DNA Cassette Exchange in ES Cells Mediated by FLP Recombinase: An Efficient Strategy for Repeated Modification of Tagged Loci by Marker-Free Constructs†

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ABSTRACT: The repeated modification of a genomic locus is a technically demanding but powerful strategy to analyze the function of a particular gene product or the role of cis-regulatory DNA elements in mammalian cells. The initial step is "tagging" a site with a selectable marker which is done by homologous recombination (HR) to modify a known locus or by random integration to study cis-regulatory elements at a reproducibly accessible genomic location. The tag is then used to target the construct of choice during an exchange step. Presented here is a novel technique in which the exchange is independent of HR and does not introduce vector sequences. It relies on our previous studies on the replacement of DNA cassettes by FLP-recombinase, whereby some common limitations can be overcome. To this end, the tag, a *hygtk* positive/negative selection marker, is integrated into the genome of embryonic stem (ES) cells. This marker is flanked by a wild-type Flp-recognition target (FRT) site on one end and by a modified heterospecific FRT site on the other. Successful Flp-mediated replacement of the *hygtk* cassette is enriched by ganciclovir (GANC) selection for cells that lack the encoded fusion protein. Thereby, the *hygtk* gene can be exchanged for virtually any sequence in a single efficient step without the need of introducing a positive selectable marker. The system can hence be used to analyze the function of either a gene product or regulatory sequences in ES cells or the transgenic mice derived thereof.

The availability of techniques for generating transgenic mice from genetically modified embryonic stem cells provides a means to analyze specific gene functions or cis-regulatory DNA elements in normally developing mammals. So far, most studies have created null mutations in order to assay the function of a gene product. During such a "knockout" experiment a coding region is replaced or disrupted by a selectable marker gene using homologous recombination techniques. Current extensions of this approach are aimed at more subtle, multiple and independent changes either in the gene product or the DNA sequences regulating the locus, a concept referred to as a "knock-in".

Efficient repetitive modification of a particular locus is desired not only in association with HR to introduce subtle mutations in a known locus, but also for randomly integrated constructs where the expression characteristics of each integration site can be exploited to express transgenes in a defined and predictable manner. So far, the study of cis-regulatory elements that are randomly integrated into cell lines has been limited by the powerful effects of genomic position on transcription of the integrated construct. Transient transfection, the available alternative, is clearly unable to analyze elements that are functional only after integration into the genome (*1*). Every advanced strategy for introducing secondary modifications into predefined loci is based on first tagging the locus with a selectable marker. Once the initial tag has been inserted there are systems available that facilitate secondary modifications to the site, but each of these systems has certain limitations.

Limitations of Present Systems and How They Are Remedied. Two types of procedures have been described for repetitive modifications of genomic loci; one is based on HR and the other on site-specific recombination (SR). In the first case, the efficiency is limited by the frequency of the HR[1] which differs widely among loci and cell lines. Moreover, common procedures such as the "plug-and-socket" approach (*2*) leave behind a selectable marker causing a potential problem since an increasing number of examples is reported where the presence of an expressed selectable marker deregulates a locus (*3*−*8* and references therein). Finally, HR cannot be used for modifying sites tagged by random integration unless these have been cloned and characterized.

Published methods based on SR circumvent some of these limitations. Up to now, however, all SR-based systems also leave behind a selectable marker, and if a single recombinase site is used as a target, unwanted vector sequences are inserted. The technical goal we have pursued here is the

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[1] Abbreviations: FRT, FLP-recombinase target site; GANC, ganciclovir; HR, homologous recombination; HYG, hygromycin; RMCE, recombinase-mediated cassette exchange; SR, site-specific recombination.

