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Site-specific Chromosomal Integration in Mammalian Cells: Highly Efficient CRE Recombinase-mediated Cassette Exchange

Yong-Qing Feng[1], Jost Seibler[2], Raouf Alami[1], Andrew Eisen[1] Karen A. Westerman[3,5], Philippe Leboulch[3,4,5], Steven Fiering[6] and Eric E. Bouhassira[1]*

[1]*Department of Medicine/ Division of Hematology, Albert Einstein College of Medicine Bronx, NY, USA*

[2]*GBF National Research Center for Biotechnology D-38124, Braunschweig Germany*

[3]*Department of Medicine/ Division of Hematology Harvard Medical School Cambridge, MA, USA*

[4]*HST Division, Massachusetts Institute of Technology Cambridge, MA, USA*

[5]*Genetix Pharmaceuticals Inc. Cambridge, MA, USA*

[6]*Department of Microbiology Dartmouth Medical School Hanover, NH, USA*

**Corresponding author*

Expression of experimental constructs in mammalian cells or transgenic animals is difficult to control because it is markedly influenced by position effects. This has limited both the analysis of *cis*-DNA regulatory elements for transcription and replication, and the physiological analysis of proteins expressed from transgenes. We report here two new methods based on the concept of recombinase-mediated cassette exchange (RMCE) to perform site-specific chromosomal integration. The first method permits the exchange of a negative selectable marker pre-localized on the chromosome with a transgene *via* a CRE-mediated double recombination between inverted Lox sites. Integration efficiency is close to 100% of negatively selected mouse erythroleukemia cells and ranges from 10 to 50% in embryonic stem cells. The second method allows RMCE with no selection at all except for cells that have taken up plasmid transiently. While less efficient, this technique permits novel experimental approaches.

We find that integration of a transgene at a given genomic site leads to reproducible expression. RMCE should be useful to develop artificial genetic loci that impart specific and reproducible regulation of transgenes in higher eukaryotes. This should facilitate the analysis of *cis*-regulatory DNA elements governing expression and position effects, improve our control over the physiological effects of transgenes, and accelerate the development of animal models for complex human diseases.

Keywords: recombinase; CRE/Lox; gene transfer; RMCE; chromosomal integration

Introduction

There are many techniques available for stable integration of transgenes in mammalian cells (Kriegler, 1990; Wolf, 1994). However, these methods result in integration at random chromosomal locations of an uncontrolled number of transgene copies that express at levels that generally cannot be predicted or reproduced with precision because of position effects. The inability to control the site of integration, the number of integrated copies and the level of expression of transgenes has impeded progress in studies of both gene expression and the physiological effects of transgenes.

Systems which can perform site-specific chromosomal integration efficiently therefore have wide utility. The first site-specific chromosomal integrations in mammalian cells were based on integration of a single Lox or FRT site on a chromosome, followed by trapping of rare integration events (O'Gorman *et al.*, 1991; Sauer, 1994). These pioneering methods had three limitations: (1) they were quite inefficient; (2) the entire plasmid was integrated; and (3) a positive selectable marker was left in the chromosome after the integration. The low efficiency of these methods is due to the reversibility of the recombination reaction: after integration the transgene is re-excised if the

Abbreviations used: RMCE, recombinase-mediated cassette exchange; MEL, mouse erythroleukemia cells; GFP, green fluorescent protein; Gan^R, gancyclovir resistant; AGL, artificial genetic loci.

E-mail address of the corresponding author: bouhassi@aecom.yu.edu

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two identical Lox sites that flank the transgene recombine with each other. Since the excision reaction is intra-molecular and the insertion reaction is inter-molecular, excisions are favored. To overcome this re-excision problem, we and other research groups have developed a range of new methodologies based on the concept of recombinase-mediated cassette exchange (RMCE) using either the CRE or the FLP recombinases (Bouhassira *et al*., 1996, 1997; Seibler & Bode, 1997; Seibler *et al*., 1998; Bethke & Sauer, 1997).

