

Use of Mutated FLP Recognition Target (FRT) Sites for the Exchange of Expression Cassettes at Defined Chromosomal Loci

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Received July 15, 1994; Revised Manuscript Received August 31, 1994*

ABSTRACT: Using the FLP/FRT system for site-specific recombination and the wild-type recognition site (FRT) in conjunction with certain mutant FRT sites, it becomes possible to provoke, with high yield, a double-reciprocal crossover event in cultured mammalian cells. It is demonstrated that this technology enables a targeting of expression cassettes to appropriate chromosomal reference sites in the recipient cell to improve the concepts of reverse genetics. The design of mutant FRT sites promoting such a process will be delineated. Our results show that the five spacer mutations tested are functional as the wild type but differ in the extent of their cross-recombination, which has to be minimized for their simultaneous usage.

For higher eukaryotes, homologous recombination is an essential event participating in processes like DNA repair and chromatid exchange during mitosis and meiosis. Recombination depends on two highly homologous, extended sequences and several auxiliary proteins, only part of which has been identified. Strand exchange can occur at any point between the regions of homology, although particular sequences may influence efficiency. These processes can be exploited for a targeted integration of transgenes into the genome of certain cell types like embryonic stem cells. On the other hand, cultured cell lines relevant for genetic engineering purposes have lost the potential to perform homologous recombination at the efficiency that would be required to incorporate it into routine procedures (S. Karreman, GBF, unpublished). We chose BHK, which is one of the two most frequently used lines in biotechnology and has a long track record for the safe production of vaccines.

For the above reasons, the major problem associated with the conventional gene transfer techniques has remained essentially unresolved as these tend to yield highly variable expression levels due to chromosomal position effects and copy number variation among different clones. Recently, it has become feasible to adopt an auxiliary enzymatic machinery from phage or yeast which has the potential to provide the required innovation for a targeted gene transfer into mammalian cell lines [reviewed by Craig (1988), Kilby et al. (1993), and Barinaga (1994)].

Bacteriophage P1 and most strains of *Saccharomyces cerevisiae* encode systems catalyzing a reciprocal, site-specific recombination (cre/loxP1 and FLP/FRT,¹ respectively). With the appropriate positioning of loxP1 or FRT sites, both recombinases enable the experimenter to insert, excise, invert, or translocate DNA molecules. Using the FLP recombinase from yeast, we will demonstrate below that the scope of applications can be extended considerably by the simultaneous use of wild-type and/or differently mutated FLP recognition target (FRT) sites. These mutants of a complete (48-bp) FRT site allow a precise exchange of expression cassettes *in*

vivo, even in case the transgene has integrated and hence assumed its final chromatin structure.

MATERIALS AND METHODS

(a) *Plasmids.* pBSIIF, a plasmid containing the FRT site (F) in its polylinker, was prepared from pBSIISK (Stratagene) by inserting the 150-bp *EcoRI*–*HindIII* fragment from pNeoβ Gal into the respective sites. Plasmids pBSIIF_n containing FRT mutants (F₁–F₅) were derived correspondingly by ligating the *EcoRI*–*HindIII* fragments arising from the respective mutagenesis (see below).

HygTk, the basic vector coding for a fusion protein from hygromycin B–phosphotransferase × HSV–thymidine kinase, controlled by the Tk-promoter, was constructed and kindly provided by C. Karreman (GBF, Braunschweig). The vector is suited for positive (hygromycin) and negative (gancyclovir) selection.

FHygTk was derived from this plasmid by inserting the 150-bp *ClaI*–*XhoI* fragment (comprising FRT) from pOG45 (O'Gorman et al., 1991) into the respective sites. The FRT site is located 5' to the selector gene. Plasmids F_nHygTk were constructed by excising a 100-bp *BamHI*–*ClaI* fragment (containing the respective FRT mutant, F₁–F₅, from pBSIIF_n) and ligating it into the *ClaI* site of HygTk.

HygTkF was obtained by ligating the *HindIII*–*SpeI* fragment (including FRT) from pBSIIF into the 5.3-kb *HindIII*–*PvuII* fragment from HygTk; the FRT site is localized 3' to HygTk. Plasmids HygTkF_n were constructed by excising the mutagenized 100-bp *BamHI*–*HindIII* fragments from pBSIIF_n and ligating them into the corresponding sites of HygTk.

F₅HygTkF (Figure 1a) was derived in a way analogous to F_nHygTk but using HygTkF in place of HygTk.