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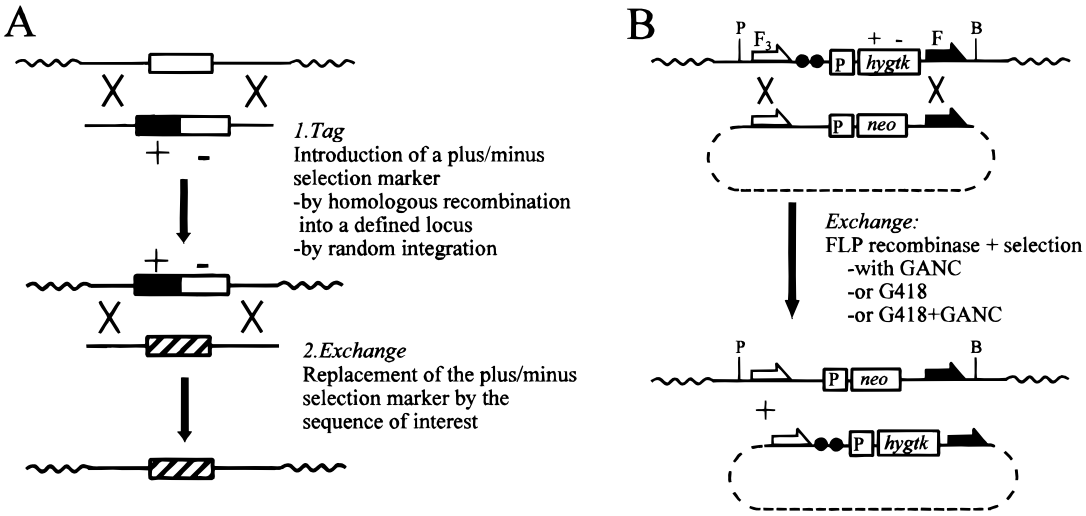

FIGURE 1: (A) Outline of a tag-and exchange experiment. (B) Use of the Flp-RMCE to exchange a genomically anchored plus/minus selection marker (*hygtk*) for an expression cassette. This can be done by only selecting against the presence of the negative marker, by only positive selection and by a combination of both. Half arrows mark the positions of heterospecific FRT-sites (solid, wild type; light, mutant F_3). Circles next to the F_3 site indicate two copies of the polyoma enhancer (see Materials and Methods). Note that the target F_3*hygtk*F but not the exchange construct F_3*neo*F contains the P (*Pvu*II) and B *(Bgl*I) sites next to the FRT sequences. These sites have to be maintained during an authentic, FLP-mediated cassette exchange.

development of an SR-based strategy for making repeated modifications to a given locus that would have the following characteristics: (1) no requirement for an incoming selectable marker; (2) independence of HR; (3) no insertion of vector sequence; (4) high efficiency; (5) functional in different genomic locations. These requirements can be met by Flp recombinase in conjunction with sets of heterospecific Flp recombinase target (FRT-) sequences. Using an incoming positive selection marker, the principle has previously been used for Flp (*9, 10*) or, more recently, for the Cre recombinase (*11*), and it is now commonly termed "recombinase mediated cassette exchange" (RMCE).

The RMCE Reaction. Recombinases such as Flp and Cre have emerged as powerful tools to manipulate the eukaryotic genome (*12*−*14*). These enzymes mediate a recombination between two copies of their target sequence and have mainly been used for deletions. We show here that Flp-RMCE can be applied to introduce secondary mutations at a locus which has been previously tagged by a positive/negative selectable marker and that these secondary mutations can be produced without depending on a selectable marker on the incoming DNA. Flp-RMCE utilizes a set of two 48 bp Flp target sites, in this case, wild-type (F) and F_3, one out of two efficient mutants that emerged from a systematic mutagenesis of the 8 bp spacer localized between the Flp binding elements (*9*). Flp effects recombination between the F_3/F_3 couple which is as efficient as between the wild-type sites (F/F) but it does not catalyze recombination between an F/F_3 pair (*10*). Thereby Flp-RMCE enables the specific exchange of an expression cassette in the genome which is flanked by an F_3-site on one end and an F-site on the other for an analogous cassette which is provided on a plasmid (Figure 1). Nothing else in the genome is altered and no plasmid sequences are inserted. In contrast to approaches using a single recombination site, the targeting product is stable even under the permanent influence of the recombinase unless it is exposed to an exchange plasmid (*10*).