The target site of the CRE recombinase is a 34 bp sequence that consists of two inverted 13 bp CRE-binding sites separated by an eight base spacer within which the recombination occurs (Hoess & Abremski, 1984). CRE-based RMCE relies on mutated heterospecific Lox sites (L1 and L2) that inefficiently react with each other because they contain sequence changes in their spacer regions (Hoess *et al*., 1986). When a chromosomal cassette is flanked by two such mutually incompatible Lox sites, it can readily be exchanged for another cassette (located on a plasmid) by a double reciprocal recombination (Figure 1(a)).

We previously reported that RMCE can easily be performed in mouse erythroleukemia cells (MEL) if a functional selectable marker is produced by the exchange (Bouhassira *et al*., 1997). We report here two new complementary methods to perform CRE-based site-specific chromosomal integration into a previously tagged genomic site in a single-step without leaving a selectable marker on the chromosome. The first method is based on the use of inverted Lox sites and is the first report of a CRE-mediated cassette exchange that does not depend on a gene trap system with its associated limitations. The second method is the first report of an RMCE that can be performed without any selective marker at all at the genomic site before or after the exchange.

Results

Since the RMCE reaction involves the removal of a cassette at a tagged locus, clones having undergone a site-specific integration should be identifiable by selecting against a negative marker pre-integrated at the target site (Figure 1(b)). To test this idea, we used the CMV-HYTK gene, a gene that can be selected positively by hygromycin and negatively by gancyclovir. A plasmid (pL1-HYTK-L2) containing the CMV-HYTK gene flanked by L1 and L2 Lox sites was created, transfected into MEL cells, and stable transfectants were selected with hygromycin B. Two clones containing a single copy of the L1-HYTK-L2 cassette were then identified by Southern blots and co-transfected with a CRE expression plasmid plus a plasmid containing a green fluorescent protein (GFP) reporter cassette flanked by L1 and L2 Lox sites (pL1-CMVEGFP-L2). Gancyclovir resistant clones (Gan^R) were selected and tested for the exchange of the HYTK by the GFP cassette. Unexpectedly, this approach proved inefficient: all but one of the 24 Gan^R clones analyzed had lost the HYTK gene as determined by PCR or Southern blot but had not integrated the GFP gene (data not shown), suggesting that an excision reaction was occurring between the L1 and L2 sites. Although unexpected, these results do not contradict our previous report that RMCE can easily be performed in MEL cells by reconstitution of a selectable marker, since when the exchanges are selected positively, excision reactions cannot be detected.

To confirm this finding, plasmids containing the GFPuv reporter gene flanked by either two identical L2 sites or by L1 and L2 sites (pL2-GFPuv-L2 and pL1-GFPuv-L2) were created and tested *in vitro* for recombination. After incubation with or without purified CRE recombinase, the plasmids were transformed into bacteria and colonies were scored for the presence or absence of the GFPuv gene by visual inspection under UV light. The percentage of white colonies observed was <1 % when no CRE was added, 28.05(+/− 3.8) when the plasmid with two L2 sites was tested, and 5.49(+/− 0.2) with the plasmid containing the L1 and L2 sites. Restriction mapping and sequencing of plasmid extracted from white colonies confirmed that the loss of the GFPuv gene was due in almost all cases to CRE-mediated recombination (data not shown). This demonstrated that the L1 and L2 sites are not entirely incompatible. A recent report confirmed this finding (Lee & Saito, 1998). The *in vivo* frequency of recombination between the L1 and L2 sites is unknown, but is probably lower than the *in vitro* frequency since RMCE with these two sites is quite efficient (Bouhassira *et al*., 1997).

RMCE with inverted Lox sites

We reasoned that the problem caused by recombination between the L1 and L2 Lox sites could be eliminated by replacing the L2 site with an inverted L1 site (termed 1L), since intra-chromosomal recombination between two inverted Lox sites would lead to inversion of the HYTK gene rather than to its excision, and would therefore not remove the sensitivity to negative selection by gancyclovir (Figure 1(c)). In this system, the cassette should integrate in one orientation in half of the clones and in the reverse orientation in the other half.

