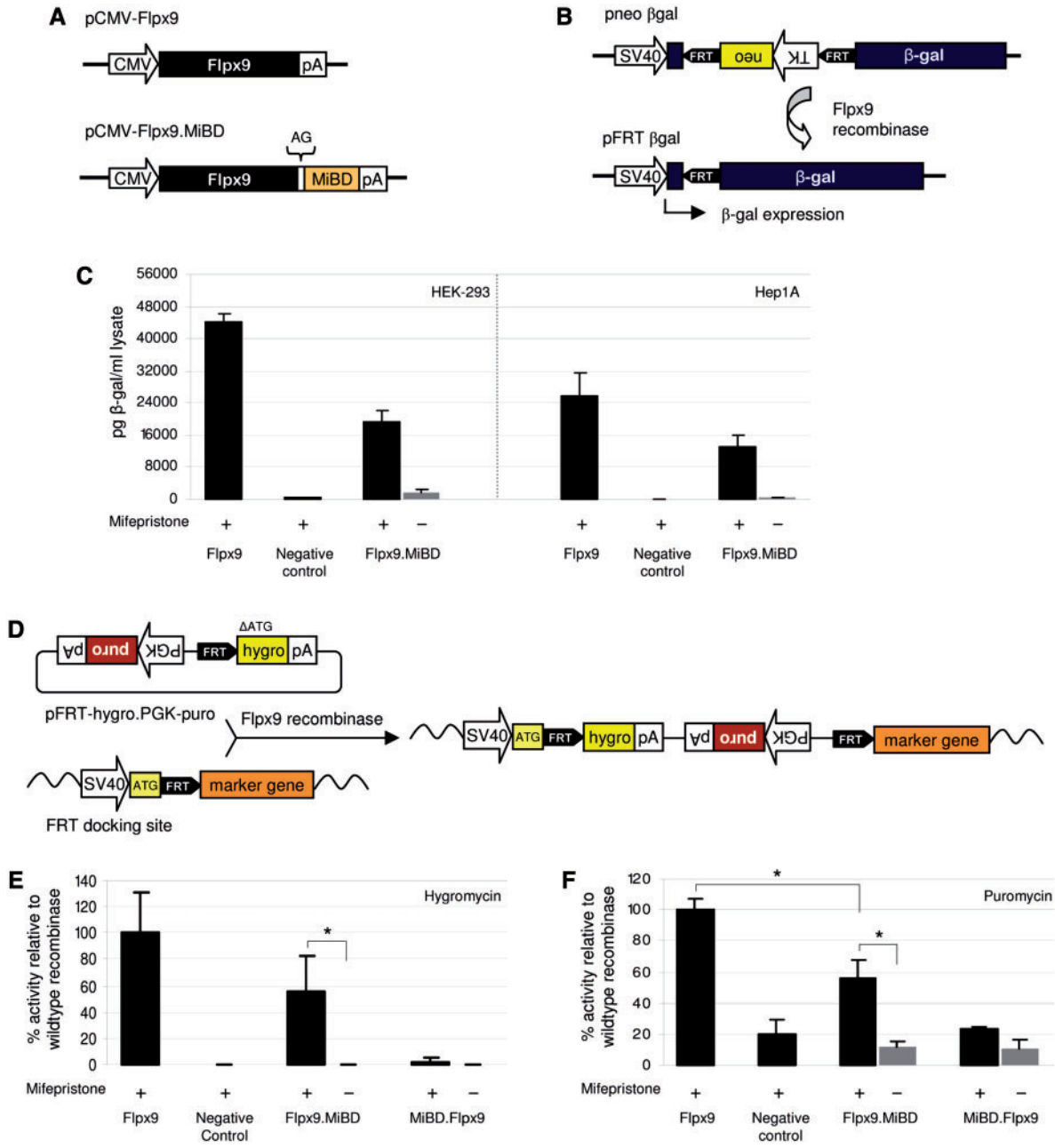


Figure 1. Mifepristone-inducible Flp recombinase mediates gene excision and insertion. (**A**) Schematic representation of the expression cassette encoding Flpx9 and Flpx9.MiBD. In the Flpx9-MiBD fusion construct, the two domains were separated by a short 6-bp linker (encoding Ala and Gly), indicated by the white box. (**B**) Schematic representation of β-gal reporter construct used for detection of Flp activity. The pneoβgal contains a neomycin resistance expression cassette (driven by the TK promoter) flanked by FRTs indicated by black arrows. Upon Flp-directed recombination between the two FRT sites a functional β-gal-FRT gene is established, and β-gal protein can be detected by ELISA. (**C**) Mifepristone-induced Flp recombination in HEK-293 and Hep1A cells. Two micrograms of pneoβgal was cotransfected with 17 μg of the indicated Flp variant (or pUC19 in case of the negative control) in the presence of 300 mM mifepristone. Presence and absence of mifepristone is indicated below the columns by + and −, respectively. The β-gal concentration in cell lysates, harvested 24 h posttransfection, was determined by ELISA and normalized to the total protein content in the sample. (**D**) Schematic representation of Flp-mediated integration of FRT donor plasmid into the FRT docking site in Flp-In T-Rex 293 cells. pFRT-hygro.PGK-puro contains both a promoterless, ATG-deficient hygromycin B resistance gene and a puromycin resistance gene driven by a PGK promoter. Upon Flp-directed recombination between the FRT site in the donor plasmid and the FRT docking site in the HEK-293-derived cells a functional hygromycin B resistance expression cassette is reconstituted, allowing selection for Flp-mediated insertion events with hygromycin B. (**E**) and (**F**) Mifepristone-induced gene insertion by the Flp recombinase in FRT-tagged HEK-293-derived cells. One microgram of pFRT-hygro.PGK-puro was co-transfected with 10 μg Flp-encoding plasmid into Flp-In T-REx 293 cells. Cells were selected by treatment with hygromycin B or puromycin for 10 days before counting colonies. A total of $1.9 \times 10^3 \pm 5.7 \times 10^2$ hygromycin B-resistant colonies and $8.9 \times 10^3 \pm 6.6 \times 10^2$ puromycin-resistant colonies was obtained per transfection with pCMV-Flpx9. The activity of the Flp variants tested is presented as percentage of activity relative to the wild-type recombinase. All experiments were performed in triplicates. The asterisks indicate significant differences between relevant groups ($P < 0.05$).