F₅NeoF (Figure 1b) was obtained after deletion of the primer binding sites in F₅HygTkF (by cleaving with *BamHI*–*NarI* or *ClaI*–*PvuII*, respectively, fill up, and religation). HygTk was excised by *NcoI*–*XhoI* and replaced by the 2-kb *PvuII* neo^r fragment from pAG60 (Colbère-Garapin et al., 1981) that was recovered from a partial digest.

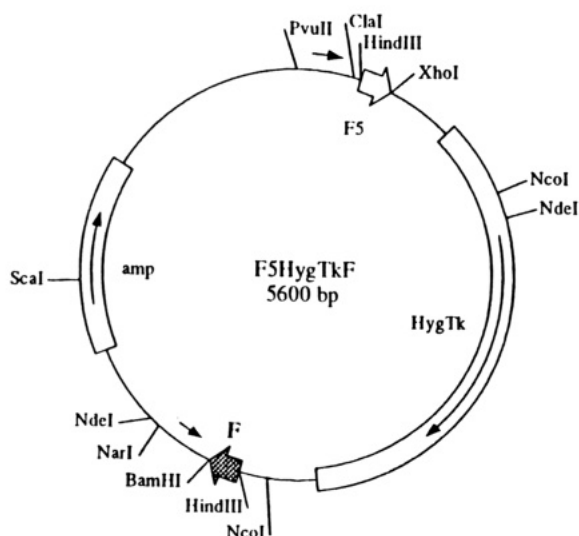
(b) *Preparation of the Mutants (F₁–F₅).* The following primers were designed according to the pNeoβ Gal sequence (4): (i) primer adjacent to the direct 13-bp repeats of FRT and provided with an *EcoRI* site, 5'-CGCATCCGAATTC-

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© Abstract published in *Advance ACS Abstracts*, October 1, 1994.

¹ Abbreviations: FRT, FLP recombinase target; SAR, scaffold-attached region.

a



b

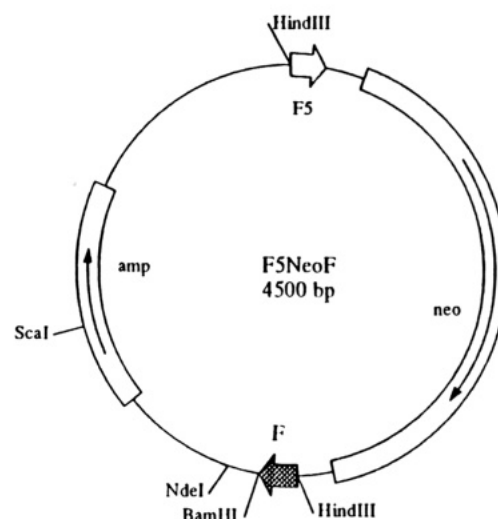


FIGURE 1: Plasmids F₅HygTkF (a) and F₅NeoF (b) used for a double-reciprocal crossover. F (large dark arrow) is the wild-type FRT site and F₅ (large light arrow) the selected spacer mutant. Small arrows represent PCR primers (cf. Figure 2).

CTTCGCCAGGG^{3'}; (ii) mutagenesis primer starting at the HindIII site and extending into FRT, 5'CGCCTATCGAT-AAGCTTGAAGTTCCTATAC(N)₈GAATAG^{3'} [(N)₈, mutated spacer sequence].

pNeoβ Gal template (200 ng) was provided with 50 pmol each of the respective primers and amplified in a Perkin-Elmer Cetus thermal cycler by 30 cycles of 90 °C (1 min), 45 °C (1 min), and 72 °C (3 min). Following amplification, the fragments were cloned into Bluescript pBSISK (Stratagene) and sequenced.

(c) *Cell Culture and Transfection.* For stable expression, BHK or CV-1 cells were seeded at a density of 2×10^5 per 25-cm² flask. On day 2, the medium (DME containing 10% FCS, 20 mM glutamine, 60 μg/mL penicillin, and 100 μg/mL streptomycin) was changed 4 h prior to the addition of DNA precipitates.