To test this novel RMCE strategy, a plasmid (pL1-HYTK-1L) containing the HYTK gene flanked by two inverted but identical Lox sites was created, and MEL cell lines with single integrated copies of this plasmid were produced as described above. Three clones, termed RL4, RL5 and RL6 were selected for further studies. Exchange reactions were performed as above using a plasmid in which inverted L1 Lox sites flank a GFP reporter (pL1-HS234GFP-1L). After the transfection, 12 Gan^R clones per cell line were picked and analyzed by Southern blots. At loci RL4 and RL5, all 12 Gan^R clones tested had undergone an exchange of the

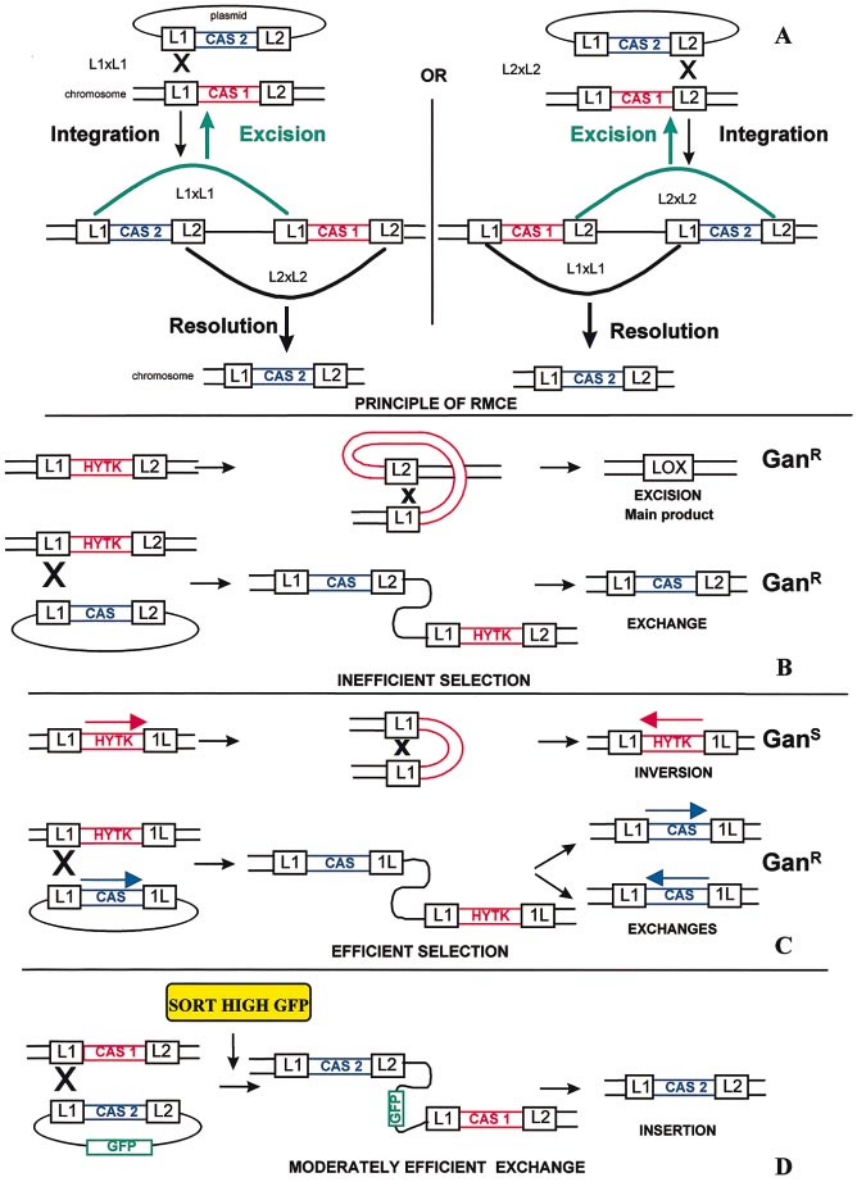

Figure 1. (a) Principle of recombinase-mediated cassette exchange. Site-specific chromosomal integration can be performed by exchanging a pre-integrated chromosomal cassette (cas1) with a plasmidic cassette (cas2). Recombination through the L1 sites leads to plasmid integration; recombination through L2 leads to resolution of the array thus created. The end result of the RMCE reaction is therefore an exchange of cassette 1 by cassette 2. This exchange is stable because the cassette is flanked by Lox sites that cannot recombine with each other. Symmetrical integration through L2 and resolution through L1 also lead to the cassette exchange. After integration the resolution step is in competition with an excision step that is the reverse of the integration step. (b) Selection for the loss of the HYTK cassette to identify RMCE clones is inefficient because the L1 and L2 Lox sites are not entirely incompatible: selection against the HYTK gene yields mostly excision products (upper recombination) rather than exchange products. (c) RMCE with inverted Lox sites. The L2 Lox sites are replaced by inverted L1 sites (1L). Excision products are eliminated because recombination between Lox sites located in *cis* leads to inversion of the HYTK gene rather than its excision (upper recombination). Gancyclovir selection