background insertion activity in the absence of the drug, which most likely resulted from the high-transfection rate and resulting random gene insertion in HEK-293 cells. Nevertheless, the number of puromycin-resistant colonies was significantly higher in the presence of mifepristone ($P = 0.003$) confirming that the Flpx9.MiBD recombinase was robustly induced by the drug. In summary, our data confirm tight steroid-regulated activity of genetically engineered tyrosine recombinases, as shown here for the first time with a mifepristone-induced variant of Flp, and demonstrate efficacy of posttranslationally regulated gene insertion by site-directed nonviral recombinases.

Limited mifepristone-mediated induction of MiBD-containing DNA transposase fusion variants

Tyrosine recombinases fused to steroid-binding domains appear to possess structural and functional properties that allow specific binding of steroids and efficacious induction of catalytic activity in the presence of the relevant drugs. We were attracted by the possibility of regulating other prominent nonviral gene insertion technologies at the posttranslational level using a similar steroid-based regulation approach. We therefore set out to test the potential to induce activities of four different transposases derived from the *SB*, *FP*, *Tol2* and *PB* transposons, which are all highly transposable in mammalian cells. The transposase coding sequences of all four elements were inserted either upstream or downstream from the MiBD sequence in the context of expression cassettes driven by the cytomegalovirus (CMV) promoter, allowing expression of transposases fused C- and N-terminally, respectively, with MiBD. In all constructs, the fused transposase and MiBD were separated by a 6-amino acid flexible linker (Linker 1, L1, PGGGTS) consisting of three glycine residues flanked by amino acids encoded by the restriction sites separating the two domains (Figure 2A).

For studies of Tc1/mariner transposases, we fused the coding sequence of HSB3, a hyperactive version of the *SB* transposase (25), with MiBD, generating pCMV-HSB3.L1.MiBD and pCMV-MiBD.L1.HSB3. In separate transfections, plasmid DNA encoding the different transposase variants was introduced into HeLa cells together with pSBT/PGK-puro, a plasmid containing a PGK-driven puromycin resistance gene cassette flanked by *SB*-derived inverted repeats. After transfection, the cells were selected with puromycin. As evident in Figure 2B neither HSB3.L1.MiBD nor MiBD.L1.HSB3 protein showed significant transposition activity in the presence or absence of mifepristone. The activity of MiBD.L1.HSB3 was ~10% of unfused HSB3 but the fusion protein did not respond to mifepristone. Likewise, N- and C-terminal fusion variants of the *FP* transposase, did not catalyze efficient insertion of a *FP* transposon carrying the PGK-puro cassette (data not shown).