DNA precipitates were prepared as follows: 1 μg of the linearized plasmid was mixed with 25 μL of 2.5 M CaCl₂ and diluted to 250 μL. The suspension was added, on a Vortex mixer, to 250 μL of 2× HEBS (280 mM NaCl, 50 mM HEPES, 1.5 mM Na₂HPO₄, pH 7.1). The final precipitate was added to the cells. The medium was changed on day 1 after transfection and replaced by selection medium on day 2. On day 6, 100–500 surviving clones (consisting of 50–100 cells each) became visible, which could be picked on day 10 (corresponding to 11 population doublings) at a time when episomal copies are no longer present, i.e., detectable in a Hirt extract. For selective media, G418 was applied at a concentration of 1000 μg/mL and hygromycin B at a concentration of 500 units/mL (BHK) or 300 units/mL (CV1). Gancyclovir was applied at a concentration of 30 μM. Media were replaced every third day.

For transient expression experiments, 2×10^5 cells were seeded per 25-cm² flask. Four hours prior to transfection the medium was changed and transfection was performed as above but using supercoiled plasmids. Then 48 h later cells were collected; DNA was prepared and subjected to PCR analyses as described below.

(d) *Recombination Prior to Integration (Transient State).* For a single recombination event, 1 μg of each of the respective plasmids was transfected into BHK cells (in case of Figure 3: 1 μg of F_nHygTk + 1 μg of HygTkF_n + 2 μg of the recom-

binase plasmid pOG44). After 48 h the products of a recombination were analyzed by PCR using the appropriate primers and verified by restriction analysis (cf. Figures 2 and 3).

In the case of a double-reciprocal crossover, the relative concentrations of the components were varied as described in the legend to Figure 4.

(e) *Recombination Subsequent to Integration (Stable State).* One microgram each of the respective plasmid HygTkF_n and the recombinase plasmid (pOG44) was transfected into CV-1 cells (strain E25B2) (Table 1). The hygromycin-resistant clones were subjected to a color reaction in situ (blue, nonspecific integration, β Gal reading frame intact; white, specific integration, reading frame disrupted, cf. Table 1). BHK cells containing a single copy of F₅HygTkF (Figures 2 and 4) were cultured continuously for 4 weeks (40 population doublings) before they were transfected with 1 μg of F₅NeoF and 2 μg of pOG44. G418-resistant clones isolated after 2 more weeks were characterized using PCR primers according to the scheme in Figure 2 (cf. Figure 4).

(f) *PCR Analyses.* The 2×10^6 cells were collected in 400 μL of TEP, diluted to 1 mL with culture medium, and pelleted by centrifugation (5 min at 500g). The pellets were collected in 1.5 mL of PBS and centrifuged again. The final pellets were suspended in 0.1 mL of PBS, diluted to 500 μL with water, and heated (95 °C) for 5 min. After the addition of 200 μg of proteinase K in 20 μL of H₂O the solution was kept at 37 °C overnight. The DNA preparation remaining after inactivating the protease (10 min at 95 °C) was subjected to PCR: 50–150 ng of chromosomal DNA or 1–100 pg of plasmid was supplied with 50 pmol of each of the primers (5'CCATGATTACGAATTCATCG^{3'} and 5'TGTTGGGAA-GGGCGATCG^{3'}; cf. primers 1 and 2 in Figure 2, respectively), 4 μL of nucleotide mixture (2.5 mM each of dATP, dCTP, dGTP, and dTTP), 4.5 μL of 10× buffer (100 mM Tris-HCl, pH 8.2, 500 mM KCl, 15 mM MgCl₂, 0.01% gelatin), and water to a total volume of 45 μL. After being heated to 95 °C (10 min) and to 72 °C (8 min), 5 μL of Taq polymerase (0.6 unit) was added. Amplification proceeded during 30 cycles of 94 °C (1 min), 56 °C (1 min), and 72 °C (3 min). PCR products were analyzed on 0.7% or 2% agarose gels in TAE buffer. The identity of bands was verified by digests