Here, we report use of Flp-RMCE to modify random genomic loci in ES cells. In all three individual sites examined, this modification can be efficiently done by only selecting for the loss of the *hygtk* positive/negative selectable marker (Figure 1B). Since there is no functional requirement for the incoming DNA, except for the two different FRT sites, this strategy permits an efficient marker free targeting of a tagged locus in a single step.

MATERIALS AND METHODS

Plasmids. Construction of plasmids F_3*hygtk*F and F_3*neo*F precisely followed the procedures described for F_5*hygtk*F and F_5*neo*F (*9*), but using the F_3 spacer sequence TtcAaAtA. Homologies between the target (F_3*hygtk*F) and the exchange vector F_3*neo*F were kept at a minimum in order to discourage homologous recombination. Moreover, while F_3*hygtk*F contains specific PCR primer binding sites next to the FRTs, the corresponding sequences have been deleted in case of F_3*neo*F by restriction, fill up, and religation (*9*). These steps led to the loss of the *Pvu*II(P) and *Bgl*I(B) sites in case of F_3*Neo*F proving the site-specific recombination event (see Figure 2 legend).

The *hygtk* gene in F_3*hygtk*F is driven by the HSV-*tk* promoter and two copies of the polyoma enhancer, which together confer robust expression in ES cells (*15*). The *neo*^{r} gene in F_3*neo*F is under the control of the HSV-*tk* promoter only and yields no G418 resistant clones. Therefore, a different construct, F_3P^{pgk}*neo*F, had to be used for positive selection in which the *neo*^{r} gene is controlled by the *pgk*-promoter. For its construction, *pgkneobpA* (a gift of Phil Soriano, Seattle) and pBS2SKF3 (*9*) were cut by *Eco*RI/*Not*I and ligated. The resulting F_3PGKNeo was cut by *Xho*I and cloned into pBS2SKF (ibid) to obtain F_3P^{pgk}*neo*F.

Cell Culture and Transfection. Murine ES cells (CCE) were cultured in DME medium containing 15% FCS, 10^4 units/mL LIF (BRL) and 1.75 ng/mL, monothioglycerol. Cells were grown without feeders on dishes coated with 0.1% gelatine. For transfection, logarythmically growing, semiconfluent cells were dispersed with trypsin, 3×10^6 cells were collected by centrifugation, resuspended in

