Double-Reciprocal Crossover Mediated by FLP-Recombinase: A Concept and an Assay[†,‡]

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ABSTRACT: FLP recombinase induces a double-reciprocal crossover event between sets of different FLP recognition target (FRT) sites. Therefore, if these sites flank an expression cassette at a given genomic locus, it can be exchanged for another cassette that has been constructed in the analogous way [Schlake & Bode (1994) *Biochemistry 33*, 12746−12751]. Here we demonstrate that an integrated expression cassette, flanked by a wild type and a mutated site, remains completely stable in the presence of constitutive FLP activity, obviating the need for a timing of this parameter. Therefore the only variable left for optimization is the initial concentration of the exchange plasmid. Since the exchange plasmid lacks a promoter, random integration is not expected to confer resistance to the selection marker, the expression of which requires the acquisition of the SV40 promoter provided at the predetermined integration site. Due to the presence of a luciferase reporter in a specific bicistronic expression cassette, recombination generates bioluminescence upon recombination, indicating the extent of the exchange reaction. This principle is utilized to compare the potential of various cell lines to support the exchange reaction and to adjust the optimum parameters.

The functions of certain regulatory elements are difficult to approach by current gene transfer techniques. This is particularly true for elements which cannot be investigated by transient expression assays as they become active only after their linkage to genomic sequences and after packaging into authentic chromatin structures. Scaffold- or matrix-attached regions (S/MAR elements) which are thought to constitute the borders of independently-regulated gene domains can serve as a paradigm (Bode et al., 1995). After the integration of S/MAR constructs interferences can arise from the common integration of multiple copies in the form of concatenates, and this may be due both to a limited set of available S/MAR binding sites on the nuclear matrix and to unwanted S/MAR-S/MAR interactions (Bode et al., 1996). Moreover, information is commonly derived by averaging expression data for several hundred clones to cancel out position effects, while the information from a few clones with predefined integration sites would be clearly superior to solve the relevant questions.

The finding that FLP recombinase, originally encoded by the 2μ plasmid of *Saccharomyces cerevisiae*, can be introduced into mammalian cells to perform site-specific recombination reactions (O'Gorman et al., 1991) has enabled entirely new concepts to overcome these limitations [cf. Walters et al. (1996) and reviews by Kilby et al. (1993) and Sauer, (1994)]. The enzyme excises any piece of DNA that is flanked by two FLP recognition target (FRT)[1] sites of identical orientation, and, even more important, it also performs the reverse reaction, i.e., integration of an FRT-labeled plasmid into an FRT tag placed in the genome (Schlake & Bode, 1994, and references therein). For thermodynamic reasons, the monomolecular excision event is largely favored over integration which has to be driven by an excess of the second recombination partner and by a precise timing of recombinase activity to disfavor the re-excision of integrants [cf. Logie and Stewart (1995)].

Recently, we could extend the system's use by the simultaneus application of wild type and mutagenized FLP recognition target (FRT) sites by which a double-reciprocal crossover event, i.e., a recombinase-mediated cassette exchange (RMCE) reaction is induced between two pieces of DNA that are each flanked by the same set of sites [Schlake and Bode (1994) and below]. The present communication refines the conditions for such an exchange reaction which proceeds to equilibrium in the presence of constitutive FLP activity. A novel assay is developed to adjust the optimum parameters.

There are several site-specific recombination systems available and these are used with different success for different cell types. While the cre/loxP1 system from bacteriophage P1 is preferred for embryonal stem (ES) cells in which FLP/FRT has caused problems (Barinaga, 1994; Westerman & Leboulch, 1996), the latter system has proven superior in certain other cultured cell lines (O'Gorman et al., 1991). These observations call for a convenient test by which the recombination potential of various cell lines can be assessed. While a colorimetric in vitro test is available for a classical excision reaction, so far the efficiency of an

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[1] Abbreviations: CMV, cytomegalovirus; FRT, FLP recognition target; HygTK, fusion gene encoding hygromycin-B-phosphotransferse and HSV-thymidine kinase; IRES, internal ribosome entry site; PAC, puromycin-*N*-acetyltransferase gene; RMCE, recombinase-mediated cassette exchange; SEAP, secretory alkaline phosphatase gene.

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authentic integration is generally established indirectly by referencing the number of specific integrants to the total number of drug-resistant clones which arise both by specific and random integration processes [blue−white screening, see O'Gorman et al. (1991)]. This assay is largely restricted to cell lines for which it is possible to establish an accessible single-copy target, which is most conveniently done by a retrovirus-mediated infection pathway (Schübeler, 1997, and unpublished).

We will demonstrate that for the RMCE approach testing is straightforward since our exchange vectors contain a promoterless luciferase reading frame which comes under the control of an SV40 promoter only in case of a correct insertion. This test provides information about the potential of a cell line to support RMCE and about the equilibrium which is established between the respective expression cassettes.

MATERIALS AND METHODS

(a) FRT mutants were as described by Schlake and Bode (1994). For mutant F_3 the wild type spacer (TCTAGAAA) was mutated, by PCR-based mutagenesis, to TTCAAATA and for mutant F_5 to TTCAAAAG.