therefore lead predominantly to the identification of exchange products (lower recombination). Exchange products in both possible orientations are obtained. Only one of several exchange pathways is depicted. (d) FACS-based RMCE. A plasmid containing the CMV-EGFP reporter gene inserted outside of the exchange cassette is co-transfected (with a CRE expression plasmid) and cells that express the highest amount of GFP are sorted. Among those cells, RMCE frequency is moderately high. No selectable marker on the chromosome is required before or after the exchange.

HYTK cassette for the GFP cassette (Figure 2(a)). At RL6, 11 out of the 12 clones tested had an RMCE. As expected, about half of the clones were in reverse orientation. These results demonstrate that RMCE with inverted Lox sites can be efficiently selected using only negative selection in MEL cells.

The same experiments were then repeated in an embryonic stem cell line: two single-copy L1-HYTK-1L-tagged loci were created and tested for RMCE using plasmid pL1-CMVEGFP-1L. At the first locus tested, about 50 % of the 60 Gan^R clones tested by Southern blot had undergone an exchange (Figure 2(b)). At the second locus the frequency of RMCE was about 10 %. As in MEL cells, clones in both orientations were obtained in approximately equal proportion.

RMCE with no selectable marker

RMCE with inverted Lox sites is extremely efficient, but an active negative selectable marker has to be present at the integration site prior to the exchange. Since in many situations it would be advantageous to perform RMCE at loci that do not contain any active gene at all, we have also developed a method to perform RMCE that does not require the presence of an active gene before or after the exchange.

When no selection is applied, about 1 % of the MEL cells that survive the transfection undergo a RMCE (data not shown). To increase this frequency we tested the hypothesis that placing a GFP reporter gene in the exchange plasmid outside