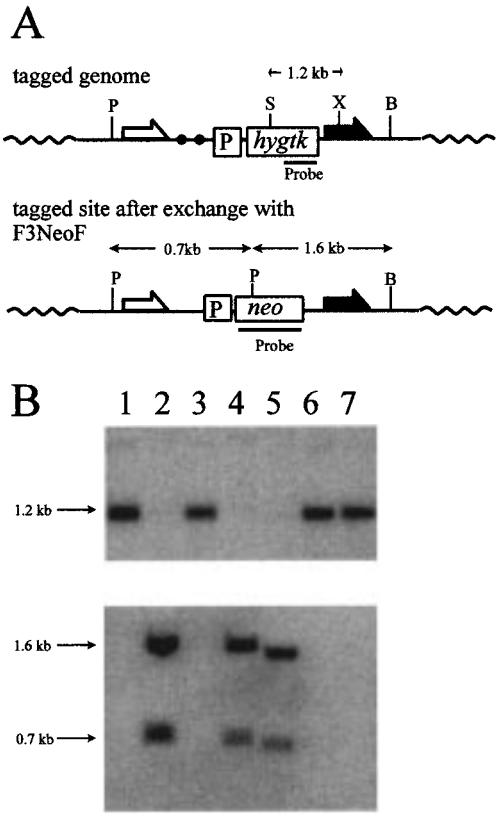

FIGURE 2: Analysis of GANC^{r}clones arising from Figure 1B-type experiments. (A) Restriction maps of a tagged locus before and after the exchange. The *hygtk* and the *neo*-constructs contain the HSV-*tk*-promoter (P) but only *hygtk* contains two copies of the polyoma enhancer elements in addition. Cleavage sites: S, single symbol for the adjacent *Sph*I and *Sac*I sites; X, *Xba*I; B, *Bgl*I; P, *Pvu*II. Presence of a unique copy of F$_3$*hygtk*F was concluded from a single bordering fragment and the absence of a transition fragment on a Southern blot. The blot was prepared from genomic DNA cut with *Sac*I and hybridized to the *hygtk* probe. See Materials and Methods for our definition of bordering/transition fragments. (B) After an RMCE reaction on clone F21 the DNA of subclones was digested at the S and X sites (upper) or B plus P sites and analyzed on a Southern blot. Lanes 1, 3, and 6 show clones resulting from spontaneous inactivation of the *hygtk* gene and lane 7 the parental clone. The 0.7 and 1.6 kb bands expected for an authentic cassette exchange are found in lanes 2, 4, and 5. Note that exchange construct F$_3$*neo*F does not contain the P and B restriction sites, respectively (Figure 1B and Materials and Methods). Their maintenance after cassette exchange confirms that this process is driven by specific crossover events between the F and F$_3$ target sites, respectively, and not by HR at vector sequences beyond the P and B sites.

700 μL medium and electroporated with the appropriate plasmid(s) at 500 μF and 250 V/cm using a Bio-rad gene pulser. Electroporated cells were seeded on four 100 mm dishes.

Cell Lines with a Single Copy "Tag". To obtain independent integration events of F$_3$*hygtk*F, 3×10^6 cells were electroporated with 10 μg of the linearized plasmid and medium containing 167 units/mL hygromycin (Calbiochem) was added the next day. About 100 clones could be isolated 10 days post transfection. Presence of single copies was established by Southern blotting. Criteria were the presence of a unique bordering fragment (starting in the integrate and ending in genomic DNA at the integration site) and the absence of a transition fragment (starting in the integrate and ending in its next neighbor) which would be characteristic for tandem integration events. The procedure can be deduced from the Figure 2A.

Table 1: Spontaneous Resistance[a]

	F8	F18	F21	F22	F1	F5	F9	F19	F20	F23
-HYG	0	0	0	0	30	100	1000	500	20	40
+HYG	nd	nd	nd	nd	0	2	300	80	0	0

[a] Number of spontaneously resistant clones arising from 10^4 cells kept in 3 μM GANC selection for 7 days. −HYG, cells have been cultivated in normal medium prior to GANC selection. +HYG, cells have been cultivated for four passages in the presence of HYG prior to GANC selection.

RMCE. A total of 100 μg of recombinase expression plasmid *flp* (16) and 30 μg of F$_3$*neo*F (or F$_3$P^{pgk}*neo*F) were co-electroporated as circular plasmids into cell clones with a single copy of F$_3$*hygtk*F. Negative selection in 3 μM of ganciclovir (Cymeven, Syntex, Germany) was applied on day 4 or 5. Cells were seeded 1 day prior to selection at low densities (10^5 per 100 mm dish) to avoid metabolic cross-feeding. Clones were isolated between day 8 and 10. For F$_3$P^{pgk}*neo*F, RMCE-modified clones could be enriched by negative and/or by positive selection. Positive selection was done in the presence of G418 (500 μg/mL, GIBCO) 1 day after electroporation. For positive *plus* negative selection GANC selection started when the first G418 resistant clones emerged (after about 7 days).

Genomic DNA. Genomic DNA was prepared according to (*17*) with small modifications. ES cells were harvested from 24-well plates, and proteinase K digestion and DNA precipitation was performed in 1.5 mL tubes. Southern and Northern blotting procedures followed (*18*).

RESULTS

Tagging Different Loci. Our experiments are based on a single copy target, F$_3$*hygtk*F, containing the *hygtk* fusion gene which encodes both hygromycin-B-phosphotransferase and HSV-thymidine kinase (HSV-Tk) activities. This marker allows positive selection for hygromycin (HYG) resistance and negative selection in the presence of GANC, which is converted into a cytotoxic drug in the presence of HSV-Tk. The *hygtk* cassette is flanked by a wild-type FRT site (F) and a mutant (F$_3$) which together permit the application of the RMCE principle (Figure 1B). F$_3$*hygtk*F was introduced into CCE embryonic stem cells by electroporation and 22 HYG resistant clones were individually analyzed for the number of integrated copies. A total of 15 clones harbored a single copy, i.e., they revealed a unique bordering but they were free from a transition fragment (see Materials and Methods). Prior to RMCE, these clones were subjected to a number of additional tests.