We next investigated the transposition activities of the *Tol2* transposase fused C- and N-terminally to MiBD by cotransfecting plasmid DNA encoding *Tol2* transposase variants with pTol2T/PGK-puro carrying the PGK-puro expression cassette in the context of a *Tol2* transposon. The C-terminal fusion of Tol2, Tol2.L1.MiBD, retained ~40% the activity of the wild-type *Tol2* transposase in the presence of mifepristone (Figure 2C). In the absence of mifepristone, a reduced level of transposition was observed, which differed statistically from the level obtained with mifepristone ($P = 0.004$). However, the protein retained substantial drug-independent activity and the level of inducibility was low. The N-terminal fusion variant of the *Tol2* transposase was completely inactive and not inducible by mifepristone. In a similar fashion, low levels of induction were monitored with *PB* transposases fused N- and C-terminally with MiBD and cotransfected with plasmid DNA, pPBT/PGK-puro, containing the PGK-puro cassette in the context of a *PB* transposon (data not shown).

In summary, of the tested transposase fusion variants with the L1 linker, only the Tol2 fusion was significantly induced in the presence of mifepristone. Although this variant, in particular, showed some activity, the level of induction was at best modest due to robust activity of the protein in the absence of the drug.

Gene insertion by a ΦC31 recombinase C-terminally fused with MiBD is inducible and does not possess activity above background level in the absence of mifepristone

The ΦC31 recombinase belongs to the family of serine recombinases, which acts through mechanisms of catalysis (involving the catalytic serine) that differ from the mechanisms of the tyrosine recombinases like Cre and Flp (51). The ΦC31 recombinase is a high molecular weight serine recombinase with a catalytic domain situated very close to the N-terminus, a flanking DNA-binding domain and a region containing several conserved cysteines required for recombinogenic activity of the protein. For comparison, the catalytic domains of Cre and Flp are located in the C-terminal part of the proteins. Despite structural and mechanistic differences we wanted to explore whether MiBD-containing variants of the ΦC31 integrase could be induced when treating target cells with mifepristone. We initially constructed fusion variants containing a 2-amino acid linker (Linker 2, L2) encoded by the cloning linker situated between the integrase and MiBD sequences (Figure 2D). We cotransfected plasmids encoding these variants with a plasmid, pAttB/PGK-puro, carrying both attachment site B (*attB*) and the PGK-puro gene cassette, into HeLa cells and counted colonies formed after puromycin selection. Both MiBD-tagged versions of the integrase, ΦC31.L2.MiBD and MiBD.L2.ΦC31 were completely inactive in the absence of mifepristone (Figure 2E). Whereas the activity of MiBD.L2.ΦC31 did not improve in the presence of mifepristone, ΦC31.L2.MiBD proved to be highly inducible when adding mifepristone to the medium. Hence, the presence of mifepristone triggered a 28-fold increase in activity over background ($P = 0.003$), resulting in a protein that possessed ~40% the activity of the unfused ΦC31 integrase (Figure 2E). In addition, a dose–response experiment demonstrated that the ΦC31.L2.MiBD protein is responsive to even small amounts of mifepristone, indicating that the system is highly sensitive (Figure 2F).