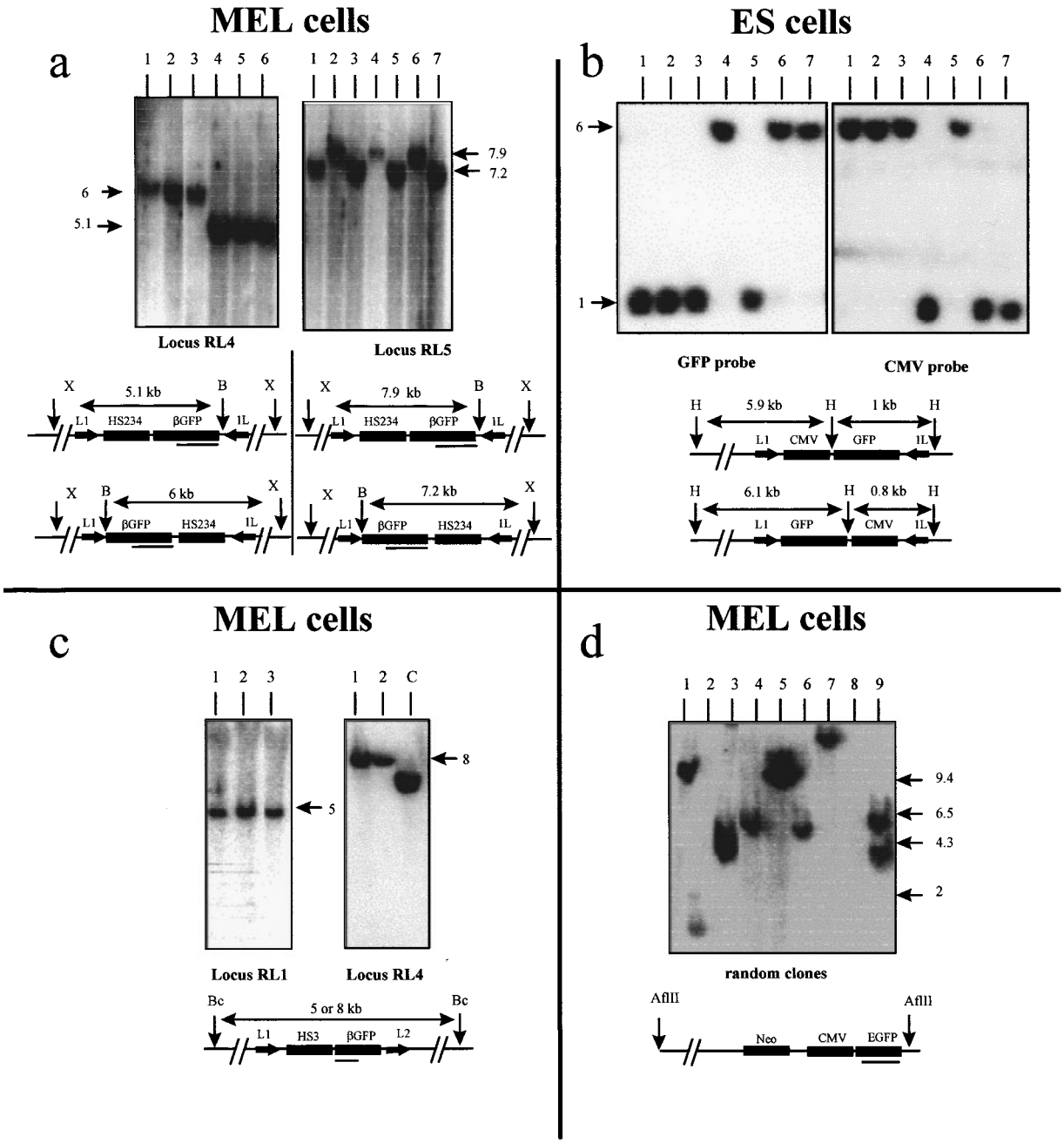

Figure 2. Southern blots demonstrating RMCE. (a) RMCE with inverted Lox sites in MEL cells. Autoradiogram illustrating the exchange of the HYTK gene with the HS234βGFP cassette at two loci in MEL cells. Lanes 1 to 6 in left panel and 1 to 7 in right panel: genomic DNA from Gan^R resistant clones digested with *Eco*RV and *Bgl*II and probed with a fragment encompassing the EGFP coding sequence. Two bands (6.1 and 5.1 kb at RL4 or 7.9 and 7.2 kb at RL5) were observed per locus demonstrating insertion in both possible orientations. B, *Bgl*II; X, *Eco*RV or *Bgl*II. (b) RMCE with inverted Lox sites in ES cells. Left panel: lanes 1 to 7, genomic DNA from Gan^R resistant clones digested with *Hin*dIII and probed with a fragment encompassing the EGFP coding sequence. Right panel: the blot of the left panel was stripped and re-probed with a fragment encompassing the CMV promoter. As expected, clones in both orientations were obtained. (c) RMCE with no selectable marker in the chromosome by selecting the cells that have taken up the most DNA during the transfection. Left panel, lanes 1 to 3, and right panel, lanes 1 and 2: genomic DNA was digested with *Bcl*I and probed with a fragment encompassing the human β-globin promoter; lane C, control DNA. As expected, a band with a size specific for each locus is observed. Since L1 and L2 sites were used all the integrations occured in the same orientation. Bc, *Bcl*I. (d) Uncontrolled integration of cassette CMV-EGFP. Lanes 1 to 9: genomic DNA digested with *Afl*II was probed with the EGFP coding sequence. Bands of all sizes and intensity characteristic of uncontrolled integration are observed.