(b) Plasmids. "pΔP" plasmid with a wild type FRT site (F) and a mutant (F_3) flanking a cassette from the luciferase and the puromycin genes. The F_3 site was inserted as a 80 bp *Sma*I-*Hin*dII fragment (Schlake 1994) into the pMCLuPF2 vector opended by *Bst*1107I (D. Schübeler, unpublished). The CMV promotor was removed by excision (*Afl*II/*Xho*I), a filling-in reaction, and religation.

pΔP-pA: pΔP was cleaved by *Ssp*I and ligated to the SV40 polyadenylation signal from pSBC2 (Dirks et al., 1993) which had been excised by *Sma*I/*Bst*1107I.

pΔP-pA$_i$: derived in a way analogous to pΔP-pA but inverting the pA-signal.

pΔP$_i$: plasmid with an inverted backbone relative to pΔP-pA$_i$. The backbone of pSBC2, cleaved by *Cla*I, filled in, then cleaved by *Sma*I, and was ligated to the *Xmn*I/*Ssp*I fragment from pΔP which contains the genes and the FRTs.

pP$_{control}$: prepared by ligating the SV40 enhancer/promotor from pSBC2 (*Not*I/*Ssp*I) into the pΔSLP vector which had been opened by *Not*I/*Ssp*I.

"pP" refers to plasmid with a wild type FRT site (F) and a mutant (F_3) flanking the bicistronic cassette containing the secreted alkaline phosphatase (SEAP), linked by an IRES element to the HygTk fusion gene (prepared from sequences encoding hygromycin resistance and tymidine kinase). The bicistronic cassette is under the control of the SV40 promotor: The *Not*I/*Xmn*I fragment of pSBC1-SEAP (Dirks, 1994) was ligated to the *Not*I/*Xmn*I fragment of pTCH (Kuhnert et al., 1996) including HygTk. The F_3-site was inserted as a 80 bp *Sma*I/*Hin*dII fragment, excised from pBSIIF3 (Schlake, 1994) into the *Bst*1107I site 3′ of the bicistronic cassette. The 5′ F site, excised from pMCLuPF0 (Schübeler, unpublished), was ligated to the vector that had been linearized by *Xba*I.

For plasmids FHygTkF, F_5HygTkF, F_3HygTkF, F_5NeoF, and F_3NeoF, see Schlake and Bode (1994).

(c) Cell Culture and Gene Transfer. For stable expression, BHK cells were seeded at a density of 1×10^5 per 9.1 cm^2 plates. 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.

Transfection (Stable Expression). DNA precipitates were prepared as follows: 1 μg of the plasmid was mixed with 25 μL of 2.5 M $CaCl_2$ and diluted to 250 μL. The plasmid was linearized if it was to be integrated to serve as the parent construct (Mielke et al., 1990). The suspension was added, on a Vortex mixer, to 250 μL of 2× HEBS (280 mM NaCl, 50 mM HEPES, 1.5 mM Na_2HPO_4, pH 7.1), and 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.

Transfection (Transient Expression). 1×10^5 BKK, NIH 3T3, CV-1 or 293 cells were seeded per 9.1 cm^2 plates, respectively. Four hours prior to transfection, the medium was changed and transfection was performed as above but using supercoiled plasmids and a corresponding amount of a reference plasmid encoding the human β-galactosidase gene under the control of the human β-actin promoter. 48 h later cells were collected and DNA was prepared and subjected to either luciferase/β-galactosidase measurements or to PCR analyses as described below. Deviations of β-galactosidase from an average value were taken to reflect the variability of the cell's competence to process DNA and were taken into account if the correction factor was below 2. Experiments requiring larger corrections were repeated [see Klehr et al. (1991)].

Electroporation. Logarithmically growing, semiconfluent cells were trypsinized, washed in PBS (137 mM NaCl, 2.7 mM KCl, 8 mM sodium phosphate, 1.5 mM potassium phosphate, pH 6.8), and collected by centrifugation (400*g*, 5 min). The cellular pellet was distributed in 500 μL of electroporation buffer (20 mM HEPES, pH 7.05, 137 mM NaCl, 5 mM KCl, 7 mM sodium phosphate, 6 mM dextrose) containing 10 μg of the linearized plasmid and treated in a Bio-Rad Gene Pulser, typically at 1100 μF and 200 V/cm. After treatment, there remained about 35% of surviving cells as determined by trypan blue exclusion. The cellular pellet was left on ice for 10 min, dissolved in 15 mL of DME/10% FCS, and seeded into a 75 cm^2 flask (day 1). The medium was changed on day 2. The further selection procedure was as for transfection.

(d) Recombination Prior to Integration (Transient State). For recombination, 2 μg of each of the respective plasmids was transfected into BHK cells (in case of Figure 2, 2 μg of the ΔP construct + 2 μg of the P construct + 1 μg of the recombinase plasmid pOG44). After 48 h the cells were processed for determination of luciferase activities. The products of a recombination were analyzed by PCR using the appropriate primers (cf. Figure 4 and below) and verified by restriction analysis.

(e) Luciferase Tests. Extracts were prepared directly from a defined number of cells. Usually, 10^5−10^6 cells were lysed in 300−1000 μL of extraction buffer [0.1 M KH_2PO_4 (pH 7.8) and 1 mM dithiothreitol]. The bioluminescence of 50 μL was quantified in a Berthold Biolumat model LB950l by integrating the output over the first 10 s and correcting this value for the number of cells in the assay.