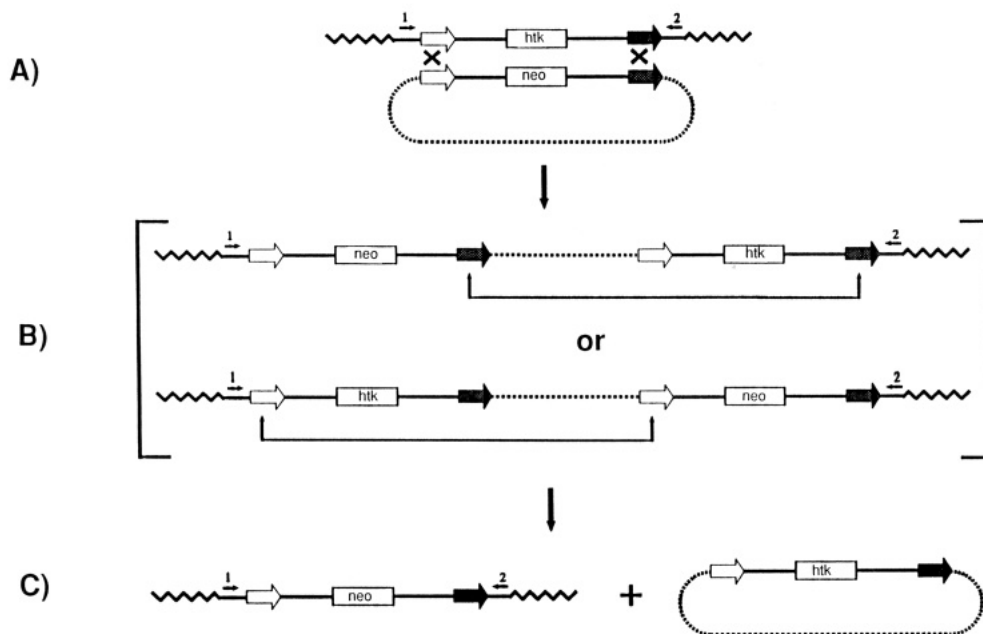


FIGURE 2: Sets of FRT sites enable a double-reciprocal crossover. (A) Recombination between an integrated (htk) expression cassette (from F₃HygTkF; cf. Figure 1a) and a circular plasmid (F₃NeoF; cf. Figure 1b) is guided by two sets of FRT sites, consisting, for example, of wild-type (dark arrows) and mutant sites (light arrows). (B) Depending on the set of FRTs involved in the first crossover, two intermediates will arise which are subject to a subsequent crossover reaction. If the second step reuses the initial set of sites, situation A will be reestablished. (C) The product of a double-reciprocal crossover arising from the successive use of both sets of FRTs. Situations A and C can be distinguished by PCR via primers 1 and 2 that will yield amplification products differing in size.

with typical restriction enzymes to either prevent amplification or subdivide the product into two bands of predictable size. Fidelity of PCR analyses was examined by performing, in parallel, amplifications on the DNA of nontransfected cells and the same DNA with the applicable amount of the linearized vector added.

RESULTS

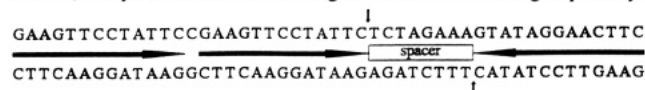
The Concept. Site-specific recombinases like FLP cleave and religate DNA at specific target sequences, resulting in a precisely defined recombination between two identical sites. Since no auxiliary factors are needed and the entire system can be transplanted into higher eukaryotic cells, its importance for genome engineering is increasing exponentially (Craig, 1988; Kilby et al., 1993; Barinaga, 1994). Given its efficiency, we reasoned that the system might be applicable for a reciprocal exchange of DNA, which requires two crossover events within target sequences that are localized on both sides of the DNA segment(s). The desired event would be extremely rare in case the cassettes are flanked by identical sites since, subsequent to integration, only one out of three possible excisions would lead to the desired product and even this would be unstable during the burst of recombinase activity [see Sauer and Henderson (1990) for the respective use of cre in yeast]. However, the same process would become efficient if sets of target sites could be engineered to enable two independent recombination events in a single enzymatic reaction. This approach would lead to a double-reciprocal crossover with a second construct carrying the same combination of sites. Figure 2 shows that this is the simple consequence of an equilibrium between the six forms of DNA (parts A–C) established by the recombinase from the two recombining educts. Using a lox mutant in the cre/loxP1 system, a related approach has recently been followed for shuffling, in phage-infected bacteria, the heavy and light antibody chain genes encoded by two different plasmids (Waterhouse et al., 1993).