Clones Analyzed for Spontaneous Resistance to GANC. During RMCE, the F$_3$*hygtk*F is exchanged for F$_3$*neo*F or its derivative. Cells undergoing this event loose Tk activity, and thereby, they gain resistance to ganciclovir. However, GANC resistance can also arise from spontaneous inactivation or loss of the *hygtk* gene. Since this loss of Tk activity should be determined by the site of integration, we decided to test a number of clones for the development of resistance in the presence of 3 μM GANC. Table 1 shows the number of GANC^r subclones arising after 7 days from 10^4 cells. Four clones underwent no spontaneous inactivation (F8, F18, F21,

Table 2: Targeting Efficiency for a F_3*hygtk*F target[a]

clone	F18	F21	F22	exchange construct
GANC^r colonies		24	63	F_3*neo*F
	16			F_3P^{pgk}*neo*F
clones analyzed	16	24	26	
clones targeted	6	5	6	
targeting frequency (%)	38	21	23	

[a] Exchange constructs are either F_3*neo*F or F_3P^{pgk}*neo*F as indicated. Note that the experiment for F18, using F_3P^{pgk}*neo*F, is continued in Table 3 with other selection conditions. Number and frequencies refer to targeted clones, isolated from 10^5 cells by negative (GANC) selection.

Table 3: Number and Frequency of Clones Obtained Using Positive and Positive *Plus* Negative Selection

clone	F18	F21	F22	exchange construct
G418^r colonies	38	24	2	F_3P^{pgk}*neo*F
clones analyzed	17	16	2	
clones targeted	4	1	1	
targeting frequency (%)	24	6	(50)	
G418^r + GANC^r colonies	4	20	21	F_3P^{pgk}*neo*F
clones analyzed	4	13	14	
cones targeted	4	7	14	
targeting frequency (%)	100	54	100	

and F22) and were chosen as immediate candidates for an RMCE reaction. F8 showed evidence for a deletion at the 3′ end and was omitted from the study.

The propensity of the remaining six clones in Table 1 to develop ganciclovir resistance varied widely, probably reflecting the different characteristics of the integration sites. Since our study is based on a fusion gene which permits both positive and negative selection, loss of thymidine kinase and hygromycin-B-phosphotransferase activities should be coupled. Therefore, inactivated clones should not accumulate during positive selection. To verify this assumption, these six parental clones were expanded in the presence of hygromycin prior to GANC selection (line "+HYG" in Table 1). Under these conditions, three clones (F1, F20, and F23) yielded no GANC^r subclones. For F5, the number of subclones was reduced 50-fold and for the remaining ones 6- or 3-fold, respectively. These data clearly demonstrate that the rate and mode of inactivation is unpredictable for a given locus. However, the accumulation of inactivated clones can be significantly reduced if a fusion enzyme harboring positive *and* negative selectable activities is used and positive selection is maintained before GANC addition. For our present purposes, we decided to rely on clones F18, F21, and F22 for the following experiments.

RMCE Enriched by Negative Selection. The exchange vector (F_3*neo*F or F_3P^{pgk}*neo*F) was transfected together with the recombinase expression construct (*flp*), both in circular form. Circularity is required for transient expression in ES cells and it also reduces the possibility of random integration. For 10^5 seeded cells, selection with GANC yielded 16−63 resistant subclones from each of the three clonal lines (Table 2) which were analyzed for a successful exchange of the *hygtk* by the *neo*^{r} cassette on Southern blots (see examples in Figure 2). Hybridization with a *neo*^{r} and a *tk* probe revealed 1.6 and 0.7 kb fragments for subclones in which RMCE had taken place (6 for F18, 5 for F21, and 6 for F22). The occurrence of these fragments proves the maintenance of the *Pvu*II and *Bgl*I sites of the original target arguing in favor of the authentic exchange (see Figure 2 legend). A 1.2 kb fragment occurs if RMCE has not taken place (10 subclones of F18, 19 of F21, and 20 of F22). Therefore, the targeting frequency ranged between 21 and 38% (Table 2). These results show that even those clones which developed no spontaneous resistance under the conditions of the Table 1 test system were not absolutely free from this property. An analysis revealed two types of background colonies.