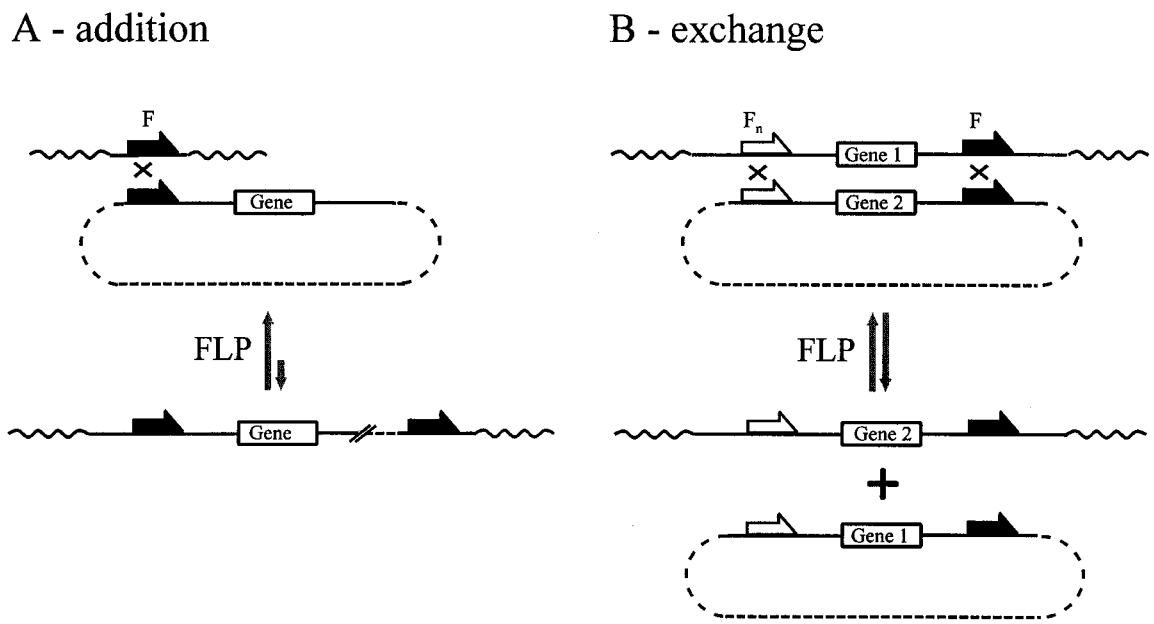

FIGURE 1: Concept: Principle of the cassette exchange reaction (RMCE, part B) as compared to the conventional addition reaction (part A).

(f) PCR Analyses. 2×10^6 cells were washed with PBS, collected in 400 μL of TEP (6 mM EDTA, 0.1% trypsin in PBS), diluted to 1 mL with culture medium, and pelleted by centrifugation (5 min at 500*g*). The pellets were dispersed in 1.5 mL 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 H_2O the solution was kept at 37° overnight. The DNA preparation remaining after inactivating the protease (10 min at 95 °C) was subjected to PCR as follows: 50−150 ng of chromosomal DNA or 1−100 pg of plasmid was supplied with 50 pmole of each of the primers ($^{5'}$CCATGATTACGAATTCATCG$^{3'}$ and $^{5'}$-TGTTGGGAAGGGCGATCG$^{3'}$; cf. Figure 5), 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 $MgCl_2$, 0.01% gelatine), and water to a total volume of 45 μL. After the solution was heated to 95 °C (10 min) and to 72 °C (8 min), 5 μL of Taq polymerase (0.6 units) was added. Amplification proceeded during 30 cycles of 94 °C (1 min), 62 °C (1 min), and 72 °C (3 min). PCR products were analyzed on 1.5% agarose gels in TAE buffer. Fidelity of PCR analyses was examined by performing, in parallel, amplifications on the DNA of non-transfected cells and the same DNA with the applicable amount of the linearized vector added. Finally, the identity of amplification products was established by Southern analyses (see Figure 4 for an example).

(g) Northern Blots and Internal Controls. See Schübeler et al. (1996).

RESULTS

The Concept

FLP recombinase mediates a site-specific recombination event between two FRT sites of identical sequence composition. With the appropriate relative orientation and location of these target sites, it is possible to invert, excise, or insert DNA at a given genomic location. For insertion, a genomic FRT site is recombined with an analogous site located on a transiently transfected, circular vector. As a result, the entire vector sequence, including its prokaryotic part, will be integrated in a process called a "additive recombination" (Figure 1A). During the lifetime of FLP recombinase, which is usually generated by transient expression from a separate plasmid, integration is easily reversed since the monomolecular excision reaction is sterically and entropically favored over integration. Subsequent attempts to enrich for authentic integration events usually also reveal the presence of nonspecific insertions since these also mediate resistance to a linked selection marker.

In contrast, an exchange reaction (RMCE, cf. Figure 1B) is based upon the simultaneous application of a wild type FRT site (F) and an FRT that had been mutated in its 8 bp spacer (F_n). The spacer is involved in DNA−DNA pairing during strand exchange, and thereby it determines the identity and orientation of the site. However, since these particular eight base pairs are not in direct contact with the recombinase, their mutation results in a functional FRT variant which will recombine with a second site of the same composition but not with a wild type one [see Schlake and Bode (1994)]. If sites F and F_n are in a strategically favorable position, they can mediate the exchange of expression cassettes, i.e., a double-reciprocal crossover event which precisely deletes prokaryotic vector sequences.

