

Flp recombinase promotes site-specific DNA recombination in embryonic stem cells and transgenic mice

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ABSTRACT Site-specific recombinases are being developed as tools for “*in vivo*” genetic engineering because they can catalyze precise excisions, integrations, inversions, or translocations of DNA between their distinct recognition target sites. Here it is demonstrated that Flp recombinase can effectively mediate site-specific excisional recombination in mouse embryonic stem cells, in differentiating embryonal carcinoma cells, and in transgenic mice. Broad Flp expression is compatible with normal development, suggesting that Flp can be used to catalyze recombination in most cell types. These properties indicate that Flp can be exploited to make prescribed alterations in the mouse genome.

Site-specific recombinases are being developed as tools for genetic engineering because of their simplicity and precise activity in a variety of organisms. Two well-studied recombinases include Flp, from *Saccharomyces cerevisiae*, and Cre, from bacteriophage P1; both have been shown to catalyze excisions, integrations, inversions, or translocations of DNA between their distinct recognition target sites without requiring added cofactors (1–6). The type of recombination reaction is determined by the orientation of target sites relative to each other on a segment of DNA; in particular, directly repeated sites specify excision of intervening DNA.

Controlled recombinase expression in an organism carrying appropriately placed target sites can be exploited to alter the genotype of subsets of cells within an otherwise normal embryo or adult. Such mosaic animals bearing clones of genetically distinct somatic cells have been most extensively generated in *Drosophila* using Flp, providing the means to address previously intractable problems. For example, Flp-mediated excisional recombination has been used to irreversibly activate a marker gene in specific cell populations and their descendants, allowing cell lineages to be studied (7, 8); similarly, genes have been ectopically expressed to study their effects on pattern formation (9). By promoting mitotic exchange between target sites on homologous *Drosophila* chromosomes, Flp has provided an effective methodology for F₁ genetic screens (10–12). In mammalian cell culture, Flp has been shown to effectively catalyze both excision and integration of DNA at specific chromosomal sites (13–16). By catalyzing recombination between target sites on the same DNA molecule or by promoting translocations between target sites on different DNA molecules, site-specific recombinases can be used to study a variety of biological processes. Importantly, such recombination schemes can be used to generate tissue- or stage-specific mutations that would be lethal if generated in the whole organism.

To establish some of these methods in the mouse, it may require using both homologous (gene replacement)- and site-specific recombination in embryonic stem (ES) cells to precisely place target sites in the genome. Consequently, the

properties of a given recombinase should be delineated in both ES cell culture and the mouse. While Cre-mediated recombination has been successfully employed (17–21), the utility of Flp recombinase in ES cells and the mouse has not been established. Developing the technology to engineer multiple recombination reactions (independent gene activation or deletion events) using both Flp and Cre should significantly augment the tools available for molecular studies in mice. Here the utility of Flp to excise DNA in ES cells, differentiating embryonal carcinoma (EC) cells, and in transgenic mice is investigated.

MATERIALS AND METHODS

Plasmid Constructions and Production of Transgenic Mice.

The *lacZ* target vector containing Flp recombinase target (FRT) sites (pFRTZ; Fig. 1A) was generated by inserting the *Hind*III/*Sal*I fragment from pSLh β APr-*lacZ*-pA (22) containing human β -actin gene (*hACTB*) sequences [3-kb 5' flank, 78-bp 5' untranslated region, and 832-bp first intron; ref. 23] into the unique *Hind*III and *Sal*I sites of pFRT₂neo.*lacZ* (24). The control plasmid pFRTZ-product was constructed by inserting the same *hACTB Hind*III/*Sal*I fragment into pFRT-*lacZ* (24). A variant of pFRTZ (designated pFRTZ.2) was generated by inserting the 1.9-kb *Xho*I/*Sal*I fragment from pIC19R-MC1TK (25) containing the herpes simplex virus thymidine kinase (HSV-tk) gene between the FRT sequences of pFRTZ. The prototype plasmid pNEO β -GAL (ref. 13; Stratagene) was also used as target DNA. The *FLP* transgene expression vector, phACTB::FLP (Fig. 1B), was constructed by inserting the 3.9-kb *Xba*I/*Sal*I fragment from pSLh β APr-*lacZ*-pA into the unique *Xba*I site of pFLP (24). A nonexpressing, negative control *FLP* vector (pRevhACTB::FLP) was constructed, which contains identical *hACTB* sequences in reverse orientation. To generate pWnt1::FLP, the 2-kb *Sal*I fragment from pFLP, containing a synthetic intron, the sequence encoding Flp (ref. 13; Stratagene), and simian virus 40 early polyadenylation (pA) sequence, was inserted into the unique *Eco*RV site of pWEXP2 (26). To produce transgenic mice, transgenes were purified away from plasmid sequences and injected into fertilized eggs from B6SJL_{F1} × B6SJL_{F1} mice as described (27).