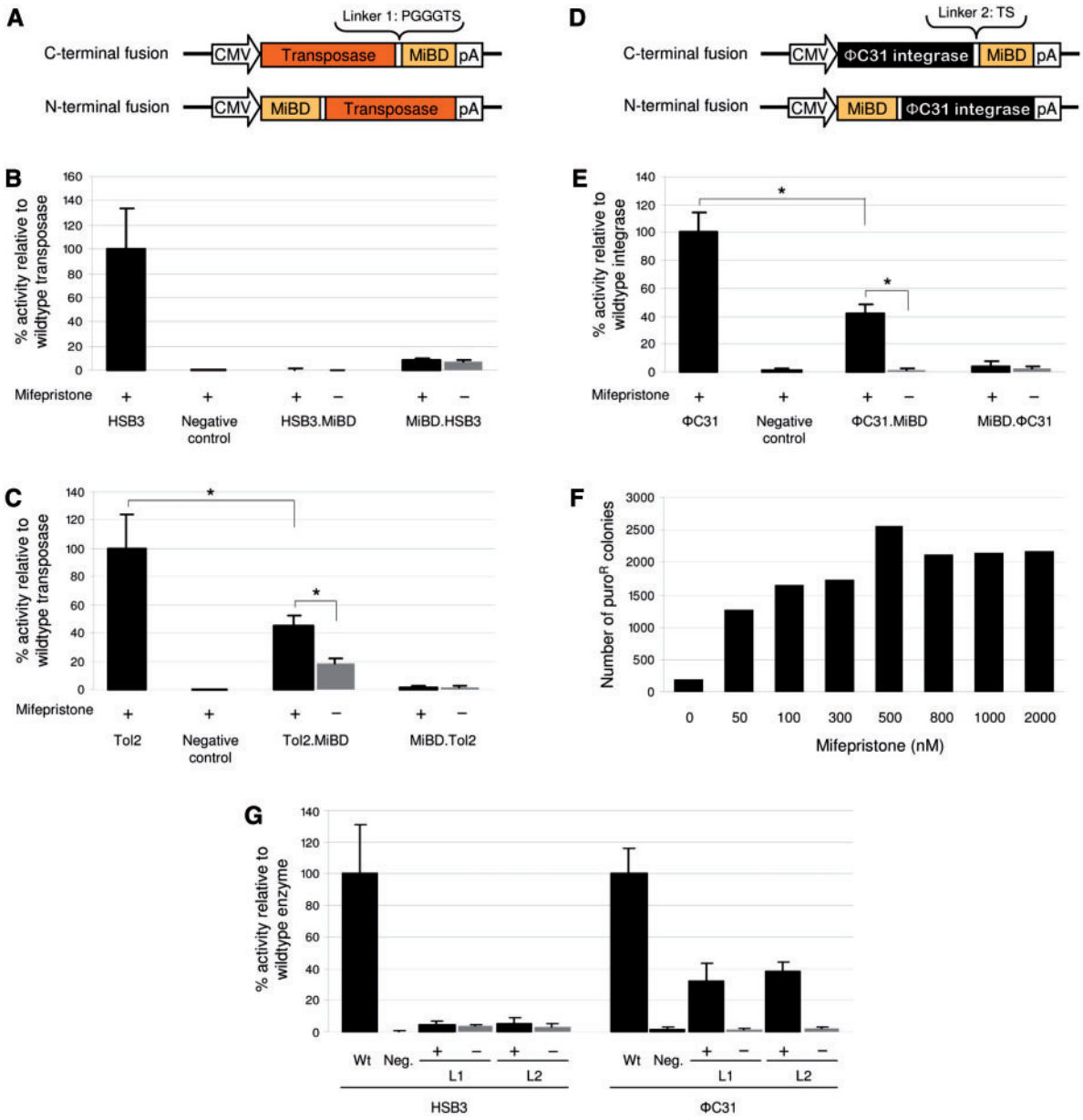


Figure 2. Variable levels of mifepristone-induced gene insertion by posttranslationally induced transposases and recombinases. (**A**) Schematic representation of transposase fusion constructs containing the transposase open reading frame fused 3′ or 5′ with the sequence encoding the MiBD. Expression of C- or N-terminally tagged fusion variants was driven by a CMV promoter (white arrow). The two protein domains were separated by a short flexible 6-amino acid linker (linker 1, Pro-Gly-Gly-Gly-Thr-Ser), indicated by the white box. (**B**) and (**C**) Activity of fusion variants, derived from the hyperactive *Sleeping Beauty* transposase HSB3 and the *Tol2* transposase, in HeLa cells. One microgram of plasmid DNA encoding CMV-driven *SB* or *Tol2* transposase variants was cotransfected with 1 μg pSBT/PGK-puro or pTol2/PGK-puro, respectively. MiBD-fusion variants were analyzed in the presence of 500 nM mifepristone (indicated by '+') or in the absence of mifepristone (indicated by '−'). The activity of each variant is presented relative to the activity of the wild-type transposase. A total of $1.3 \times 10^4 \pm 4.5 \times 10^3$ and $1.3 \times 10^4 \pm 3.1 \times 10^3$ puromycin-resistant colonies were obtained per transfection with pCMV-HSB3 and pCMV-Tol2, respectively. All experiments were performed in triplicates. The asterisks indicate significant differences between relevant groups ($P < 0.05$). (**D**) Schematic representation of ΦC31-based fusion constructs containing the ΦC31 gene fused 3′ or 5′ with the sequence encoding the MiBD. Expression of C- or N-terminally tagged fusion variants was driven by a CMV promoter (white arrow). The two protein domains were separated by a short 2-amino acid linker (linker 2, Thr-Ser), indicated by the white box. (**E**) Activity of fusion variants, derived from the ΦC31 integrase, in HeLa cells. One microgram of plasmid DNA encoding CMV-driven ΦC31 integrase variants was cotransfected with 1 μg pAttB/PGK-puro. MiBD-fusion variants were analyzed in the presence of 500 nM mifepristone (indicated by '+') or in the absence of mifepristone (indicated by '−'). The activity of each variant is presented relative to the activity of the wild-type recombinase. A total of $1.3 \times 10^3 \pm 2.0 \times 10^2$ puromycin-resistant colonies were obtained per transfection with pCMV-ΦC31. All experiments were performed in triplicates. (**F**) Mifepristone dose–response assay in HeLa cells. One and a half microgram of plasmid DNA encoding the inducible ΦC31 integrase (pCMV-ΦC31.L2.MiBD) was cotransfected with 1.5 μg pAttB/PGK-puro in the presence of increasing concentrations of mifepristone. Levels of induction are presented by the exact number of puromycin-resistant colonies obtained after selection. (**G**) Activity of HSB3 transposase and ΦC31 integrase fusion variants containing the L1 or L2 linker. One microgram of plasmid DNA encoding MiBD-HSB3 or ΦC31-MiBD fusion proteins containing either L1 or L2 was cotransfected in HeLa cells with 1 μg pSBT/PGK-puro or pAttB/PGK-puro, respectively. The fusion variants were analyzed in the presence of 500 nM mifepristone (indicated by '+') or in the absence of mifepristone (indicated by '−'). The activity of each variant is presented relative to the activity of the wild-type (wt) recombinase. All transfections were performed as three independent experiments.