the exchange cassette, and selecting for the cells that expressed the highest levels of GFP, would increase the proportion of cells with a RMCE because the sorted cells would be the cells that have taken up the highest amount of the exchange plasmid during the transfection (Figure 1(d)). A plasmid containing a GFP reporter gene located outside of a LacZ exchange cassette (pL1-HS3βglobZ-L2-GFP) was therefore created and co-transfected with a CRE expression plasmid in two lines of MEL cells (RL1 and RL3) containing pre-integrated target L1 and L2 Lox sites flanking the HYTK gene. After 48 hours post-transfection, the cells expressing the highest amount of GFP (0.1 percentile) were sorted individually into 96 well plates, expanded without applying any selection and tested for RMCE. A total of 74 clones were obtained for the RL1 line and 32 for the RL3 line. The clones that had lost the HYTK gene were identified by culture in hygromycin, and the Hyg^S clones were then tested by Southern blots. All clones that express GFP produced bands of the expected size for an RMCE (Figure 2(c)). At locus RL1, 4% (3/74) of the clones obtained had an exchange. At locus RL3, 16% (5/32) of the clones had an exchange. No evidence of random integration of plasmid HS3βglobZ-L2-GFP was found. This demonstrates that RMCE with no expressed selectable marker in the genome can be performed at relatively high frequency by simply sorting the cells that have been transfected with a large number of exchange plasmids.

Site-specific integration greatly improves reproducibility of expression

We then determined whether site-specific chromosomal integration leads to expression that is more reproducible and predictable than expression of the same cassette randomly integrated. MEL cell clones with integration of the CMV-EGFP cassette at random sites were generated by transfection of plasmid pEGFP-N1, and compared with clones containing the CMV-EGFP cassette integrated by RMCE at locus RL4. Southern blots revealed that most of the random clones contained multiple integrated copies (Figure 2(d)) and that as expected the RMCE clones had integration in both possible orientations. Nine random clones and nine RMCE clones with integration in one of the two possible orientations were analyzed for expression by flow cytometry (Figure 3). Mean levels of green fluorescence for the random clones varied from 5.3 (which is similar to untransfected controls) to 357.5. The average was 94.1(+/− 103.5). Mean levels of fluorescence for the RMCE clones varied from 100.1 to 147.4. The average was 127.8(+/− 11.9). Although the average expression of the two types of clones were similar, the tenfold difference between their standard deviations clearly demonstrates that expression levels of clones integrated at the same locus by RMCE are much less variable than those of randomly integrated clones. Similar results were obtained at the five other RMCE loci that we have tested so far (data not shown).

Discussion

We report here on two new CRE-based cassette exchange techniques. The inverted Lox sites method is extremely efficient and is very easy to implement. It should prove the method of choice for most applications. The FACS-based approach is less efficient but has the advantage of not requiring any active gene at the locus before or after the exchange.

The highly reproducible expression observed when independent RMCE clones were tested in the absence of any selective pressure at the locus suggests that controlling the integration site and therefore eliminating position effects permit meaningful comparisons of multiple transgenes individually integrated at a particular genomic site. This confirms and extends the finding of Fukushige & Sauer (1992) who have shown reproducible expression of site-specifically integrated transgenes in a different cell line at loci where minimum expression was obligatory since positive selection was used to select for the recombination event.