(1) After negative selection, all non-RMCE subclones arising from F21 and F22 still revealed a complete copy of the *hygtk* gene on Southern blots. Northern blot analyses showed that while the parental F21 and F22 clones produced detectable amounts of the *hygtk* transcript, the present ones did not (data not shown). Therefore, epigenetic inactivation of expression appears to happen for certain clones which survive the selection but have retained the *hygtk* gene.

(2) GANC^r subclones from F18 showed no Southern blot signal for the *hygtk* gene. Such a result would be expected for a cross-recombination between the two heterospecific FRT-sites (F and F_3) or for a physical loss by chromosomal aberration. To resolve these alternatives, the blots in Figure 2 were reprobed with a fragment from the vector backbone upstream of the F_3-site. This probe would yield a specific 250 bp *Pvu*II-*Bgl*I signal in case of a cross-recombination. The lack of any signal of this type strongly suggests the loss of the whole vector and argues against a cross-recombination between F and F_3. Therefore, for F18, GANC resistance in the absence of RMCE seems to arise from chromosomal aberration as reported in previous repetitive modification studies (*19*, *20*).

RMCE Enriched by Positive Selection. We next utilized the encoded activity of the *neo*^{r} gene to study details of the exchange reaction and to test the efficacy of Flp-mediated RMCE when positive selection for the incoming marker is applied. This marker was provided on a construct (F_3P^{pgk}*neo*F) which mediated expression of the neomycin resistance gene in ES cells. Although negative selection is to be preferred wherever feasible, the application of a positive selection system extends application of the RMCE principle to sites that either give a high level of spontaneous GANC resistance or that have not been tagged with a positive/negative selectable marker gene. We selected the same three clonal lines used above with G418 after electroporation of the circular Flp expression vector and the F_3P^{pgk}*neo*F exchange construct. In this experiment, subclones were obtained by G418 and G418 *plus* GANC double selection (Table 3). The characterization of these clones is illustrated in Figure 3, taking clone F18 as an example.

In case of the double selection (G418 + GANC), clones which have undergone RMCE yielded a 1.7 kb fragment if hybridized with a *neo*^{r} probe. When reprobed with a *tk* sequence, there was no 1.2 kb band verifying the complete exchange. Figure 3 (lanes 6−9) shows an example obtained after hybridization to the *neo*^{r} and *tk* probes. If the selection was done with G418 only, two classes of subclones were observed (Figure 3, lanes 1−5). Class 1 exhibited a 1.2 kb signal for the *tk* probe and a 3.1 kb (but no 1.7 kb) signal for the *neo*^{r} probe. The 1.2 kb fragment indicates the persistence of the F_3*hygtk*F tag while the 3.1 kb band results from a random integration of the complete F_3P^{pgk}*neo*F