Construction of FRT Mutants. The minimum FRT site consists of two inverted 13-bp repeats separated by an 8-bp

Table 1: Cross-Reactivity for Homologous (F × F) and Heterologous (F × F_n) FLP-Mediated Recombination: Integration of Vector B (Figure 3a) into a Stably Transfected Copy of pFRTβ Gal [Cf. O'Gorman et al. (1991)]^a

	spacer	white/total	%	98% conf interval
F ^{0.75}	TCTAGAAA	397/838	47.4 (100)	
F ₁ ^{0.75}	TCTAGATA	11/643	1.74 (3.61)	7.1
F ₂ ^{0.75}	TCTACTTA	3/629	0.48 (1.01)	2.8
F ₃ ^{0.88}	TTCAAATA	2/849	0.24 (0.51)	1.5
F ₄ ^{0.63}	TCTAGAAG	3/604	0.50 (1.05)	2.9
F ₅ ^{0.75}	TTCAAAG	1/645	0.15 (0.32)	1.3

^a Superscripts in column 1 designate the spacer's AT content (%) and underlined letters in column 2 the mutated base pairs, respectively. A scheme of the complete wild-type FRT site (F) is shown below. In Figures 1 and 2, the symbol of this site is a large dark arrow indicating its polarity.



asymmetric spacer (cf. the representation in Table 1) and will still permit excision *in vivo* while it appears to be refractory in the reverse process of integration which requires a third 13-bp direct repeat (Lyznik et al., 1993). The 8-bp spacer is involved in DNA–DNA pairing during strand exchange. Its asymmetry determines the direction of site alignment in the recombination event, which will consequently lead to either inversion or excision. Footprinting techniques have defined two 12-bp FLP recombinase binding sites that include 11 bp of each of the inverted repeats but only the outermost bases of the spacer, suggesting that most of the spacer can be mutagenized without a loss of function. For a FLP recombinase expressed in *Escherichia coli*, this idea has been verified, and some principles for the construction of mutants which are functional in an *in vitro* assay have been delineated (Umlauf & Cox, 1988). On the basis of the efficiency of *in vitro* recombination and the strength of enzyme–site interaction, it was concluded that the function of a given set of sites remained largely unaffected by a mutation in almost any single