While the feasibility of this concept could easily be demonstrated by PCR techniques (Schlake & Bode, 1994), the subsequent isolation of clones that had undergone the desired exchange proved to be tedious owing to the fact that, again, both specific and random integration events had conferred resistance to the selection drug. Attempts to select for authentic exchange events, i.e., for the loss of the HygTk cassette (which would convert gancyclovir to a toxic metabolite by the kinase activity of the fusion gene product) failed due to the bad performance in BHK cells (J. Seibler, unpublished). We therefore developed a positive selection system which strongly favors the expression of correctly integrated constructs. The system involves recombination between a promoter-free (ΔP) and a promoter (P) construct (Figure 2). The P construct contains one of the FRT sites interposed between the SV40 promoter (P_{SV40}) and a bicistronic expression unit consisting of the SEAP and HygTk

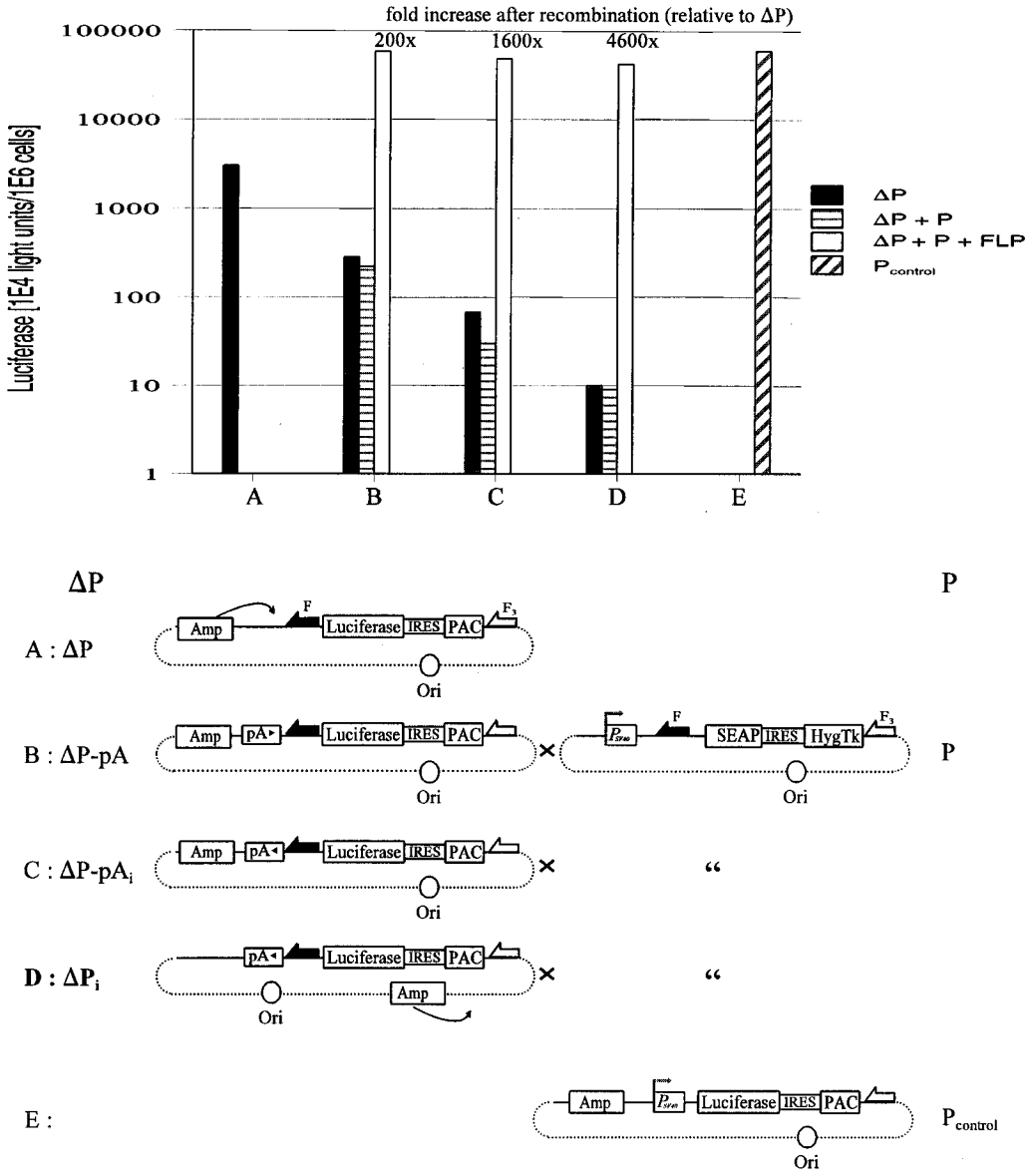

FIGURE 2: Promoter-free reporter plasmids (ΔP) for monitoring site-specific recombination events mediated by FLP recombinase. Construct A (ΔP) demonstrates the necessity of separating the luciferase gene from cryptic promoter functions in the Amp^r gene and construct E ($P_{control}$) is used as a reference for the level of luciferase activity directed by the SV40 promoter in a given cell line. Optimum performance was achieved by recombining ΔP_i with the promoter construct P (situation D) which forms the basis of the test used henceforth. All data are derived from transient expressions in BHK cells (see Materials and Methods).

genes. The promoter-free exchange plasmid (constructs called ΔP in Figure 2) contains a bicistronic luciferase-PAC cassette which should only mediate resistance to puromycin in case it has recombined with the corresponding set of FRT sites present on the P vector. While P is ultimately to be used as the integrated copy which recombines with an incoming ΔP construct, Figure 2 describes a series of pilot transient expression experiments designed to optimize the system.

The luciferase gene in the exchange plasmid provides a sensitive test for monitoring the activity of FLP recombinase in the exchange reaction and at the same time for the co-expression of the selection marker. In the following we will describe the performance of this assay in various common cell lines and we will thereby demonstrate their widely differing potential to support an exchange reaction between sets of FRTs. The test will also be used to monitor the stable, constitutive expression of FLP recombinase in a cell line which has been established for further simplification of the exchange reaction (see below).

The Assay

To be both convenient and quantitative, the assay should meet two major criteria. First, it should be based on transiently transfected reaction partners (i.e., the promoter substrate P, the promoter-free second substrate ΔP, and the FLP expression vector) to be fast and independent of position effects. Second, an expression reference should be provided to enable an estimate about the extent of the exchange.