In summary, our data show highly variable levels of mifepristone-directed inducibility of transposases and recombinases. As the linkers that were used to couple the hormone-binding domain with the catalytic protein differed in length and composition, we constructed a MiBD-HSB3 fusion variant with an intervening L2 sequence. As shown in Figure 2G, both MiBD.L1.HSB3 and MiBD.L2.HSB3 variants were inactive and did not respond to mifepristone. In contrast, the ΦC31-derived fusion variants (ΦC31.L1.MiBD and ΦC31.L2.MiBD) containing either of the two linkers were both inactive in the absence of mifepristone and highly induced in cells treated with the drug (Figure 2G). These data suggest that the composition of the linkers used in the fusion proteins was not the prime cause of the different responses to mifepristone.

As shown by differences in inducibility (relative number of colonies obtained in the presence and absence of mifepristone) (Figure 3), the C-terminal fusion variant of the ΦC31 recombinase proved in our hands to be the most tightly mifepristone-regulated gene-inserting protein and was studied, therefore, in further detail.

Induced ΦC31.MiBD recombinase mediates site-directed gene insertion into a known hotspot in human cells

The ΦC31 integrase mediates site-specific recombination between the *attB* site on the vector plasmid and pseudo-*attP* sites dispersed throughout the human genome (18,20). Hotspot insertion sites have been identified in human cells including HEK-293 cells (20,21). To investigate whether the mifepristone-induced ΦC31.L2.MiBD recombinase has retained properties of site-directed gene insertion into hotspot sites, we analyzed puromycin-resistant clones for insertions into the 19q13.31 pseudo site (21). We pooled colonies obtained by ΦC31- and ΦC31.L2.MiBD-directed gene insertion and screened genomic DNA from these pools by PCR analysis using primers annealing to the inserted vector and to sequences flanking the 19q13.31 site (Figure 4A). Among 10

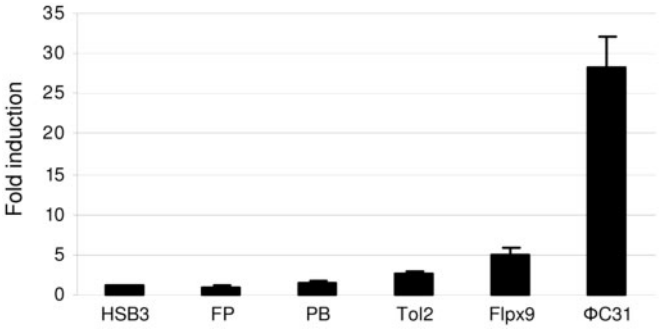
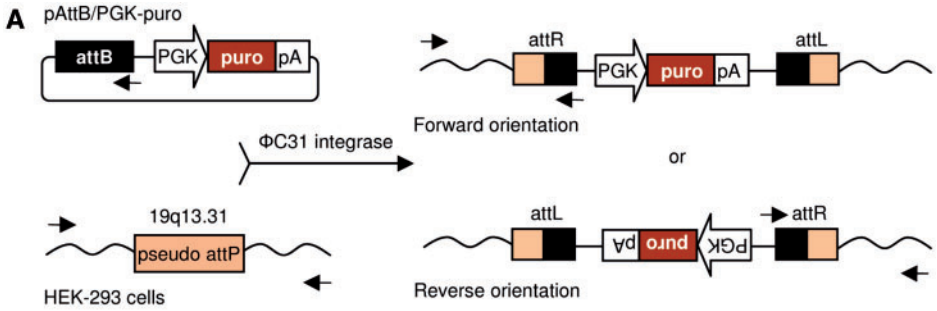


Figure 3. Inducibility of each of the six tested integration systems. The index value for each system is calculated by dividing the number of puromycin-resistant colonies obtained in the presence of mifepristone with the number of colonies obtained in the absence of mifepristone (data taken from Figures 1F and 2). All index values were determined by colony-forming assays in HeLa cells, except for the activity of Flp that was analyzed in HEK-293-derived cells.