Cell Culture. CCE ES cells (28) were plated onto mitomycin C-treated STO fibroblasts (29) in DMEM supplemented with 15% fetal bovine serum (FBS), 2 mM glutamine, 0.1 mM 2-mercaptoethanol, 2000 units/ml of leukemia inhibitory factor (ESGRO, GIBCO/BRL), 0.1 mM MEM nonessential amino acids, 30 μ M nucleosides. Primary embryonic fibroblasts (EF) were prepared from hemizygous transgenic embryos 13.5 days post coitum as described (29). P19 EC cells

Abbreviations: FRT, Flp recombination target; FRTZ, FRT-disrupted *lacZ* transgene; *hACTB*, human β -actin gene; RA, retinoic acid; X-Gal, 5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside; β -Gal, β -galactosidase; ES, embryonic stem; EC, embryonal carcinoma; EF, embryonic fibroblast.

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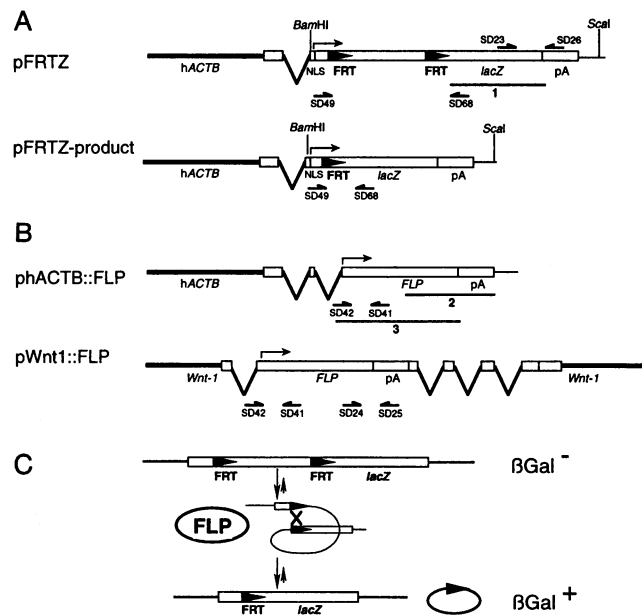


FIG. 1. DNA constructs and the Flp-mediated recombination event. (A and B) Structure of target and recombinase transgenes. FRTs are depicted as black triangles. Rectangles represent exons; heavy lines, introns and flanking regulatory sequences; thin lines, vector sequences; arrows, translation start sites. Hybridization probes are represented by numbered lines. PCR oligonucleotide primers are represented by half arrows. (A) Structure of target transgenes. Plasmid pFRTZ (for FRT-disrupted *lacZ* transgene) contains 3.9 kb of sequence from the human β -actin (*hACTB*) gene (22, 23) inserted into the target vector pFRT₂neo.lacZ (24); a nuclear localization signal (NLS) and simian virus 40 early polyadenylation (pA) sequence are also included. Although not shown, pFRTZ.2 is an alternative target plasmid that contains the HSV-tk gene inserted between the FRT sequences of pFRTZ and is relevant to transfections shown in Fig. 3. Control plasmid pFRTZ-product represents the product of Flp-mediated excisional recombination. Restriction sites and probe 1 used in the Southern blot analysis of Fig. 4B are shown on pFRTZ. (B) Structure of *FLP* transgenes. Plasmid phACTB::FLP contains the 3.9-kb *hACTB* fragment inserted into the expression vector pFLP (24), which contains a synthetic intron, Flp-encoding sequence, and simian virus 40 late pA sequence from pOG44 (ref. 13; Stratagene). Although not diagrammed, pRevhACTB::FLP contains the *hACTB* sequences in reverse orientation and serves as a negative control. Plasmid pWnt1::FLP contains the synthetic intron, Flp-encoding sequence, and the simian virus 40 late pA from pFLP inserted into the polylinker of the *Wnt-1* expression vector pWEXP2 (26). Probe 2 is relevant to the whole mount *in situ* hybridization analyses shown in Fig. 3; probe 3 is used in Northern blot analyses of Fig. 3. (C) Diagram of the *FLP*-mediated excisional recombination reaction.

were maintained in a 1:1 mixture of DMEM and Ham F2 medium supplemented with 7.5% FBS/2 mM glutamine.

Transient Transfections. Transient transfection of ES cells (2×10^5 ES cells in 3.5-cm dishes) was by lipofection (Lipofectamine, GIBCO/BRL) using either 0.5, 2, or 4 μ g of plasmid phACTB::FLP (or negative control vector pRevhACTB::FLP) and 0.5 μ g of either pFRTZ or pFRTZ-product, as indicated (Fig. 2). β -Galactosidase (β -Gal) activity was detected *in situ* using 5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside (X-Gal) (30). Primary EF cultures were plated (5×10^4 cells/ml) in 3.5-cm dishes and transfected by calcium phosphate precipitation (31) with 3 μ g of the target pFRTZ or target pNEO β -GAL (13) followed by X-Gal stain 48 hr later. P19 EC cells were plated (5×10^4 cells/ml) in 10-cm dishes. The next day, pairs of duplicate dishes were transfected by calcium phosphate precipitation (31) with 5 μ g of target pFRTZ.2 alone or with 5 μ g of phACTB::FLP or pWnt1::FLP as indicated (see Fig. 5). Twenty-four hours later one-half of the dishes were treated with either 0.5 μ M of