As an expression reference, we constructed a vector $P_{control}$ which poses luciferase under the control of an SV40 promoter and thereby simulates the state after recombination. The development of promoter free vectors (ΔP series) started with a construct ΔP which was to be recombined with the promoter construct P. Although devoid of an eukaryotic promoter, ΔP exhibited significant expression levels even in the absence of the P plasmid and of recombinase. Since this expression could be eliminated by restriction cuts between the Amp^r (Amp) and FRT (F) portions (not shown), it is obviously directed by some cryptic promoter functions

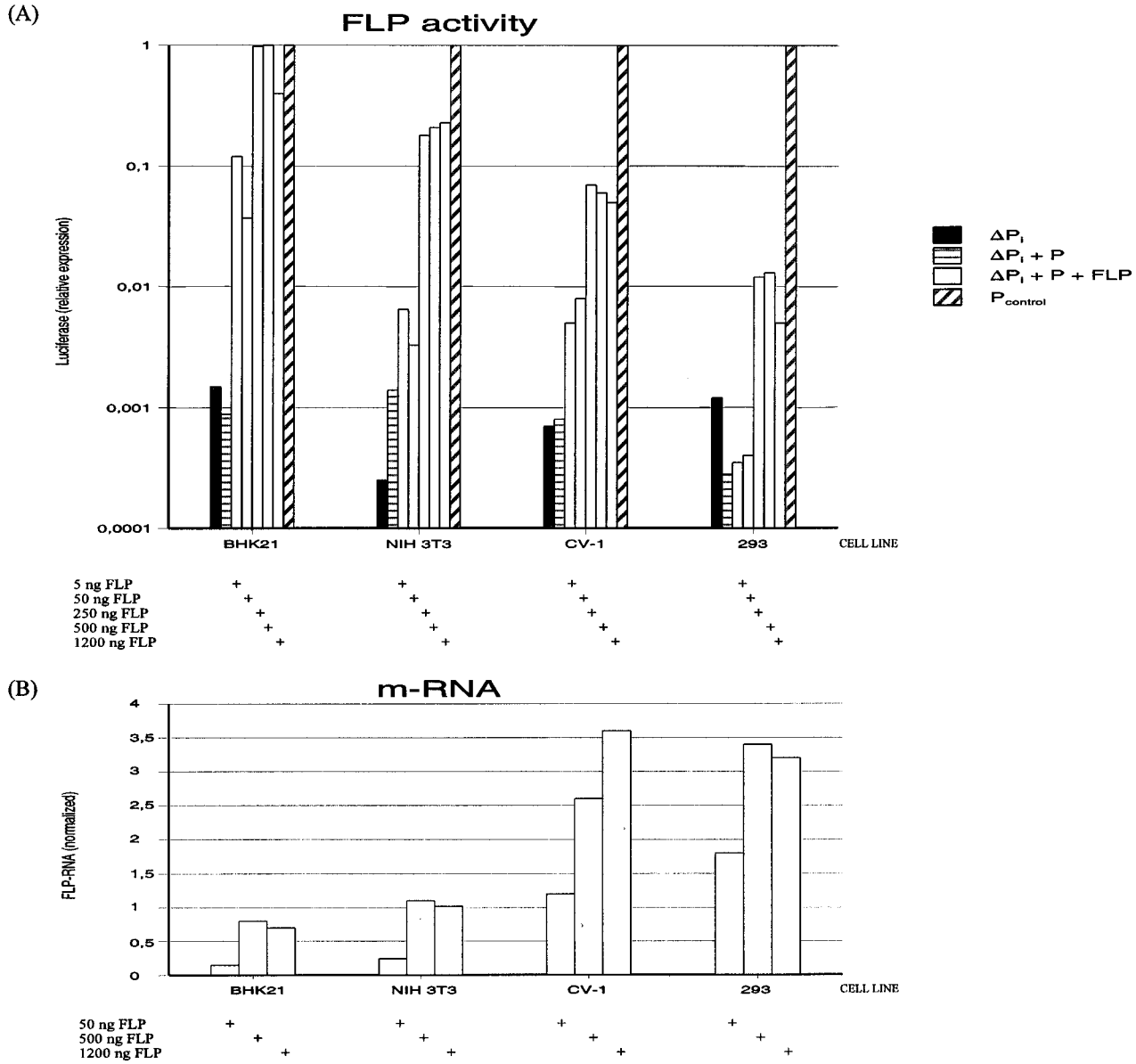

FIGURE 3: Performance of FLP recombinase in various cell lines. (A) Activity data according to Figure 2D. The promoter-free reporter plasmid ΔP_i was transfected either in isolation or together with the promoter construct P plus/minus the FLP expression vector (pOG44). Expression data were referenced to the respective level of an authentic SV40 promoter−luciferase construct ($P_{control}$). (B) FLP-mRNA levels. Four cell lines, transiently transfected with the respective plasmid(s), were subjected to the isolation of RNA which was subsequently quantitated on Northern blots using a signal generated from pyruvate kinase transcripts as the internal standard. Varations in the amount of FLP plasmid were compensated by additions of HMW-DNA.

in the ampicillin-resistance gene. This phenomenon reminds of observations by Boshart et al. (1992) and was solved in the analogous way, i.e., by inserting an SV40 polyadenylation signal between the Amp^r region and the luciferase structural gene. An increasingly better separation was obtained if this signal was inserted in its direct orientation (ΔP-pA), in its inverse orientation (ΔP-pA_i) or by also moving the Amp^r sequences to a different part of the vector (ΔP_i) which is in accord with unpublished observations by A. Fendt (GBF Braunschweig). On the basis of these results (Figure 2), ΔP_i was chosen as the promoterless construct to be used henceforth.