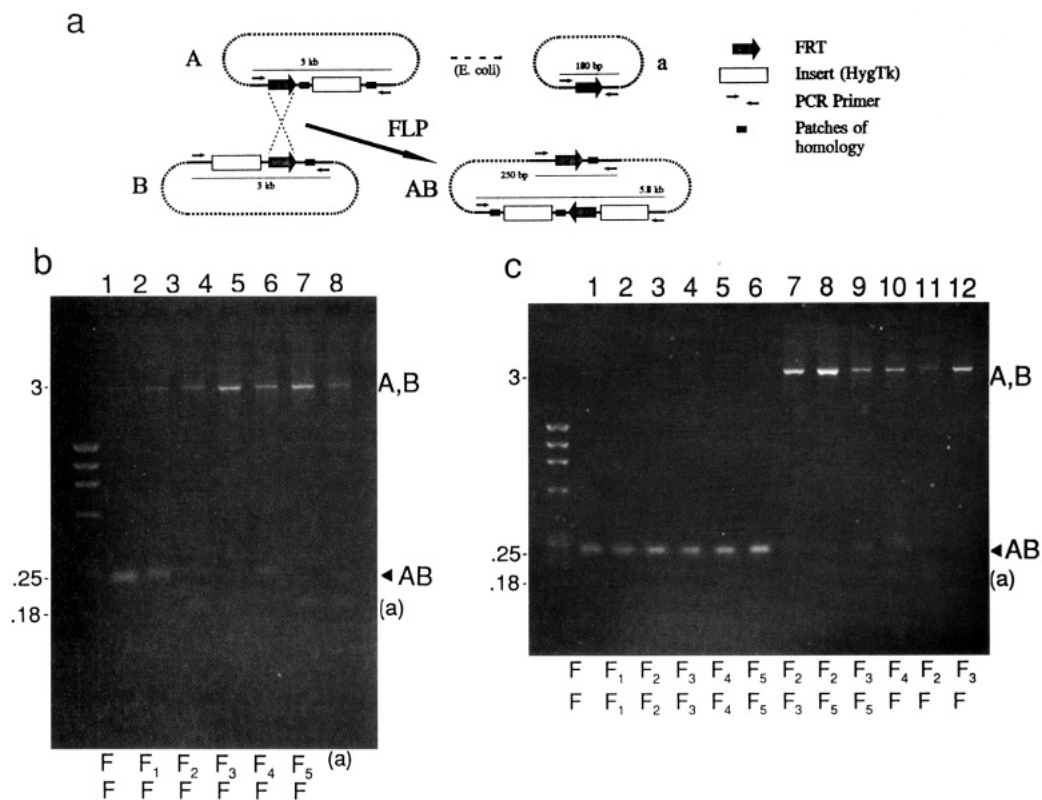


FIGURE 3: Homologous ($F \times F$; $F_n \times F_n$) and heterologous ($F \times F_n$; $F_n \times F_m$) FLP-mediated recombination efficiencies. (a) The assay. A basis construct (HygTk) was provided with a 48-bp FRT site either upstream (plasmid A, i.e., FHygTk and the corresponding mutants F_n HygTk) or downstream from the insert (plasmid B, i.e., HygTkF and the corresponding mutants HygTk F_n). Plasmids A and B were cotransfected together with a vector coding for the FLP recombinase [pOG44 from O'Gorman et al. (1991)]. After the transient expression phase the product of a recombination between plasmids A and B (AB) could be detected by PCR via a short 0.25-kb amplification fragment. The educts of the reaction produce a 3-kb amplification fragment, and the same is true for the products of a self-recombination (AA or BB, respectively). A band of 0.18 kb (a) is a result of an intramolecular recombination between two inverted repeats during the cloning procedure in *E. coli* in the A-construct containing a wild-type FRT site. (b) Recombination potential between wild-type F and mutant F_n sites as judged from the intensity of the 0.25-kb fragment visualized on a 2% agarose gel (marker: *Hae*III digest of ϕ X174 DNA). The 0.18-kb fragment (arising from a) is amplified only in mixtures where AB is a minor component as demonstrated in lane 7 where a nonrecombined mixture of A and B has been submitted to PCR directly. (c) Recombination potential for $F_n \times F_n$ (self-recombination; lanes 1–6) or $F_n \times F_m$ (cross-recombination; lanes 7–9). Lanes 10, 11, and 12 contain the respective samples from part b and have been added for comparison.

base of the central 6 bp of the spacer. Multiple replacements became deleterious if they affected the polypyrimidine and polypurine tracts which extend from the spacer's center into the 13-bp repeats or if they reduced the high (75%) AT content of the spacer to any major extent. The importance of AT content and of negative supercoiling (accelerating the recombination 2–5-fold) suggests that the affected step involves DNA unwinding.

We have constructed, by PCR-based mutagenesis, the series of spacer-mutants (F_1 – F_5) listed in Table 1. Among these, F_1 equals pJFS40 (Umlauf & Cox, 1988) and F_4 is Mu5T2 [described by McLeod et al. (1986)]. F_2 , F_3 , and F_5 include and extend the mutations of F_1 and F_4 , respectively. Some of the rules outlined by Umlauf and Cox (1988) are compromised in that an outer base pair of the spacer is included (F_4 , F_5), the AT content is decreased (F_4), or the polypurine tract is interrupted (F_3).

Our initial assay has been adapted from O'Gorman et al. (1991) using their CV-1 cell line (strain E25B2) harboring a single copy of pFRT β Gal. The expression of β -galactosidase is blocked in case an integration occurs by site-specific recombination. Vector HygTk F_n (B in Figure 3a) was transfected into these cells; hygromycin-resistant clones were grown and classified as shown in Table 1. According to these figures, 47% of hygromycin-resistant cells integrated at least one copy in a site-specific manner if plasmid B had the same 48-bp FRT wild-type sequence present in pFRT β Gal. Only

1.7–0.15% of white clones arose in the case of a mutated spacer, revealing an increasing discrimination in the order $F_1 < F_4 = F_2 < F_3 < F_5$. These observations leave unresolved the question as to the extent of $F_n \times F_n$ self-recognition.

Searching for an appropriate test, we have initially studied the monomolecular process of FLP-mediated excision, which is usually more efficient than integration. In fact, plasmids of the AB-type (FHygTkF/ F_n HygTk F_n ; see Figure 3 for nomenclature) with the same FRT sites in both locations generated sizable amounts of A- and B-type plasmids; however, careful controls showed that this was not only due to the exclusive action of the FLP enzyme but also due to the potential of the bacterium to perform homologous recombination via any homologous sequences of FRT size. Therefore, the strategy was changed and two sets of A- and B-type plasmids (FHygTk/ F_n HygTk and HygTkF/HygTk F_n) were cloned; the respective partners were cotransfected together with the FLP plasmid and tested for recombination to yield AB (procedure detailed in Figure 3). The PCR test was based on the fact that recombination brought two primer sites in close juxtaposition, generating a 250-bp fragment which could be amplified in preference to alternative fragments of 3 kb (educts; products of $A \times A$ and $B \times B$ recombination) and 5.8 kb (second primer binding site in AB; cf. Figure 3a). A minor contaminant a (180-bp fragment), was only coamplified if FHygTk was one partner and in cases where the recombination was inefficient; its origin is clarified in Figure 3a.