In addition to the L1/L2 pair of sites used in this study, other pairs of heterospecific Lox and FRT sites have been described (Hoess *et al.*, 1986; Sauer, 1996; Lee & Saito, 1998). These sites could further extend RMCE in two possible ways. (1) Pairs of Lox sites that would be more mutually incompatible than L1/L2 might permit CRE-based RMCE to be performed with no incoming selectable marker and in a defined orientation. (2) Multiplex integration of transgenes (Sauer, 1996) sequentially at the same site or at multiple independent sites could be accomplished by RMCE using multiple pairs of Lox sites, either as heterospecific pairs or inverted as shown here. Combination of FLP (Seibler *et al.*, 1998) and CRE-based RMCE could further expand the possibilities. Since there is a paucity of negative selectable markers available, the method of choice for multiplex experiments would presently be the FACS-based approach.

Potential uses of RMCE

There are two broad types of applications for RMCE: Lox sites can be integrated in the genome either at random sites or at known chromosomal locations *via* homologous recombination. Lox sites at random locations can be used to generate reference loci. Of particular interest would be loci with characteristic position effects. For instance, one could find reference loci favorable for strong expression in a particular tissue, or loci that confer a *pan*-cellular or a variegated pattern of expression. RMCE sites at known genomic locations should facilitate the systematic characterization of endogenous *cis*-regulatory elements and the genetic

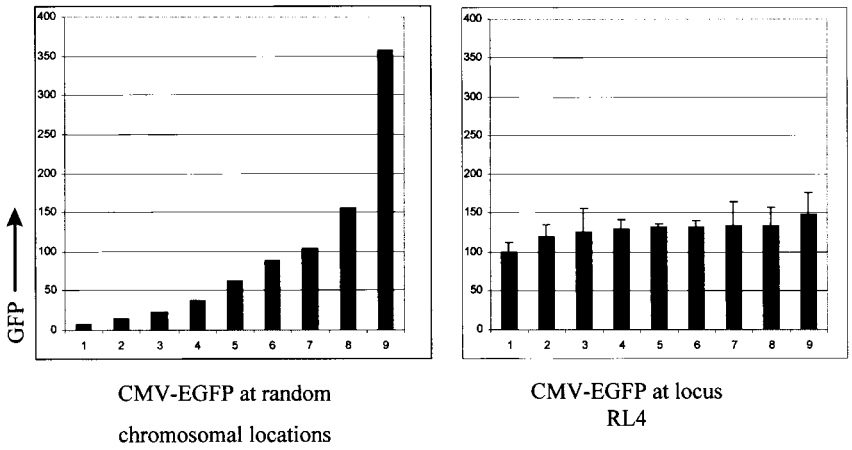

Figure 3. Expression of GFP at pre-selected and random locus. GFP expression of nine independent clones with the CMV-EGFP cassette integrated at nine uncontrolled random loci (left panel) or at the same pre-selected locus (right panel) was analyzed by FACS. The *y*-axis represents the mean linearized GFP fluorescence. Site-specific integration considerably diminishes the variability of expression typically observed in stable transfection studies.

dissection of protein function *via* the generation of mutated versions of endogenous gene products.

Site-specific integration by RMCE or by other means (Ow & Medberry, 1995; Araki *et al*., 1997) will prove useful for: (1) the discovery and analysis of *cis*-acting DNA regulatory elements controlling expression and position-effects; (2) the production of polypeptides of biological value by insertion of transgenes at reference loci at which expression is high or optimally inducible; (3) the development of designer proteins by insertion of multiple versions of a gene at the same locus in order to test variants in a context in which they are produced in the same amount and have the same tissue distribution; (4) creation of animal models of human diseases (particularly those in which multiple genes must be expressed at well regulated levels); and (5) creation of improved agricultural organisms.

Artificial genetic loci

Classical gene transfer technology often involves cumbersome screening procedures to identify clones or animals with appropriate level and developmental pattern of expression. While these first generation technologies are sufficient to perform simple genetic manipulations, they are inadequate for the implementation of complex genetic strategies to modify plants and animals by introduction of large numbers of carefully regulated transgenes to achieve a desired phenotype. For such complex genetic engineering purposes the development of rules for the creation of artificial genetic loci (AGL) will be required. We envision AGL as arrays of genes and regulatory elements integrated at reference genomic sites whose chromatin structure and influence on gene expression can be controlled at will by insertion of known *cis*-acting elements. Transgenes making up an AGL would be expressed in a tissue and developmental stage-specific manner at levels that are totally predictable. RMCE will facilitate the development of AGL by both permitting the careful characterization of the site with different *cis*-regulatory elements and mediating the convenient exchange of multiple transgenes into the AGL.