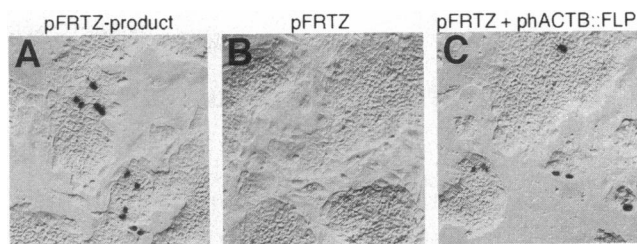


FIG. 2. Cotransfection assay for Flp function in ES cells. Flp-mediated recombination was detected by the gain of β -Gal activity as assayed by histochemical X-Gal staining (13, 30). (A) Positive control transfection (pFRTZ-product). ES cells were transiently transfected with 0.5 μ g of pFRTZ-product plus 0.5 μ g of phACTB::FLP. Although not necessary for β -Gal activity, phACTB::FLP was included to maintain equivalent amounts of *hACTB* sequences and DNA between control and experimental transfections. (B) Negative control transfection (pFRTZ) included 0.5 μ g of pFRTZ and 0.5 μ g of the negative control Flp plasmid, pRevhACTB::FLP. (C) Experimental transfection (pFRTZ plus phACTB::FLP) contained 0.5 μ g of pFRTZ and 0.5 μ g of phACTB::FLP. To define a dose-effect relationship, ES cells were transiently transfected with 0.5 μ g of pFRTZ (or pFRTZ product) and 0.5, 2, or 4 μ g of phACTB::FLP. Following X-Gal staining for β -Gal expression blue-staining cells were counted. Cells staining blue after transfection with pFRTZ-product reflect transfection efficiency. The number of X-Gal-positive cells observed following transfection with pFRTZ plus phACTB::FLP were normalized to the pFRTZ product positive control values. On the basis of this estimation, 30 to 78% of the cells transfected with pFRTZ plus phACTB::FLP underwent a recombination event. Neither pFRTZ, phACTB::FLP or pRevhACTB::FLP generate β -Gal activity when transfected alone.

all-trans retinoic acid (RA; Sigma) or control diluent for an additional 5 days after which cells were stained with X-Gal.

Transcript Detection. Whole mount *in situ* hybridization to 9.5 days post coitum embryos was performed as described (32) using single-strand digoxigenin-UTP-labeled RNA probes. The *FLP* probe (antisense probe 2, Fig. 1B) was a 1386-bp *EcoRV/ApaI* fragment from the 3' end of the *FLP* transgene; control probe (sense) was a 648-bp *XbaI/EcoRV* fragment. For Northern blot analyses, fresh tissue or EF cells were homogenized in 6 M guanidinium isothiocyanate and RNA isolated using acid:phenol (33). Total cellular RNA (20 μ g) was separated and assayed for hybridization to *FLP* sequence as described (34). Ethidium bromide staining of the gel and filter was used to confirm equivalent RNA loading.

Molecular Analysis of Transgenic Mouse Genotypes. Mouse tails were lysed with NaDodSO₄/proteinase K and treated with phenol/chloroform, 1:1 (vol/vol), precipitated with ethanol, and dissolved in 10 mM Tris-HCl, pH 8/1 mM EDTA. For PCR analysis, DNAs were amplified with the following primers: SD42 (5'-GGTCCAAGTGCAGCCCAAGCTTCC-3') and SD41 (5'-GTGGATCGATCCTACCCCTTGCG-3'), for the *FLP* transgene (a 0.75-kb amplified fragment); SD49 (5'-GACTGCTCCAAAGAAGAAGCGTAAGG-3') and SD68 (5'-GCTATTACGCCAGCTGGCGAAAGG-3'), for the *FRTZ* transgene (a 1.4-kb amplified fragment) and FRTZ-product (a 0.25-kb fragment). The 0.25-kb PCR amplification product was cloned into plasmid pCR (TA cloning, Invitrogen) and sequenced. Genomic DNA isolated from freshly harvested tissues (35) was subjected to *BamHI/ScaI* digestion, and Southern blot analyses. Radiolabeled DNA fragments (specific activity of $2-5 \times 10^8$ cpm/ μ g) for use as probes were prepared by random priming (36). Transgene copy number was estimated by including standard amounts of the injected transgene in parallel. Quantitation of radioactivity in specific bands was performed with a Molecular Dynamics PhosphorImager.

RESULTS

Strategy Used to Assay Flp Function in Cell Culture and the Mouse. To generate a test recombination substrate for Flp

function, a *lacZ* gene was disrupted by inserting an FRT cassette that contains stop codons in all three reading frames (24). This target transgene is referred to as *FRTZ*, for FRT-disrupted *lacZ* (Fig. 1A). Because the two FRT sequences flanking the cassette are in the same orientation, Flp activity should excise the intervening DNA leaving a single residual FRT in-frame with *lacZ* (Fig. 1C). Because there are no ATG codons to initiate translation of functional β -Gal downstream of the FRT cassette, β -Gal activity is strictly dependent on Flp-mediated excisional recombination in a manner similar to previously described β -Gal gain-of-function systems (9, 13).