An inspection of the expression level obtained after recombining ΔP_i with P (column D in Figure 2) relative to that monitored for the respective amount of the reference ($P_{control}$) indicates that the equilibrium between the two educts (present at 2 μg each) and the two recombination products is a favorable one. By this token the exchange reaction is clearly superior to an insertion by the conventional addition approach which is easily reversed during the lifetime of the FLP recombinase.

The Exchange Reaction, Studied in Various Cell Lines

We tested a panel of cell lines for their capacity to support an exchange reaction according to Figure 1 but in the transient state. The data of Figure 3A show that only baby hamster kidney (BHK21) cells generated a signal comparable to the $P_{control}$ reference whereas in all other lines recombination turned out to be much less efficient.

BHK is a standard cell line which is well established for biotechnological and basic research purposes and has been used before for FLP-based recombination studies (Schlake & Bode, 1994; Schübeler, 1997). It should be noted that in the transient state these cells mediate, to a minor extent, a recombination even in the absence of FLP ($\Delta P + P$ situation in Figures 2 and 3A) which, however, is clearly surpassed by the cotransfer of an increasing amount of FLP expression vector (bars designated $\Delta P_i + P + FLP$).

Table 1: FLP Activities Obtained by Stable Expression and G418 Selection of/for a Bicistronic Construct Consisting of FLP and Neo^r Reading Frames[a]

Ca-phosphate transfection, pool	500 ± 50×
Ca-phosphate transfection, clones	100−900×
electroporation, clones	50−100×

[a] Activities are expressed in terms of bioluminescence readings relative to blank values from the promoter-free ΔP_i-construct (fold values as defined in Figure 2D).

NIH3T3 is a standard cell line which lends itself to gene transfer by murine ecotropic vectors. Usually, the infection process can be performed under conditions yielding the integration of a single, intact provirus. The incorporation of recombinase target sites into this type of vectors opens an entirely new avenue for manipulating cells and tagging integration sites as targets for possible subsequent integrations (Choulika et al., 1996; Schübeler et al., 1997). Unfortunately, while excisions proceed with eases, the same cells appear highly refractory to integration (Karreman et al., 1996). The reasons for this may be multifold but it is reassuring that the exchange reaction proceeds to a reasonable extent, indicating the potential of FLP recombinase for this type of recombination event.

O'Gorman et al. (1991) have established, in CV-1 monkey cells, a single-copy FRT target for which integrations are obtained with an unparalleled efficiency. Therefore, this cell strain (E25B2) has frequently served for routine tests on the functionality of FRT constructs [see Schlake and Bode (1994)]. The moderate performance of CV-1 cells in the exchange reaction was unexpected. It is therefore concluded that, although these cells offer an unfavorable environment for the expression of FLP activity, a highly accessible target site may be the reason for their successful use. Finally, a significantly impaired performance of FLP in the exchange reaction was monitored for human 293 cells which in the studies by O'Gorman et al. (1991) and Logie and Stewart (1995) had served for a systematic study of excision or integration and excision, respectively.

To learn about the level at which FLP expression is regulated, we have also quantified the steady state concentration of FLP transcripts for the four cell lines by Northern blotting. This study (Figure 3B) showed a relationship that was almost inverse to Figure 3A, and the cell line that did badly support FLP activity (293) was the one generating the most transcripts.

Exchange in the Presence of Permanently Expressed FLP Recombinase

An FLP-mediated addition reaction according to Figure 1A requires sufficient FLP activity during the time the circular recombination substrate is present at a high concentration. When the plasmid's concentration decreases due to its degradation or dilution during the cell cycle, FLP activity has to be terminated to prevent re-excision. In contrast, an exchange reaction (Figure 1B) can only take place in the presence of the exchange plasmid and will simply stop after its dilution, even if FLP recombinase is permanently present. These predictions were verified in the set of experiments shown in Figure 4 and are based on BHK cells which express a stably integrated construct encoding FLP at an activity level 500(±50)-fold above the reference (500×, cf. Table 1). Since the set of vectors developed in Figure 2 was used for monitoring the persistance of this activity over time, the exchange reactions in Figure 4 are based on a different series of constructs and were monitored by PCR methods in a way analogous to Schlake and Bode (1994).

For several clonal strains FLP activity was shown to be stable over the period of at least three month. In order to demonstrate that this activity suffices for an effective exchange, we first performed a series of pilot experiments in the transient state (Figure 4, lanes 1−3). In this milieu, a vector containing two identical FRT sites (F-HygTk-F, abbreviated F-F) readily underwent intramolecular recombination (excision) which caused the complete conversion of the educt into a 250 bp fragment (lane 1). Under the same conditions, a vector F_n-F remained almost stable (lane 2; contribution of the 250 bp fragment <5%) demonstrating a virtual lack of cross-interaction between the wild type (F) and mutated (F_3) FRT sites. If the same vector was cotransfected with a matching plasmid F_n-neo-F, occurrence of the exchange reaction was readily documented by the appearance of a new 2.2 kb band which contributed about 30% to the respective equilibrium mixture (lane 3).