Materials and Methods

Plasmid constructions

Constructions were performed using standard procedures. Sequences of the plasmids used in this study are available on request.

RMCE plasmids

The L1 and L2 Lox sites are as described (Bouhassira *et al*., 1997). They were initially described by Hoess *et al*. (1986). The CMV-HYTK cassette is from plasmid pTgCmvhytk (Immunex, Seattle, Wa). The CMV-EGFP and GFPuv cassettes are from plasmids pEGFP-N1 and pGFPuv (Clontech, Palo Alto, CA). The β-GFP cassette was created by replacing the CMV promoter in plasmid pEGFP-N1 by the β-globin promoter (fragment −374 to +44 relative the cap site). Cassette HS234-βGFP was produced by linking fragment HS234 of Bouhassira *et al*. (1997) to the β-GFP cassette. Cassette HS3-β-globZ was from pCAS3 of Bouhassira *et al*. (1997). A series of plasmids containing a cassette flanked by Lox sites L1and L2 in various orientations was created. Each plasmid is designated by the Lox site 5′ of the cassette, the name of the cassette and the Lox site 3′ of the cassette. Inverted L1 Lox sites are designated as 1L. The following plasmids were used in this report: pL1-HYTK-L2, pL1-HYTK-1L, pL1-CMVEGFP-L2, pL1-Gfpuv-L2, pL2-GFPuv-L2, pL1-HS234βGFP-1L, pL1-HS3βglobZ-L2EGFP (contains EGFP 3′ of the L2 Lox site).

Cre expression plasmids (mammalian)

pBS 185 (CMV-CRE) was obtained from Clontech (Palo Alto, CA). pSSR73 (RSV-CRE) was a gift from Dr P. Leboulch (Harvard University, Cambridge MA), pMC-Cre was a gift from Klaus Rajewsky (Koln, Germany) (Gu *et al*., 1993).

Cre expression plasmid (bacterial)

A PCR generated fragment containing the coding sequence of the CRE recombinase was cloned into the *Nde*I and *Xho*I sites of plasmid pET23c (Novagen, Madison, WI), resulting in a plasmid (pET23c-CRE) that expresses a His-tagged CRE protein in induced BL21 bacteria.

CRE purification

BL21 (DE3) LysE bacteria (Novagen, Madison, WI) containing plasmid pET23c-CRE were induced with 2 mM IPTG for two hours, sonicated in basic buffer (50 mm NaPi (pH 8), 200 mm NaCl) plus 0.05% (v/v) Tween 20 and 1% (v/v) lysozyme, centrifuged at 4000 *g* for ten minutes at 4 °C, and the supernatant was loaded on a Talon metal affinity Resin column (Clontech, Palo Alto, CA). The column was washed successively with 20 ml of basic buffer containing 0, 5 and 10 mM imidazol and the CRE protein was then eluted with 5 ml of basic buffer containing 100 mM imidazol. *In vitro* CRE recombination were performed as in Abremski & Hoess (1984).

Cell culture and electroporation

Culture and RMCE reactions in MEL cells were performed as in Bouhassira *et al*. (1997). AK-7 ES cells were cultured and selected on SNL feeder cells as described (Soriano, 1997). RMCE was performed by electroporating (250 V, 500 uF, Biorad gene pulser, Biorad, Hercules, CA) 3×10^6 cells with 200 µg of L1GFP1L and 30 µg of MC-Cre (Gu *et al*., 1993). Selection with 3 µM gancyclovir was applied five days after the transfection.

GFP expression studies

A sample containing 5×10^5 cells was rinsed once in PBS and resuspended in Hank's solution containing 5% (v/v) fetal calf serum and 2 µM propidium iodide, and analyzed on a FACSCAN flow cytometer (Becton Dickinson, CA).

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