To broadly express both *FLP* and *FRTZ*, both transgenes were placed under the control of regulatory sequences from *hACTB* gene (Fig. 1A and B). These *hACTB* sequences have been shown to be active in most tissues in transgenic mice (22). A "recombined" control transgene, *FRTZ-product*, representing the predicted product of Flp recombination was also constructed (Fig. 1A).

Flp-Mediates Efficient Recombination of Extrachromosomal DNA in ES Cells. The efficacy of Flp-mediated excisional recombination in ES cells was tested by assaying for gain of β -Gal activity following cotransfection with target and recombinase plasmids. Cells were transiently transfected with either pFRTZ plus phACTB::FLP, or pFRTZ plus the negative control plasmid pRevhACTB::FLP, followed by X-Gal stain 48 hr later. Positive control cultures were transfected with the "recombined" plasmid, pFRTZ-product (Fig. 2A). Cultures transfected with target plasmid pFRTZ, alone or with pRevhACTB::FLP, showed no detectable β -Gal activity (Fig. 2B); in contrast, robust activity was observed following cotransfection with phACTB::FLP (Fig. 2C).

To estimate recombinase activity, X-Gal-positive cells in each transfection were counted and compared. The number of cells staining blue after transfection with the control "recombined" pFRTZ-product reflected transfection efficiency and, because constitutively active, the maximal number of β -Gal-positive cells. Cotransfection with a fixed amount of target plasmid and increasing amounts of *FLP* expression vector resulted in an increasing percentage of X-Gal-positive cells relative to control pFRTZ-product transfections. A comparison between experimental (pFRTZ plus phACTB::FLP) and control (pFRTZ-product) transfections showed that Flp-mediated β -Gal activation occurred in at least 30% of transfected ES cells and could be as high as 78%. This increase in recombination with increasing Flp-encoding plasmid likely reflects more Flp protein produced per cell, as well as an increase in the proportion of cells that took up both the target and Flp-encoding plasmids (and thereby had the potential to activate *lacZ*).

Flp Can Be Generally Expressed in the Mouse Without Deleterious Effects. To determine whether Flp can function in the mouse and whether Flp expression, itself, would have any adverse effects, mice carrying the *hACTB::FLP* transgene were generated. To identify mouse lines producing Flp in a wide range of tissues, F₁ mice from each founder were screened for ubiquitous *FLP* mRNA and recombinase activity. The distribution and amount of *FLP* mRNA was assessed in the embryo by whole mount *in situ* hybridization and in adult tissues by Northern blot analysis. Two of the five *hACTB::FLP* mouse lines exhibited broad patterns of *FLP* transcripts in 9.5 days post coitum hemizygous embryos (mouse lines 4917 and 4924; Fig. 3B and D) and in adult tissues (Fig. 3E and F). Flp activity was assayed in EF cultures derived from each transgenic mouse line. The EF cultures were transiently transfected with target plasmid and stained with X-Gal. Maximal Flp activity (approximately 45% of the "recombined" control) was observed in lines 4917 and 4924 (Fig. 3G), the same mouse lines that showed broad *FLP* expression (Fig. 3B and D). As shown in Fig. 3G and H, the amount of recombinase activity detected in EF cultures also correlated with the amount of *FLP* mRNA

isolated from each culture. From these experiments it can be inferred that mouse lines 4917 and 4924 are the best candidates for broadly expressed active recombinase. Because no abnormalities were detected in founders or offspring it is likely that Flp activity is nontoxic and can be used in most cell types.

Flp Is Necessary and Sufficient to Recombine Target Sequences in Transgenic Mice. To test whether Flp activity can recombine a chromosomal target *in vivo*, mice carrying *FRTZ* were generated. Five transgenic founders were obtained. F₁ mice from four of the five founders bred as expected for unique single-site integration events (one founder failed to transmit the transgene). Southern blot analysis of liver DNA isolated from each mouse line showed that three of the four mouse lines carried the target *FRTZ* in head-to-tail array: line 4999 carried an array of approximately 4 copies of the *FRTZ* transgene; line 4998, 11 copies; line 5000, 30 copies. Transgene transmission was Mendelian and no rearrangements were observed.

The ability of Flp to catalyze *in vivo* recombination of the target *FRTZ* transgene was initially examined by crossing these mouse lines with the Flp producing lines described above (4917 and 4924). Tail DNA from doubly transgenic animals was analyzed by PCR using primers (diagrammed in Fig. 1A and B) specific for detecting either the *FRTZ* transgene, the recombined target *FRTZ-product*, or the *FLP* transgene. Analyses of progeny from three distinct crosses are shown in Fig. 4A. The product of Flp-mediated excisional recombination at the *FRTZ* locus, was amplified only in DNA isolated from doubly transgenic mice and was not detected in littermates transgenic for only the recombinase or the target gene. All three *FRTZ* target lines were found to be competent for recombination by this assay. Sequence analysis of the 0.25-kb amplification product showed precise site-specific recombination.