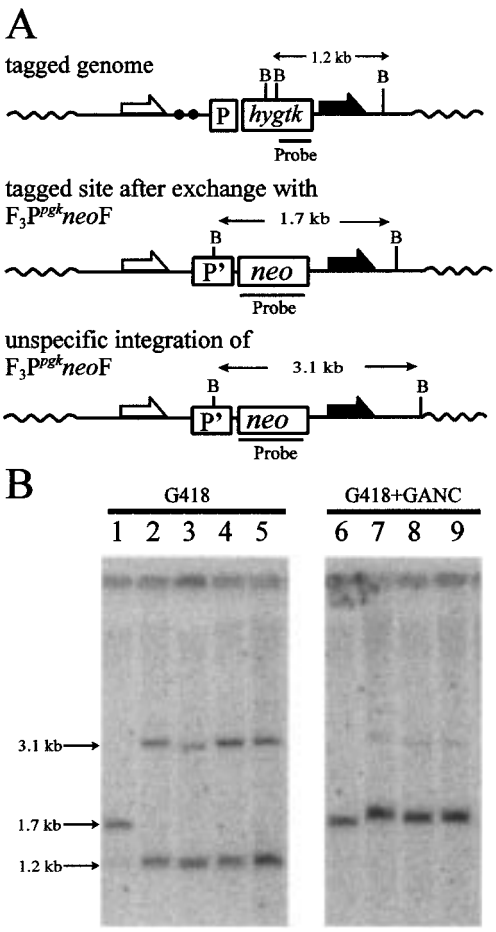

FIGURE 3: Analysis of G418 and G418/GANC-resistant subclones arising from clone F18. (A) Restriction maps (not to scale) of a tagged locus of parent clones and subclones resulting from the exchange (B, *Bgl*I). Here, the *neo*^{r} gene is driven by the *pgk*-promoter (P^{pgk}). (B) Southern blots of *Bgl*I digests. Lanes 1−5 show clones resulting from G418 selection. Persistence of the h*ygtk*-gene is documented by the 1.2 kb signal while the 3.1 kb signal derives from a randomly integrated copy of $F_3P^{pgk}neo$F. Only the exchange leads to an exclusive 1.7 kb signal. Lanes 6−9 derived from clones after G418/GANC selection which all underwent the exchange. Note: there are explanations for each of the minor bands in lane 1 (1.2 kb) and lanes 6−9 (∼3.1 kb). While the 1.2 kb band is lost by continued culture in GANC and thereby represents a sub-population of not yet eliminated cells in which no exchange has occurred, the 3.1 kb band is equally present in any sub-clone regardless of source as well as in nontransfected CCE cells. It is therefore due to an artifact rather than to a second randomly integrated copy of $F_3P^{pgk}neo$F which would otherwise have an intensity comparable to the 1.7 kb band.

construct (Figure 3). Class 2 (one out of the five depicted events) had undergone the authentic exchange as documented by the presence of an 1.7 kb *neo*^{r} signal and the absence of the 1.2 kb *tk* signal. In no case did we observe a recombination at a single FRT site with retention of the *hygtk* gene, a possible result when selection only for G418 resistance is applied. This suggests either that RMCE intermediates which would arise from singular recombination events at the F *or* F_3 sites (*9*) are efficiently resolved by F/F or F_3/F_3 recombination or that RMCE occurs via a simultaneous double-crossover mechanism as diagramed in Figure 1B.

With positive selection only, the overall efficiency of RMCE was 6% for F21, 24% for F18, and one out of two for F22 (Table 3). This difference between the sites may reflect the characteristics of the different chromosomal locations of the *hygtk* tag or a different probability of random integration for the four clones. On the other hand, the targeting efficiency for positive *plus* negative double selection was either 54 or 100% (Table 3). Such an improvement arises because clones which owe their G418 resistance to a randomly integrated copy still contain the *hygtk* gene and are therefore eliminated with GANC.

DISCUSSION

We have demonstrated the efficient recombinase-mediated exchange of a cassette that has been randomly integrated into the genome of ES cells for a vector-borne cassette of the same type. This exchange is achieved by the Flp-RMCE system, which does not insert plasmid sequences and does not depend on the frequency of HR. Since RMCE is possible without positive selection for the incoming vector, this system permits great flexibility regarding the composition of the exchange construct. This system also functions using positive selection for the incoming vector while a maximum efficiency is achieved by combining positive and negative selection. Such versatility makes Flp-RMCE unique compared to other reported systems.

The use of HR for the marker-free targeting of an endogenous locus in ES cells has been described previously, and has been named "tag and exchange" (*19*) or "double replacement" (*20*; see Figure 1A). In this strategy, HR was used to tag an endogenous sequence with a *neo*^{r}/HSV-*tk* gene cassette and also for the second step in which a mutation is introduced and selected for by the loss of Tk-activity. In one study (*19*), 1.4% of the GANC^r clones were targeted correctly, and in the other (*20*), the efficiency was 9.6%. According to our complete protocol, higher targeting frequencies can be achieved and it is therefore anticipated that Flp-RMCE will provide an improvement over existing technologies.