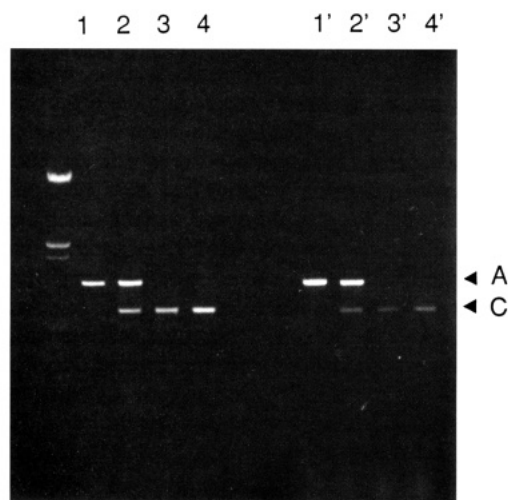


FIGURE 4: Results of a double-reciprocal crossover in the transient (lanes 1–4) and stable states of expression (lanes 1'–4'). The reactions for the transient/stable states were based on plasmids F₅HygTkF (2.5/5 μ g) and F₅NeoTkF (2.5/1 μ g). PCR fragments are analyzed on a 0.7% agarose gel and calibrated against a *Hind*III/*Eco*RI digest of λ DNA. Lanes 1/1' show the situation in the absence of recombinase (control); for the other reactions 5/2 μ g of pOG44 have been cotransfected. For lanes 3/3' and 4/4', *Nde*I and *Xho*I have been added prior to PCR. Disappearance of the upper band under these conditions clearly identifies it as the educt, F₅HygTkF.

For the initial experiments A was provided with a wild-type FRT (F) and B with the same or a mutant site (F, F_n) (Figure 3b). Here, the signal strength of the 250-bp band decreases in the order F \gg F₁ > F₄ > F₂ > F₃ > F₅, reproducing the conclusions from the Table 1 data for a different cell line (BHK). In a second series, identical FRTs were incorporated in vectors A and B to yield, after 30 PCR cycles, 250-bp bands of indistinguishable intensity (Figure 3c, lanes 1–6). Since the same is true for the whole range of PCR cycles that allowed their visualization, it is concluded that none of the mutations affect the recombination between two identical FRTs *in vivo*. The conclusion of the combined data of Figure 3 can then be stated as follows: If pairs of FRTs are needed that mediate independent recombination events with an identical partner, F + F₅, F + F₃, F₂ + F₃, and F₂ + F₅ will be possible while F + F₅ will be the preferred one.

Double-Reciprocal Crossover. Subsequently, we have used the combination F plus F₅ to construct vectors F₅HygTkF and F₅NeoF (Figure 1a/b) in order to create the situation depicted in Figure 2, part A. As a first test, both vectors were cotransfected together with the recombinase expression plasmid (pOG44), and recombination was allowed to proceed in the transient state prior to integration. Then 48 hours post transfection, cellular DNA was isolated and subjected to PCR analysis following the scheme in Figure 2. According to the position of primers 1 and 2, the educt situation (A) would yield a band of 3 kb whereas, after a successful exchange, a 2.2-kb band would occur reflecting situation C. The intermediates arising from the first crossover (B) are not expected to participate in the PCR reaction owing to their extended size. Figure 4 shows the result (lane 2) together with some specific controls. The equilibration process is seen to proceed to a ratio of about A:C = 1:0.7, which is not too far from the theoretical limit of 1:1 which would arise for a complete equilibration of educts and products in case these forms were equally stable.