We then addressed the more critical question of whether constructs of the type F_n-F would also be stable after integration and long-term cultivation of the host cell. A corresponding experiment on a plasmid F_5-F is shown in Figure 4 (lane 6) after the host cell line had been cultured for 3 months in the absence of selection pressure, i.e., in a medium not containing hygromycin B. The result clearly demonstrates that this construct remained entirely stable although FLP activity clearly sufficed to induce a complete recombination (excision) between alike sites (see trace 5). To overcome a possible objection that we may have dealt with an inaccessible integration locus, an analogous experiment was set up with a clone for which an initial construct F_5-HygTk-F (scheme in Figure 4, part a) had already been converted to F_5-neo-F (scheme in Figure 4, part b) by the exchange reaction. Since this cassette has been created by the action of recombinase in situ, it has clearly been accessible to the enzyme but, nevertheless, it remained stable in the absence of an exchange partner. The specificity of the major bands could be verified by Southern blotting while a ubiquitous but minor band of approximately 0.5 kb could clearly be identified as a mispriming artefact.

DISCUSSION

Targeted integration into predefined sites with a known genomic location has the potential to solve a major problem of current gene transfer techniques. Traditionally, these techniques tend to yield highly variable expression levels due to chromosomal position effects and copy number variation among different clones. The "addition reaction" (Figure 1A), enabled by site-specific recombinases like the yeast FLP enzyme, is a commonly used bimolecular recombination during which a circular vector is directed to and integrated at an FLP recognition target (FRT) site. This process initiates with the linear alignment of two FRT sites which are localized on the recombination partners and yields a state in which the linearized vector is flanked by two identical FRTs. Unfortunately, this state is unstable, i.e., the reaction readily reverses if FLP reactivity cannot be terminated at a time the concentration of the circular vector begins to drop. In cases of a faulty co-integration of the FLP vector we have experienced a continuous and finally complete loss of the insert (M. Iber, unpublished).