Flp Mediates Recombination in a Variety of Tissues in a Dose-Dependent Manner. The efficiency of Flp recombination at target *FRTZ* loci was assayed by Southern blot analysis. Genomic DNA isolated from doubly transgenic adult mice (target line *FRTZ-4999*; *FLP-4917*) was hybridized with a *lacZ* probe (probe 1, Fig. 1A) to allow simultaneous detection of the target *FRTZ* transgene and the product of recombination. As shown in Fig. 4B, the new 4.4-kb DNA fragment resulting from the recombined target was present only in samples from doubly transgenic animals, and absent in DNA isolated from either target *FRTZ* (Fig. 4B) or *FLP* littermates (data not shown).

The amount of recombination product detected by Southern blot analysis was found to correlate directly with the amount of *FLP* mRNA detected in each tissue by Northern blot hybridization (Fig. 3E: lane 6, liver; lane 12, muscle; lane 1, testes). Estimates of recombination efficiency were obtained from phosphorimage quantification of recombined (4.4 kb) to nonrecombined (5.6 kb) bands. In muscle, approximately 30% of the transgenes were in the recombined (4.4 kb) configuration. This represents an average of the actual recombination achieved in the various cell types isolated when dissecting muscle tissue (myofibrils, connective tissue fibroblasts, vascular endothelial cells, lymph node cells, blood cells). The value of 30% therefore represents a low estimate of the maximal recombination efficiency. This frequency is consistent with that observed in the EF cell culture assay derived from the same *FLP-4917* mouse line (45%, Fig. 3G); indeed, both cell populations showed similar amounts of *FLP* mRNA. Hybridizing with a probe specific to DNA between the FRT sites detected only the unrecombined fragment (data not shown).

A Recombined Transgene Is Stably Transmitted Through the Germ Line. A prerequisite to using Flp to genetically manipulate cell lineages is that the recombination product be stable and heritable. Germ-line transmission of the recombined transgene was demonstrated by outcrossing a doubly transgenic (*FRTZ-5000*; *FLP-4917*) male and genotyping progeny by PCR (data not shown). Both recombined and unre-