Encouraged by these results, we performed the analogous reaction with a chromosomal copy of F₅HygTkF and an episomal copy of F₅NeoTkF. Taking advantage of the HygTk

positive-negative selection system, we have therefore established, by long-term cultivation, clonal cell lines that are both hygromycin-resistant and gancyclovir-sensitive (Materials and Methods). These lines were screened, by three criteria—(i) dot blotting (hybridization intensity), (ii) Southern blotting (fragment sizes), and (iii) PCR analyses (lack of fragments indicating either tandem head-to-tail integrates or the persistence of any recircularized episomal transgene)—for the presence of a single chromosomal copy of F₅HygTkF (Schlake, 1994). For Figure 4 (lanes 1'–4') one cell line was subjected to a second transfection with F₅NeoTkF plus pOG44. G418-resistant cells were selected and subjected to gancyclovir to demonstrate that 15% of the formerly sensitive cells had been converted to gancyclovir resistance. This is in close agreement with the A:C ratio (Figure 4, trace 2') revealed by the standard PCR procedure, proving that a target that has been packaged as chromatin is still accessible for the recombination machinery. The identity of bands marked A was verified by their sensitivity to two restriction enzymes specific for the htk cassette of the educt (lanes 3/3' and 4/4' in Figure 4). Similarly, the band labeled C could be identified via its cleavage with *Pvu*II, which was typical for the neo cassette in the product (not shown). Repetitions of this experiment with different clones and various concentrations of the FLP expression plasmid yielded A:C ratios between 9:1 and 1:1, suggesting that the recombination efficiency depends on both the genomic localization of the target and an optimal timing of FLP activity (data not shown).

DISCUSSION

The present series of experiments clearly demonstrates that a double-reciprocal crossover can be mediated by two different sets of FRT sites. If these sites are in a strategically favorable position, they can mediate the exchange of expression cassettes completely excluding the prokaryotic vector sequences (Figures 2 and 4). Therefore, such a procedure avoids the negative effects these vector sequences can exert [cf. Townes et al. (1985)], leading to expression levels that are a true reflection of the chromosomal surroundings. Moreover, if a favorable chromosomal site has been detected by screening procedures or by the use of retroviral vectors, these sites can be reused.

Using a single loxP1 site, Fukushima and Sauer (1992) have clearly demonstrated that the utilization of distinct integration loci has the potential to improve the procedures that are presently used for reverse genetics, and it is anticipated that this will also pertain to the properties of production cell lines both regarding reproducibility and stability. We have initiated the present studies to gain a better insight into the function of putative chromatin-domain bordering elements, the so-called SARs (scaffold-attached regions) which for their rigorous characterization require the absence of certain vector sequences. SARs are thought to shield a transgene from the transcriptional influences of the surrounding chromatin (Schlake et al., 1994). We are therefore in the process of (i) identifying and marking integration sites of a low or high transcriptional potential and (ii) targeting these with non-SAR and SAR constructs in order to demonstrate the amount of shielding that can be achieved. Such an approach circumvents the problems of the conventional techniques (mostly based on multicopy integration events) that have been applied to this end [see discussion by Dillon and Grosfeld (1994) and Wolffe (1994)].

As a prerequisite we have studied the function of FRT spacer mutants using a full 48-bp site and an *in vivo* assay. To our knowledge, comparable *in vivo* conditions have only been

applied for yeast (McLeod et al., 1986) whereas the bulk of studies was based on *in vitro* systems applying a minimal 34-bp site. A comparison of these two lines of data suggests that the *in vivo* system is more tolerant toward the effects of mutations in the sense that all variants (F₁–F₅) listed in Table 1 are fully functional (Figure 3c, lanes 1–6) whereas the discrimination between different sites strictly parallels their difference.

What are the alternatives that could be used for an exchange of expression cassettes analogous to Figure 2? While there is no precedent in mammalian cells, some observations have again been reported for yeast, mostly using the transient expression approach. We have already mentioned the use of two sets of identical sites which results in problems of yield and stability (Sauer & Henderson, 1990). The application of identical but inversely oriented sites has also been studied, in which case one of two possible excision reactions yields the desired product (Saveliev et al., 1993). This scenario suffers from the fact that there is a competition between integration and inversion reactions which would lower the yield of the desired exchange product. Finally, one could consider the successive use of two recombinases (FLP and cre) together with the respective sites (FRT and loxP1). The approach would enable a separation of the integration (A → B in Figure 2) and excision steps (B → C transition in the same scheme). While this situation could be advantageous regarding the potential yields, it would involve a tedious procedure and necessitate several additional screening steps.

ACKNOWLEDGMENT

We appreciate the stimulating discussions of this subject with our colleagues Michaela Stengert and Dirk Schübeler

(GBF Braunschweig). Particular thanks are due to Karin Maass for expert technical assistance.

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