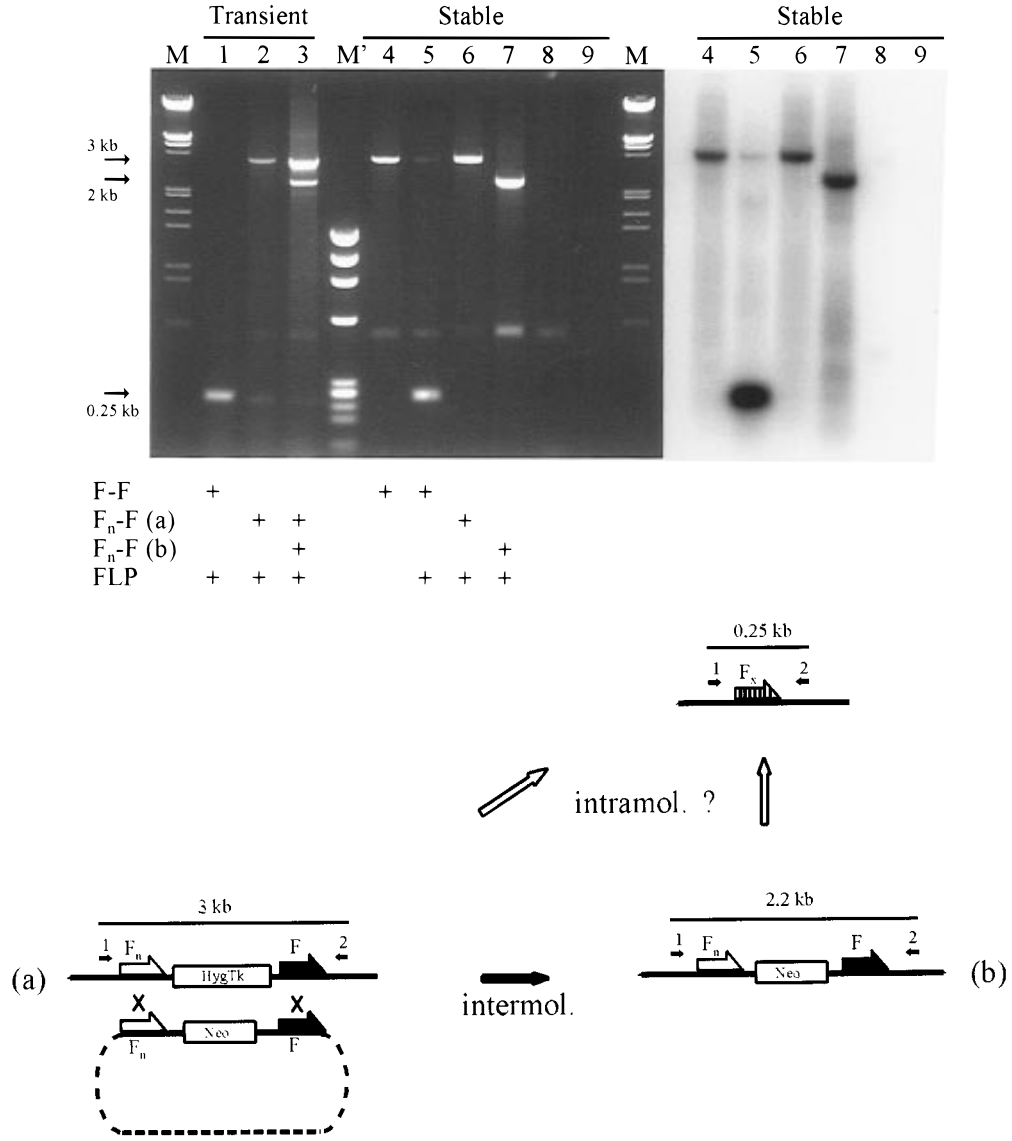

FIGURE 4: Long-term stability of combinations between different FRT sites probed by PCR reactions. Examples for F/F_n combinations in the transient (lanes 1−3) and stable (lanes 4−9) phases of expression. Lane 1 designates an experiment in which F-HygTk-F (abbreviated F-F) has been transfected into a BHK cell line stably expressing FLP recombinase at a 500× activity (see Table 1), lane 2 contains a corresponding experiment for F_3-HygTk-F (F_n-F) and lane 3 an exchange reaction for two plasmids of the F_n-F-type (F_3-HygTk-F and F_3-Neo-F according to situation "a" below). Lane 4 demonstrates the stability of F-HygTk-F transfected into "empty" BHK cells (not expressing FLP); Lane 5 is the corresponding experiment after stable transfer of an FLP expression vector. Lane 6 refers to an integrated copy of F_5-HygTk-F (F_n-F), kept for 3 months in the absence of selection pressure and in the presence of FLP recombinase, continuously expressed at 500× activity. Lane 7 shows a corresponding experiment on a cell clone subsequent to an exchange reaction (situation "b"). Lane 8 shows the background due to a PCR reaction on DNA isolated from "empty" (non-transfected) cells, and lane 9 is a water control. Signals specific for the exchange reaction have been verified by hybridizing a Southern blot of the agarose gel with a 0.18 kb probe with the approximate composition of the 0.25 kb fragment covering the F_x site above. This is shown exemplarily for the part of the gel comprising lanes 4−9 (righthand autoradiogram). Markers are a *Hin*dIII-*Eco*RI digest of λ-DNA (M) and a *Hae*III digest of φX174-DNA (M').

We have recently overcome this problem by constructing expression cassettes, each flanked by the same set of different FRTs (called F_n and F in Figures 1B and 4). These sites have been rendered incompatible by introducing mutations into the spacer region (Schlake & Bode, 1994). This novel approach has at least three obvious advantages:

(i) The "recombinase-mediated cassette exchange" (RMCE) reaction is always bimolecular, and it is only driven by the availability of the exchange plasmid. It permits a highly efficient recombination with permanent exchange of the two cassettes flanked by F and F_n sites because, once integrated, the cassette is virtually locked in place as the excised portion arises at a vanishingly low concentration;

(ii) Given the stability of F_n-F constructs in the integrated state (Figure 4), FLP may be expressed constitutively. Once a cell line with an appropriate level of FLP activity is available, the concentration of the incoming plasmid becomes the only variable in the transfection system. Moreover, a cell line with a continuous level of FLP activity enables recombination to occur at all stages of the cell cycle and it will eliminate accessibility problems which might otherwise arise during the restoration of a compact chromatin structure subsequent to S phase;

(iii) During the exchange reaction prokaryotic parts of the exchange plasmid can be precisely deleted by an appropriate positioning of F and F_n sites. Therefore, cis-effects of prokaryotic sequences which would arise from the activity of cryptic promoters (cf. Figure 2) or the presence of "poison sequences" can safely be prevented.

For the addition reaction, the equilibrium is strictly in favor of the educt side and the isolation of any products requires a stringent selection system. Among the selected clones there will always be a proportion which gained resistance due to randomly integrated copies. Random co-integration also causes problems as it would affect any interpretation of expression data which are thought to arise from integration at the predefined location.

Although one faces the same category of problems when using the exchange reaction, here the standard free energy change is not expected to favor either the educt or product side, respectively (see Figure 2). The reaction can be driven by a molecular excess of the exchange plasmid although thereby the risk of random integration events is increased. For these reasons, we have improved the method by omitting the promoter from the exchange plasmid such that random integration rarely if ever leads to expression of the selection marker whereas site-specific integration brings the incoming exchange vector under the influence of the promoter that pre-exists on the parent construct.

The performance of this system can easily be monitored since our promoter-free (ΔP_i) construct consists of a bicistronic cassette with a reporter (luciferase) and a selector gene (PAC, puromycin-*N*-acetyltransferase). Alternatively, the test can also be exploited to monitor the FLP activity that arises in the transient state or after stable integration (Figure 3A and Table 1). Studies of this type have led to the unexpected conclusion that cell lines support the activity of the FLP enzyme to a strikingly different extent and that the major level of regulation occurs post-transcriptionally (Figure 3). Relative to the $P_{control}$ construct, clearly the highest activity is found in BHK cells.

For these reasons BHK cell lines have been established which stably expresses the enzyme and it has been demonstrated that basal activities of 500−900× (Table 1) suffice to perform exchange reactions prior to and after integration of the parent construct (Figure 4). In the integrated state, there is no crossinteraction between wild type (F) and mutated (F_3 or F_5) sites and thereby an intramolecular recombination (excision) is safely prevented. We have hence demonstrated that it is possible to change a parent cassette solely by transfecting a circular vector that contains another cassette of choice. Potential uses of this system are multifold. First of all, it becomes possible to create reference integration sites and to characterize them regarding their expression potential and long-term stability via a convenient reporter. Subsequently, the molecular reasons for these properties can be established by recovering genomic flanking sequences by inverse PCR (iPCR) techniques (Mielke et al., 1996). Finally, the cassette can be exchanged for an analogous one, and if the exchange vector is provided with cis-acting sequences, these can then be studied at a given genomic location. This approach also has obvious potential for gene therapy since transgenes can be directed to loci with an established stability and not affecting vital genomic functions. A favorable side aspect arises from the fact that a repetition of identical target sites as it occurs during the common head-to-tail integration of transgenes is also recognized by the enzyme and hence reduced to the level of a single remaining cassette [M. Iber, unpublished; see also Lakso et al. (1996)]. This process automatically creates a stable situation which safely excludes phenomena commonly known as "co-suppression effects" (Bode et al., 1996).

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