The major advantage of Flp-RMCE is the ability to introduce a marker-free construct independent of HR. During the preparation of this manuscript, two papers appeared which applied Cre-based exchange systems with heterospecific lox-sites to cell lines other than ES cells (*11*, *21*). These studies elaborate Cre-RMCE, a system comparable to Flp-RMCE, but they depend on a positive selection trap system in which only the correct targeting event reconstitutes the selected marker. A trap strategy has been reported previously for the modification of ES cells where it was based on HR and therefore restricted to known loci (*2*).

As a prerequisite for the exclusive use of negative selection, the background of GANC resistant clones has to be low. This demands the absolute lack of Flp-mediated cross-recombination between the recombinase targeting sites constituting a set (here, F and F_3) which would otherwise excise the negative marker and lead to subclones surviving negative selection in the absence of the exchange. None of the clones analyzed here had undergone such cross-recombination. So far the experiments reported for Cre-RMCE do not address the question of a possible cross-recombination, because a product of such a reaction yields no surviving clone if a selection trap is used. It will be interesting to learn if our experimental strategy will also be possible for Cre with the currently used loxP mutant which

was derived from a point mutation in the spacer (*22*) or for any other potential heterospecific loxP sites.

A background due to spontaneously resistant clones is inherent in any targeting system based on the exclusive use of negative selection, irrespective of whether SR or HR is used (*19, 20*). In the clones we analyzed, this background was governed by the frequency at which expression of the *hygtk* gene is silenced or by the loss of the construct by chromosomal aberration. We demonstrate that employing HYG selection, and thereby enforcing expression of the *hygtk* gene up to the time point of negative selection, is well suited to reduce the accumulation of inactivated clones (Table 1). Our data also indicate that there exist genomic locations for which RMCE based on negative selection will be inefficient, and it will require extensive further work to determine the frequency at which silencing of the gene or loss of the construct events require to enrich RMCE by a positive selectable marker.

In cases where RMCE events are enriched by negative selection, the selectable marker is removed during the exchange step and thereby reusable for further manipulations. Since there is only a restricted number of selectable markers available for manipulating murine embryonic stem cells, this may be an advantage of its own (*23*). That a clone obtained by selecting for the loss of HSV-Tk activity can contribute to the germ line has been shown in two studies (*20, 24*). Flp-RMCE requires that the set of FRT sites (F and F_3) is present in the genome before and after the exchange. There is no data to suggest that the 48 base pair FRT sites have any activity other than those associated with Flp recombination, and even their insertion between triplet codes conserves a gene's reading frame and expression (*25,* Schübeler and Bode, submitted for publication). Their position within the locus must nevertheless be accounted for by the design of any experiment. For random integration sites, this does not cause any restriction, but for HR tagged sites, care must be taken that FRT sites are situated so as not to interfere with the coding regions of an inserted or endogenous gene.

If the initial tagging is performed by HR, the system permits the introduction of subtle mutations in an endogenous locus or gene product. Performing the initial tagging by random integration allows the selection of tagged expression sites in ES cells for repeated use either to compare different cis-regulatory elements or to construct transgenic mouse lines that express the protein of interest at reproducible levels and in the appropriate tissues. Position effects are a frequent obstacle to this goal, and often the tendency to silence varies according to tissue type. In cases where an expression pattern is typical of some restricted cell type for which no cis-regulatory elements are known, RMCE will permit the identification of appropriately regulated and stable genomic sites which can then be used for a reproducible expression of a construct in the tissue of interest.

The manipulation of ES cells and the subsequent analysis of transgenic mice has led to important insights into gene function and regulation. The development of animal models for human diseases as well as the dissection of gene action and regulation will require combinations of existing and new techniques (*12*). By necessity, gene-targeting experiments subject ES cells to long-term culture during which a progressive loss of totipotency may occur. We demonstrate here that, using Flp-RMCE, two subcloning steps are sufficient to set a genomic tag and to retarget it efficiently without selection for the incoming DNA. This may represent an important addition to the currently available techniques for repetitive genomic site modification.

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