B

Integrase variant	Average number of colonies per pool	Number of pools which contain at least one site-directed insertion into pseudo site 19q13.31	
		Forward orientation	Reverse orientation
ΦC31	50	10/10	9/10
ΦC31.MiBD	25	6/10	5/10

Figure 4. Analysis of ΦC31.MiBD-mediated gene insertion at pseudo *attP* site 19q13.31. (**A**) Schematic representation of ΦC31-mediated insertion of *attB* donor plasmid into the 19q13.31 pseudo *attP* site in HEK-293 cells. The pAttB/PGK-puro contains an *attB* site and a puromycin resistance gene driven by a PGK promoter. ΦC31-mediated recombination between the *attB* site in the donor plasmid and the 19q13.31 hotspot pseudo site in HEK-293 cells will lead to insertion of the donor plasmid into the hotspot site in either a forward or reverse orientation. The black arrows indicate vector- and locus-specific primers. (**B**) Detection of ΦC31.MiBD-mediated gene insertion at pseudo *attP* site 19q13.31. A total of 1.5 μg of pAttB/PGK-puro was cotransfected with 1.5 μg pCMV-ΦC31 or pCMV-ΦC31.MiBD into HEK-293 cells. Cells were selected by treatment with puromycin, and after 10 days of selection resistant colonies were pooled. Genomic DNA from pooled colonies was screened by PCR analysis using vector- and locus-specific primers.

analyzed pools, each consisting of ~50 clones obtained by ΦC31-mediated gene insertion, nine pools were found to contain insertions in both orientations in this particular site (Figure 4B). In one pool, we could detect an insertion in only one of the two orientations. For insertions mediated by mifepristone-induced ΦC31.MiBD protein the pools on average consisted of ~25 clones. Among 10 analyzed pools, one or more insertions in the first of the two orientations could be detected in six pools, whereas five pools contained at least one insertion in the opposite orientation. In consideration of the different numbers of screened colonies with the wild-type and the inducible integrase, our findings suggest that the specificity of ΦC31.L2.MiBD, allowing insertion in a previously identified hotspot, is unaltered in comparison with the unfused ΦC31 recombinase. However, based on these findings we cannot formally exclude the possibility that the induced ΦC31 integrase may have altered recombinogenic properties that reduce or increase its site-selectivity.

Inducible gene insertion by a ΦC31 recombinase C-terminally fused with the ER^{T2} domain

Tight regulation of tyrosine recombinases fused to the modified estrogen receptor hormone-binding domain, ER^{T2}, has previously been reported (30). We therefore wanted to develop a ΦC31 fusion variant containing a C-terminally fused ER^{T2} domain and compare induction of this variant by 4-OHT with mifepristone-based induction of ΦC31.L2.MiBD. We exchanged the MiBD sequence in pCMV-ΦC31.L2.MiBD with the ER^{T2} sequence, creating a novel fusion construct, pCMV-ΦC31.L2.ER^{T2}. In colony-forming assays using pAttB/PGK-puro as substrate, transfected cells were cultivated in medium containing 500 nM mifepristone or 500 nM 4-OHT for induction of the ΦC31.L2.MiBD and ΦC31.L2.ER^{T2} variants, respectively. Transfections with plasmid DNA encoding the wild-type ΦC31 or a mutated form, mΦC31, were included as positive and negative controls, respectively. As illustrated in Figure 5, the patterns of induction with mifepristone and 4-OHT were very similar. In the presence of the drug, we detected close to 2500 colonies with both ΦC31.L2.MiBD and ΦC31.L2.ER^{T2}. This result indicated that the fusion variants had retained activities that were comparable to the wild-type protein, although the numbers of colonies obtained with wild-type ΦC31 and the induced fusion variants did indeed vary statistically ($P = 0.008$ for ΦC31.L2.MiBD; $P = 0.034$ for ΦC31.L2.ER^{T2}). Moreover, the presence of mifepristone and 4-OHT induced function of the two fusion variants 14- and 22-fold above the background ($P = 0.025$ and $P = 0.025$, respectively). Notably, comparable numbers of colonies obtained in cells treated with mΦC31 and the uninduced fusion variants ($P = 0.11$ and $P = 0.33$ for mΦC31 versus ΦC31.L2.MiBD and ΦC31.L2.ER^{T2}, respectively) indicated that gene insertion in the absence of the drug was not facilitated by residual function of the fusion integrase but more likely originated from randomly inserted plasmid DNA. In summary, we conclude that the activity of the ΦC31 recombinase fused with steroid-binding domains can be tightly regulated at the post-translational level.