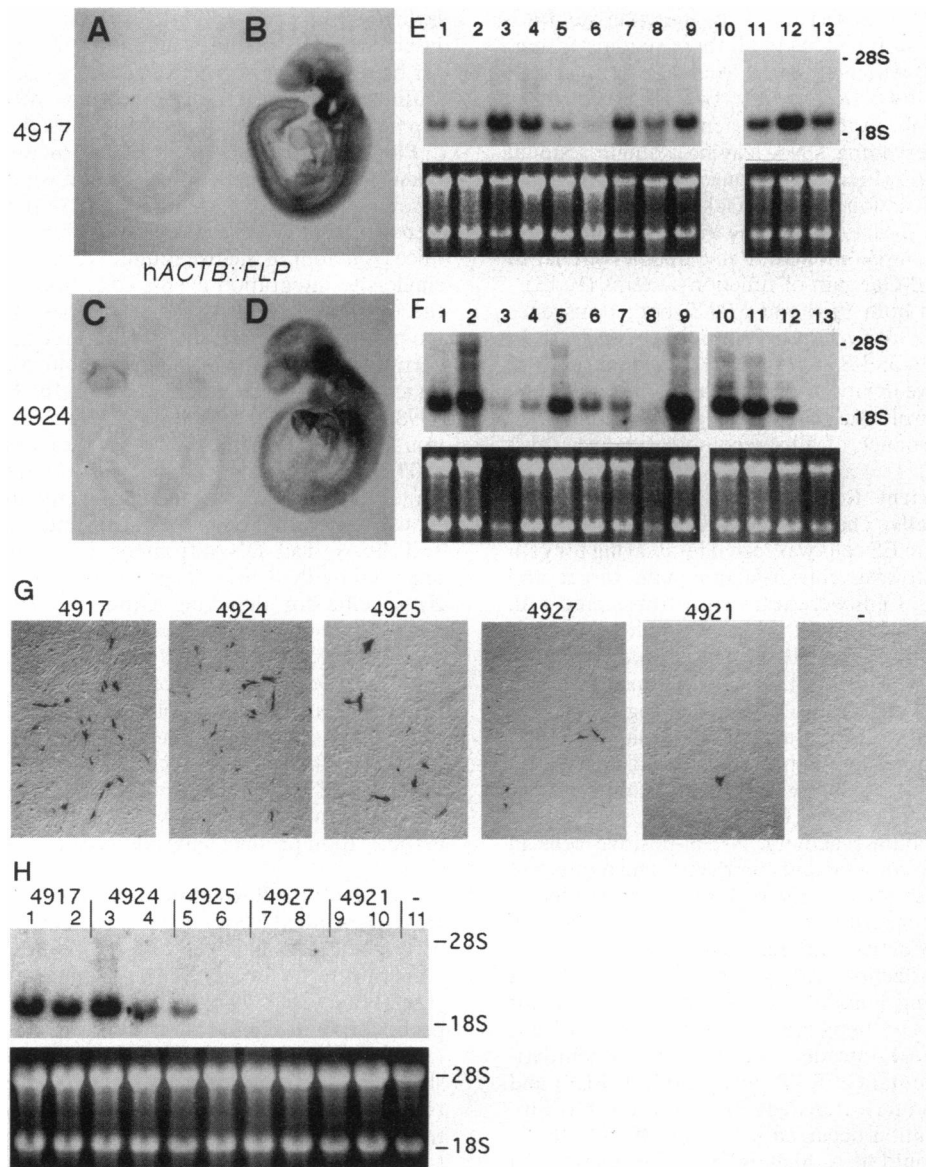


FIG. 3. Human β -actin sequences direct broad expression of *FLP* in the embryo and adult mouse without adverse affects. (A–D) Whole mount *in situ* hybridization analysis of *FLP* RNA expression at 9.5 days post coitum. Lateral views of nontransgenic (A) and transgenic (B–D) embryos from *hACTB::FLP* mouse lines 4917 (B) and 4924 (C and D). (A, B, and D) *FLP* RNA detected using antisense probe 2; (C) control sense probe. (E and F) RNA blot analyses of *FLP* expression in adult tissues from transgenic mouse lines 4917 (E) and 4924 (F). Total RNA (20 μ g) was fractionated by electrophoresis, transferred to nitrocellulose, and assayed for hybridization to ³²P-labeled *FLP* probe 3; lower panels show ethidium bromide staining to document RNA loading. (E and F) Lanes: 1, testes; 2, brain; 3, heart (degraded sample in F, therefore repeated in lane 10); 4, intestine; 5, kidney; 6, liver; 7, lung; 8, spleen; 9, ovary; 10, heart (see lane 3 in E); 11, quadriceps and hamstring muscles; 12, gastrocnemius and soleus muscles; 13, uterus. Positions of 28S and 18S rRNAs are indicated. (G) Assay for Flp function. Primary EF cultures were prepared from hemizygous *hACTB::FLP* transgenic embryos as described (29). Cultures derived from five different transgenic mouse lines (4917, 4924, 4925, 4927, 4921), and one nontransgenic line (–), were transfected with 3 μ g of target pNEO β -GAL (13) followed 48 hr later by histochemical X-Gal stain (30). Maximal activity, as indicated by the number of blue cells, was observed in cultures derived from mouse lines 4917 and 4924. Similar results were obtained following transfection with pFRTZ. (H) Expression of *FLP* in EF cultures correlates with activity observed in transfection assay. Total RNA (20 μ g) was separated and hybridized to ³²P-labeled *FLP* probe 3; ethidium bromide staining of gel in lower panel shows equivalent RNA loading. Two independent cultures from each *FLP* mouse line were analyzed: (lanes 1 and 2) *hACTB::FLP* mouse line 4917, (lanes 3 and 4) line 4924, (lanes 5 and 6) line 4925, (lanes 7 and 8) line 4927, (lanes 9 and 10) line 4921, and (lane 11) nontransgenic negative control.

combined transgenes were detected in this singly transgenic F₃ mouse indicating that recombination was incomplete; a subset of the 30 *FRTZ* transgenes in tandem array underwent recombination.

Conditional Expression of Flp Can Induce Regulated Rearrangement of Target Sequences in Differentiating EC Cells. Controlling expression of the *FLP* transgene is a way to restrict recombination, and therefore gene activation or deletion, to specific cell populations. I investigated whether Flp recombination could be induced in a differentiating EC cell culture

system by using *Wnt-1* regulatory sequences (37) to express *FLP* (see Fig. 1B for the *Wnt1::FLP* transgene). RA can induce pluripotent P19 EC cells to differentiate into a mixed population of fibroblasts, astrocytes, and neural cells (38, 39). *Wnt-1* expression is likely induced specifically in neural derivatives, paralleling that seen in embryos where *Wnt-1* mRNA is detected in differentiating neuroectoderm (40).

P19 cells were transiently transfected with target plasmid, target plus *phACTB::FLP*, or target plus *pWnt1::FLP*; 0.5 μ M RA or control diluent was added to the monolayer 24 hr later.