DISCUSSION

Site-specific recombination mediated by tyrosine recombinases—Cre or Flp—has been widely utilized to generate specific genomic DNA rearrangements in somatic cells of transgenic animals (1). In this report, we describe for the first time the development of tightly drug-regulated serine recombinases derived from the Streptomyces phage ΦC31 integrase. By fusing hPR891 and ER^{T2} ligand-binding domains to the C-terminus of ΦC31 integrase, we created variants that were, in a comparative study, inducible by a factor of 14 and 22 in the presence of mifepristone and tamoxifen, respectively. In the absence of the relevant

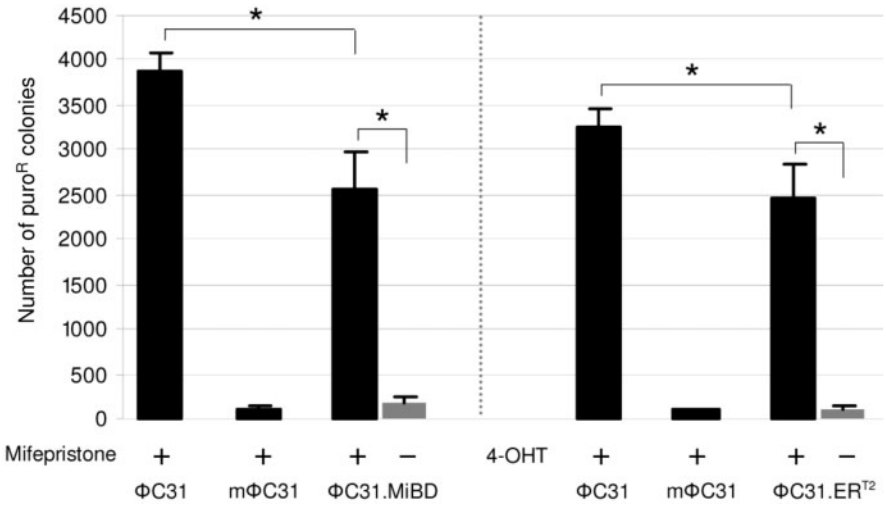


Figure 5. Steroid-directed gene insertion facilitated by ΦC31 integrase fusion variants. A total of 1.5 μg of pAttB/PGK-puro was cotransfected into HeLa cells with 1.5 μg plasmid DNA encoding the indicated variants of the ΦC31 integrase. ΦC31.L2.MiBD and ΦC31.L2.ER^{T2} were analyzed in the absence of steroid (indicated by '−') or in the presence of 500 nM mifepristone (left panel) and 500 nM 4-OHT (right panel), respectively. All transfections were performed as three independent experiments. The asterisks indicate significant differences between relevant groups ($P < 0.05$).

steroids, hPR891- and ER^{T2}-containing fusion proteins are believed to be sequestered by heat-shock proteins (52) and therefore retained in the cytoplasm. Upon drug administration, the ligands bind to the fusion proteins, facilitating the release of heat-shock proteins and translocation of active proteins to the nucleus. Induced variants retained between 66% and 75% of the activity of the unmodified ΦC31 protein and the proteins were inactive in the absence of steroid, as measured by colony formation assays. Inducible ΦC31 integrase fusion variants containing different internal linker regions could be induced to similar levels of activity, whereas a fusion variant containing an N-terminal steroid-binding domain were inactive and did not respond to the drug. Furthermore, our data suggest that mifepristone-induced ΦC31 integrase has retained the same integration profile as the uninduced wild-type version of the integrase. Altogether, drug-inducible ΦC31 integrases possessed properties and inducibilities comparable to the properties of the steroid-induced tyrosine recombinases, in this study represented by a mifepristone-induced variant of Flp that has not previously been described.

The ΦC31 integrase is active in mammalian cells and facilitates recombination between the heterotypic *attB* and *attP* sequences. The resulting recombination sites, *attL* and *attR*, are not substrates for the ΦC31 integrase, and the recombination process is therefore irreversible. For Cre and Flp, in contrast, the recognition sites generated after recombination are immediate substrates for excision, rendering an integrated gene cassette vulnerable for subsequent recombinase-directed re-excision. In this perspective, the ΦC31 integrase has become an attractive novel tool for genomic engineering. Indeed, recent findings have demonstrated that the ΦC31 integrase mediates efficient site-specific cassette exchange in mouse embryonic stem cells (53) and site-specific gene insertion in single-cell mouse embryos (54) and human embryonic stem cells (55). Moreover, expression of the ΦC31 integrase appears to be compatible with normal mouse development (53). Hence, we envision that strictly controlled inducible versions of the ΦC31 integrase could be of important use together with Cre and/or Flp in more complex schemes of genomic engineering, providing more sophisticated options for genetic alterations such as sequential deletion or insertion of transgenes (e.g. selective marker genes) in somatic cells. Such applications will require generation of transgenic mice expressing regulatable variants of the ΦC31 integrase.

Site-directed insertion of *attB*-tagged plasmid DNA into pseudo-*attP* sites in the genome has made the ΦC31 integrase-directed gene insertion an attractive option for persistent nonviral gene therapy. Indeed, the system has been shown to facilitate *in vivo* gene insertion in a range of tissues including mouse liver (56–58) and muscle (59) as well as rat retina (60) and rabbit joints (61). However, recent findings suggest that the system might be harnessed by a tendency to cause DNA rearrangement, deletions and interchromosomal translocations (21,56,62), possibly caused by integrase-directed recombination between genomic pseudo sites. It is not yet clear if such genomic abnormalities are caused by high levels of ΦC31 integrase in cell culture or if such events may occur also *in vivo* as a result of a transient boost or prolonged expression of the ΦC31 integrase. Investigations of steroid-directed induction of ΦC31 integrase activity may shed further light on these issues and suggest use of inducible nonviral systems to enhance safety. In any case, a regulatable approach will allow the protein to act in a predetermined time window, which can be determined by administration of synthetic steroids.

Binding of steroids to hPR891- and ER^{T2}-containing fusion proteins would in theory allow the use of steroid-binding domains for posttranslational regulation of a variety of proteins with catalytic activities in the nucleus. Indeed, a mifepristone-regulated fusion variant of the adeno-associated virus Rep protein and a tamoxifen-inducible variant of the *PB* transposase have been shown to facilitate drug-dependent gene insertion (63,64). In the present study, we investigated steroid-directed induction of *SB*, *FP*, *PB* and *Tol2* transposases. Using a flexible linker consisting of six amino acids, we showed that the modified Tc1/mariner transposases, derived from *SB* and *FP*, as well as the *PB* transposase could not be regulated by mifepristone, whereas the activity of the *Tol2* transposase fused C-terminally to the steroid-binding domain was upregulated in the presence of mifepristone. However, the *Tol2* fusion variant also showed a high basal level of activity in the absence of the drug, indicating that further genetic optimizations would be required to obtain tightly regulated proteins with negligible activity in the absence of mifepristone. Previous findings have suggested that the *PB* transposase appears to be structurally flexible, allowing fusion with alternative protein domains, like DNA-binding domains, without losing its catalytic activity (65,66). However, in a previous study only one of three tested *PB* transposase-ER^{T2} fusion variants, each containing different linkers between the two domains, was found to be active in the presence of tamoxifen (64). Our findings confirm that short linkers in the context of a *PB* transposase fusion result in proteins with low drug inducibility.

Recent data have demonstrated that *SB* transposases containing N-terminal fusion domains can retain transposition activity albeit with reduced efficiency (28,29,67). We have previously developed functional fusion transposases based on the hyperactive HSB3 variant and have verified their production by western blotting (Sharma,N., Moldt,B., Mikkelsen,J.G., unpublished observations). Thus, the lack of activity of MiBD-containing variants most likely reflects specific structural alterations, rendering the fusion protein inactive. In case of the *Tol2* transposase, a previous attempt to create a fusion variant was unsuccessful (66). As a demonstration of difficulties predicting the catalytic status of transposase fusions, we detected robust insertional activities of the *Tol2* transposase tagged C-terminally with MiBD. Although this fusion was also somewhat active in the absence of mifepristone and therefore not tightly regulated, our results suggest that the *Tol2* transposase is indeed flexible and may remain active as part of a fusion protein.

Our studies demonstrate efficient posttranslational induction of tyrosine and serine recombinases, which appear more functionally and structurally flexible than the

various tested transposon systems. We believe that the inducible ΦC31 integrase, in particular, will find use not only in future transgenesis applications but also in important safety studies of the ΦC31 integrase for therapeutic gene transfer.

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Conflict of interest statement. None declared.

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