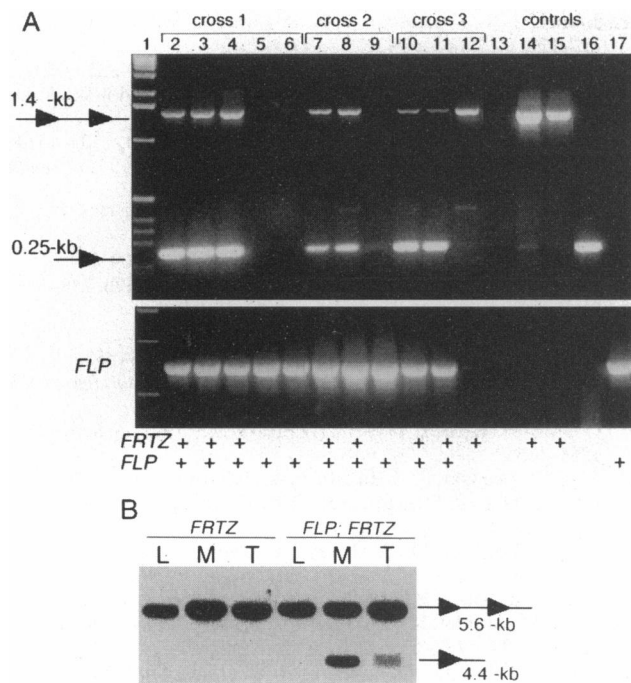


FIG. 4. Dose-dependent Flp recombination in genomic DNA isolated from tissues of doubly transgenic mice. **(A)** Identification of recombined *FRTZ-product* transgenes by PCR amplification of tail DNA. Primers (SD49/SD68) used in this assay flank the FRT-cassette (see Fig. 1A); amplification of the target *FRTZ* transgene yields a 1.4-kb fragment and *FRTZ-product*, a 0.25-kb fragment. Parallel reactions using *FLP*-specific primers (SD41/SD42) are shown below. Brackets group littermates from three distinct recombinase target crosses; control single transgenic parental samples and *FRTZ-product* DNA are shown on the right: lanes: 1, 1-kb ladder; 2–6, *FRTZ-5000* × *FLP-4917*; 7–9, *FRTZ-4999* × *FLP-4924*; 10–12, *FRTZ-4999* × *FLP-4917*; 13, no DNA; 14, single transgenic *FRTZ-5000* parental sample; 15, single transgenic *FRTZ-4999* parental sample; 16, *FRTZ-product* DNA; and 17, single transgenic *FLP-4917* parental sample. Genotypes as determined by independent PCR reactions (*FRTZ*, SD23/SD26; *FLP*, SD24/SD25; see Fig. 1) are indicated by plus signs. Sequence analysis of the 0.25-kb product showed precise site-specific recombination (data not shown). **(B)** Correlation between the amount of recombination product and the level of *FLP* RNA expressed in a given tissue. Southern blot analysis using probe 1 (see Fig. 1A) of *Bam*HI/*Sca*I-digested genomic tissue DNA (10 μ g) isolated from a doubly transgenic (*FLP-4917*; *FRTZ-4999*) mouse or singly transgenic (*FRTZ-4999*) littermate. The expected unrecombined (5.6-kb) and recombined (4.4-kb) fragments within the context of the four-copy array are depicted on the right. Tissue samples include liver (L), muscle (M), and testes (T). For the amount of *FLP* RNA detected in each tissue see Fig. 3E; lane 6, liver; lane 12, muscle; lane 1, testes.

Following 5 days of RA treatment, β -Gal activity was assessed by histochemical X-Gal staining. Neural induction was monitored by morphology (the presence of long cellular processes) and culture senescence, as well as by induction of endogenous *Wnt-1* mRNA.

β -Gal activity was detected in target plus pWnt1::FLP cotransfections only following RA induced differentiation (Fig. 5 C and F). Similarly, endogenous *Wnt-1* expression was absolutely dependent on RA. Low levels of *Wnt-1* transcripts were first detected by Northern blot hybridization after 4 days of RA treatment; no *Wnt-1* RNA was detected in untreated cells (data not shown). As predicted by the nature of the *hACTB* regulatory sequences, β -Gal-positive cells were observed in the target plus phACTB::FLP cotransfections independent of RA (Fig. 5 B and E). The target plasmid alone showed no activity (Fig. 5 A and D). In addition to demonstrating regulated rearrangement of target sequences, these results define a temporal relationship between *FLP* expression

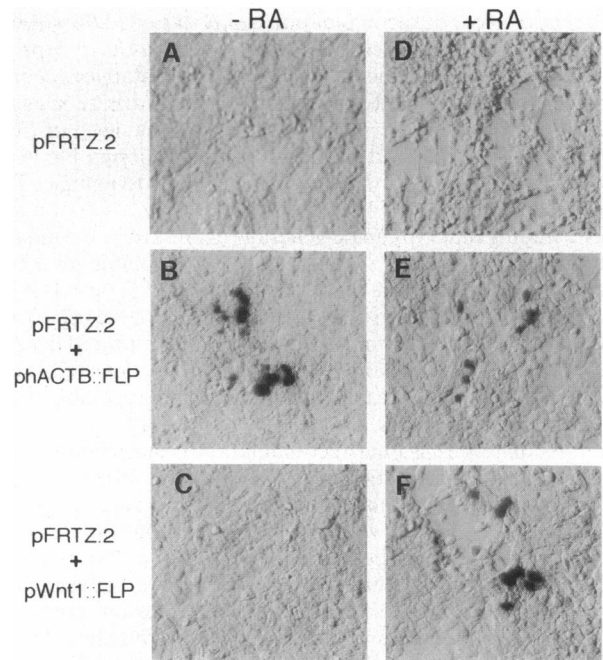


FIG. 5. Regulated Flp recombination in differentiating embryonal carcinoma cells. P19 EC cell monolayers were transiently transfected with the indicated plasmids and induced to differentiate by treatment with RA (38, 39). Target plasmid pFRTZ.2 is identical to pFRTZ except it contains the HSV-tk gene inserted between FRTs. Histochemical X-Gal staining was performed 5 days later. **(A–C)** Exposure to control diluent; **(D–F)** exposure to 0.5 μ M RA.

and completed recombination. β -Gal activity, reflecting Flp recombination, was observed in the target plus pWnt1::FLP cotransfection within 24 hr of first detecting *Wnt-1* transcripts, and by inference *Wnt1::FLP* mRNA.

DISCUSSION

This study demonstrates that Flp can effectively recombine target DNA in ES cells, EC cells, and transgenic mice. I have shown that Flp can direct site-specific and heritable DNA recombination in the mouse, and regulated (inducible) recombination in differentiating EC cells. These properties indicate that Flp can be used to make directed modifications of the mouse genome.

Using this Flp system, recombination of an extrachromosomal target can occur in ES cells with an efficiency similar to that previously observed in mouse embryonal carcinoma (F9) cells (13) and in monkey (CV-1) and human (293) embryonic kidney cells (13, 16). Because the efficacy of Flp recombination estimated here (30–78%) is comparable to that reported for Cre (40–80%; ref. 41), it is likely that this Flp system can be exploited to similarly manipulate ES cell chromosomal DNA. Toward this end, Fiering *et al.* (42) has recently employed a more elaborate two-step selection scheme where Flp-mediated deletion of an integrated selectable marker gene (*PGK-neo*) was reported to occur in 90% of Flp-expressing ES cells.

In the mouse, I have shown that Flp expression is necessary and sufficient for excisional recombination of FRT target sequences. Because recombination was detected at all three chromosomal sites assayed, it is likely that most chromosomal transgenes will be accessible to Flp function. The extent of recombination observed in a given tissue correlated directly with the overall amount of *FLP* mRNA detected in that tissue; it is important to note that this type of tissue analysis presents an average and therefore may underestimate the maximal recombination achieved in a specific cell type. Nonetheless, these results define a dose-effect relationship that suggests that

different degrees of recombination can be attained by varying the strength and specificity of the sequences used to express *FLP*. For some experiments, complete (quantitative) recombination may be needed. The results presented here suggest that one means to achieve this is to increase the level of *FLP* expression. Alternative strategies include identifying Flp variants with higher activity in mammalian cells, or to enhance the nuclear localization of Flp.

The finding that Flp can be generally expressed in the mouse without adverse effects suggests that Flp recombination between random sequences in the mouse genome is rare. If high levels of illegitimate (non-FRT) recombination were occurring due to Flp expression, abnormalities would be expected in *FLP* founders or offspring. No adverse effects were detected. This result suggests that Flp can be used to mediate recombination in a variety of cell types.

Flp-mediated excisional recombination is sufficiently dose sensitive that recombination can be regulated in differentiating EC cells in culture. This was evident from examination of RA-treated P19 cells in which the *Wnt-1* promoter was used to express *FLP*. The temporal induction of *Wnt-1* transcripts following RA-induced differentiation indicates that recombination occurred relatively quickly: *FLP* expression, recombination of the target transgene to reconstitute a functional *lacZ* gene, and subsequent β -Gal production occurred within 24 hr. These results demonstrate that regulated rearrangement of a target sequence can be achieved.

The demonstration that Flp can excise DNA in mice and that the recombination product is heritable, suggests that Flp will be useful to study cell lineages. Considering this potential application, the initial test recombination substrate was designed to indicate and "remember" a recombination event by the irreversible gain of β -Gal activity (dependent only on constitutive promoter activity). Mice transgenic for this target should have the capability of marking cell lineages following introduction of Flp by crossing. Toward this end, mice transgenic for *Wnt1::FLP* have been generated; by crossing to an "optimal" target mouse, cells originating from the dorsal aspect of the developing central nervous system are predicted to be marked. Although all three *FRTZ* target lines analyzed here were competent for recombination, none of the recombinant target alleles were sufficiently active to allow cell marking by X-Gal stain (unpublished observations). The lack of β -Gal activity associated with the observed recombination most likely reflects a position effect on transgene transcription exerted by the genomic integration site since only one in four control *FRTZ-product* mouse lines expresses β -Gal (unpublished observations). Such sensitivity to chromosomal context is also supported by the variation in transcript profiles observed when using the same *hACTB* regulatory sequences to direct *FLP* expression (two of five lines showed general expression in this study). It is likely that by screening more *FRTZ* target loci, a chromosomal integration site will be identified that can support similarly general *lacZ* expression following Flp recombination.

Together, these findings demonstrate that Flp can serve as a tool to alter the mouse genome. By employing both Flp and Cre, it should be possible to engineer multiple independent recombination reactions (gene activation or deletion events) in mice.

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